THE ROLE OF COLLAGENASE IN THE HEALING OF COLONIC

ANASTOMOSES — A REAPPRAISAL

A thesis submitted to the
University of London
for the degree of
Master of Surgery

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Collagenase, the initiating enzyme in collagen degradation, has been implicated as a contributory factor in colonic anastomotic dehiscence. Mammalian collagenase is synthesised on demand in a latent form and its activity is normally tightly controlled. Contemporary methods of investigation were developed and adapted to reassess this specific enzyme in colonic healing in rabbits.

Hydroxyproline and protein measurements confirmed that collagen concentration in the colon wall proximal to an anastomosis decreases three days after operation. Collagenase activity, assessed by culture of colonic explants and radiochemical assay of the medium, showed a corresponding increase after three days on both sides of the anastomosis and more marked distally.

Immunohistochemical localisation of collagenase and its inhibitor, tissue inhibitor of metalloproteinases (TIMP), demonstrated extracellular enzyme and secreting cells in the edges of bowel wall twelve hours after both everted and 'end-on' anastomoses. Collagenase was accompanied by TIMP within twenty-four hours and secretion was decreasing by the third day. At seven
days, only small aggregates of cells secreting enzyme and inhibitor were found within the maturing scar. The distribution of collagenase was always extremely localised and confined to the immediate vicinity of the suture line. TIMP secreting cells appeared in a protective serosal layer on the third day; TIMP distribution indicated close control of collagenase activity in the healing tissue. No collagenase or TIMP was localised in colon after sham laparotomy.

The histochemical and biochemical findings cannot be correlated but critical evaluation of the techniques indicates that immunolocalisation is more likely to represent the true 'in vivo' situation. Collagenase appears to initiate the degradation of tissue which will be removed during healing and which does not contribute to the integrity of the anastomosis. The enzyme remains tightly controlled by TIMP and there is no evidence for excessive collagenase activity in uncomplicated colonic healing.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>2</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>4</td>
</tr>
<tr>
<td>List of Illustrations and Tables</td>
<td>6</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>11</td>
</tr>
<tr>
<td>Claim to Originality</td>
<td>14</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>15</td>
</tr>
<tr>
<td>General Statement of Purpose of the Investigation</td>
<td>17</td>
</tr>
</tbody>
</table>

Chapters:

I  Historical Review and Background to the Study   18
    1 Historical Review of Intestinal Surgery ... 18
    2 Wound Healing .................................. 26
    3 Intestinal Healing ............................ 37
        i methods of assessment ..................... 37
        ii collagen dynamics ....................... 48
        iii factors in dehiscence ................. 59
    4 Collagen ....................................... 76
    5 Collagenase ................................... 91

II Detailed Statement of the Purpose of the Investigation 103
### CONTENTS

III Establishment of Methods ........................................... 105  
1 Animal Model of Colonic Healing .......................... 105  
2 Measurement of Collagen .............................. 116  
3 Measurement of Collagenase Activity ............. 129  
   i Background .................................. 129  
   ii Extraction .................................. 131  
   iii Culture ..................................... 140  
   iv Assay ........................................ 155  
4 Immunolocalisation of Collagenase and TIMP 183  

IV Investigation of Collagenase in Colonic Healing 195  
1 i Biochemical Measurements .................. 195  
   ii Results ..................................... 203  
2 i Immunolocalisation ................... 214  
   ii Results ..................................... 218  

V Discussion .................................................. 243  
1 Introduction ................................... 243  
2 Measurement of hydroxyproline ............... 246  
3 Biochemical measurement of collagenase .... 250  
4 Immunolocalisation of collagenase and TIMP 263  
5 Criticisms and implications of findings ... 272  

VI Conclusions ............................................... 286  
References .................................................. 287  
Appendix .................................................... 327
LIST OF ILLUSTRATIONS AND TABLES

Table I.4.1 Characteristics of collagen types I to IV .......................... 80

Figure I.5.1 Summary of regulation of collagenase activity ............ 97

Figure III.1.1 Diagram of blood supply to distal colon in rabbit showing site of vessel ligation and resection .... 110

Figure III.1.2 Diagram of everted and 'end-on' suture techniques .......... 115a

Table III.2.1 Concentrations of hydroxyproline and total amino acids measured in 12 segments of colon to confirm that hydroxyproline represents more than 2% of the total amino acid concentration in rabbit colon .... 126

Table III.3.1 Total collagenase activity in 7 individual explants of resected colon over 96 hours in culture ... 151

Table III.3.2 Total collagenase activity in 21 individual explants of post-operative colon over 96 hours in culture ... 152

Figure III.3.3 Graph of collagenase activity against time in culture for 7 explants of resected colon .................... 153

Figure III.3.4 Graph of collagenase activity against time in culture for 21 explants of post-operative colon .............. 154

Table III.3.5 Effect of temperature of incubation on control readings and total collagenase activity of normal colon explants ................. 164

Figure III.3.6 Effect of temperature of incubation on collagenase assay ........ 165

Table III.3.7 Effect of time of incubation on collagenase activity .......... 168
3.8 Effect of time of incubation on collagenase assay .................. 169

3.9 Demonstration of collagen breakdown products by SDS-PAGE .............. 176

3.10 Results of SDS-PAGE experiment to demonstrate the specificity of the collagenase assay ............. 179

3.11 Coefficient of variation for collagenase assay between 3 explants from each segment of colon ........ 182

4.1 Effect of short-term culture on colonic tissue ...................... 194

1  Hydroxyproline concentration of normal rabbit colon ............... 197

2  Diagram of dissection of anastomotic segment to give strips of tissue for hydroxyproline and collagenase determination ....................... 200

3  Hydroxyproline and protein concentrations one and three days after anastomosis ................. 204

1 day post-op - mean values from 5 rabbits. Total, active and latent collagenase activity ............. 207

3 days post-op - mean values from 5 rabbits. Total, active and latent collagenase activity ............. 208

1 day post-op - mean values from 5 rabbits. Log transformed collagenase activity ............. 209

3 days post-op - mean values from 5 rabbits. Log transformed collagenase activity ............. 210

Diagram of dissection of anastomotic segment to give strips of tissue for immunohistochemistry .......... 216

Normal appearance of rabbit colon 219
Figure IV.2.3  Fluorescent micrograph of inflammatory cells .............. 220
Figure IV.2.4  Anastomosis 12 hours after operation ...................... 222
Figure IV.2.5  Collagenase localisation 12 hours post-op ................... 223
Figure IV.2.6  Extracellular collagenase and inflammatory cells around suture material ............. 224
Figure IV.2.7  Collagenase secreting cells 12 hours after anastomosis ................. 226
Figure IV.2.8  Absence of TIMP 12 hours after operation ..................... 227
Figure IV.2.9  Anastomosis one day after operation .......................... 229
Figure IV.2.10 TIMP secreting cells one day after anastomosis .................. 230
Figure IV.2.11 Anastomosis three days after operation ........................ 232
Figure IV.2.12 Collagenase in submucosa and muscle layers three days after anastomosis 233
Figure IV.2.13 TIMP cells in serosal layer three days post-op .................. 235
Figure IV.2.14 Serosal coat in proximal segment three days after anastomosis ...... 236
Figure IV.2.15 Anastomosis seven days after operation ........................ 238
Figure IV.2.16 Anastomosis seven days after operation ........................ 239
Figure IV.2.17 Collagenase and TIMP secreting cells seven days after anastomosis ..... 241
Figure IV.2.18 Collagenase and TIMP secreting cells seven days after anastomosis ..... 242
<p>| Figure IV.2.19 | 'End-on' anastomosis 12 hours after operation .................................. 242b |
| Figure IV.2.20 | 'End-on' anastomosis one day after operation ...................................... 242d |
| Figure IV.2.21 | 'End-on' anastomosis three days after operation .................................. 242f |
| Figure IV.2.22 | 'End-on' anastomosis seven days after operation .................................. 242h |
| Table A.1.1-5 | Comparison of hydroxyproline concentrations in wet and dry tissue before and 3 days after colonic anastomosis ........................... 327 |
| Table A.2     | Hank's balanced salts solution ......................................................... 332 |
| Table A.3     | Dulbecco's modification of Eagle's medium ........................................... 333 |
| Table A.4.1   | 1 day post-op. Hydroxyproline concentration ....................................... 334 |
| Table A.4.2   | 3 days post-op. Hydroxyproline concentration ....................................... 335 |
| Table A.4.3   | 3 days post-op. Log transformed hydroxyproline concentration ................. 336 |
| Table A.4.4   | 1 day post-op. Protein concentration .................................................. 337 |
| Table A.4.5   | 3 days post-op. Protein concentration ................................................ 338 |
| Table A.4.6   | Ratio of hydroxyproline to protein. 1 day post-op ................................ 339 |
| Table A.4.7   | Ratio of hydroxyproline to protein. 3 days post-op ............................... 340 |
| Table A.5.1   | 1 day post-op, 24 hours culture. Active collagenase activity ................. 341 |
| Table A.5.2   | 1 day post-op, 24 hours culture. Latent collagenase activity .................. 342 |</p>
<table>
<thead>
<tr>
<th>Table A.5.3</th>
<th>1 day post-op, 24 hours culture.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total collagenase activity ......</td>
</tr>
<tr>
<td></td>
<td>343</td>
</tr>
<tr>
<td>Table A.5.4</td>
<td>1 day post-op, 48 hours culture.</td>
</tr>
<tr>
<td></td>
<td>Active collagenase activity ......</td>
</tr>
<tr>
<td></td>
<td>344</td>
</tr>
<tr>
<td>Table A.5.5</td>
<td>1 day post-op, 48 hours culture.</td>
</tr>
<tr>
<td></td>
<td>Latent collagenase activity ......</td>
</tr>
<tr>
<td></td>
<td>345</td>
</tr>
<tr>
<td>Table A.5.6</td>
<td>1 day post-op, 48 hours culture.</td>
</tr>
<tr>
<td></td>
<td>Total collagenase activity ......</td>
</tr>
<tr>
<td></td>
<td>346</td>
</tr>
<tr>
<td>Table A.5.7</td>
<td>1 day post-op, 72 hours culture.</td>
</tr>
<tr>
<td></td>
<td>Active collagenase activity ......</td>
</tr>
<tr>
<td></td>
<td>347</td>
</tr>
<tr>
<td>Table A.5.8</td>
<td>1 day post-op, 72 hours culture.</td>
</tr>
<tr>
<td></td>
<td>Latent collagenase activity ......</td>
</tr>
<tr>
<td></td>
<td>348</td>
</tr>
<tr>
<td>Table A.5.9</td>
<td>1 day post-op, 72 hours culture.</td>
</tr>
<tr>
<td></td>
<td>Total collagenase activity ......</td>
</tr>
<tr>
<td></td>
<td>349</td>
</tr>
<tr>
<td>Table A.5.10</td>
<td>3 days post-op, 24 hours culture.</td>
</tr>
<tr>
<td></td>
<td>Active collagenase activity ......</td>
</tr>
<tr>
<td></td>
<td>350</td>
</tr>
<tr>
<td>Table A.5.11</td>
<td>3 days post-op, 24 hours culture.</td>
</tr>
<tr>
<td></td>
<td>Latent collagenase activity ......</td>
</tr>
<tr>
<td></td>
<td>351</td>
</tr>
<tr>
<td>Table A.5.12</td>
<td>3 days post-op, 24 hours culture.</td>
</tr>
<tr>
<td></td>
<td>Total collagenase activity ......</td>
</tr>
<tr>
<td></td>
<td>352</td>
</tr>
<tr>
<td>Table A.5.13</td>
<td>3 days post-op, 48 hours culture.</td>
</tr>
<tr>
<td></td>
<td>Active collagenase activity ......</td>
</tr>
<tr>
<td></td>
<td>353</td>
</tr>
<tr>
<td>Table A.5.14</td>
<td>3 days post-op, 48 hours culture.</td>
</tr>
<tr>
<td></td>
<td>Latent collagenase activity ......</td>
</tr>
<tr>
<td></td>
<td>354</td>
</tr>
<tr>
<td>Table A.5.15</td>
<td>3 days post-op, 48 hours culture.</td>
</tr>
<tr>
<td></td>
<td>Total collagenase activity ......</td>
</tr>
<tr>
<td></td>
<td>355</td>
</tr>
<tr>
<td>Table A.5.16</td>
<td>3 days post-op, 72 hours culture.</td>
</tr>
<tr>
<td></td>
<td>Active collagenase activity ......</td>
</tr>
<tr>
<td></td>
<td>356</td>
</tr>
<tr>
<td>Table A.5.17</td>
<td>3 days post-op, 72 hours culture.</td>
</tr>
<tr>
<td></td>
<td>Latent collagenase activity ......</td>
</tr>
<tr>
<td></td>
<td>357</td>
</tr>
<tr>
<td>Table A.5.18</td>
<td>3 days post-op, 72 hours culture.</td>
</tr>
<tr>
<td></td>
<td>Total collagenase activity ......</td>
</tr>
<tr>
<td></td>
<td>358</td>
</tr>
</tbody>
</table>
ABBREVIATIONS

APMA 4-aminophenylmercuric acetate
αM α2-Macroglobulin
C Centigrade
cm centimetre
DAB 4-dimethylaminobenzaldehyde
DMEM Dulbecco’s modification of Eagle’s medium
DMSO dimethyl sulfoxide
dpm disintegrations per minute
EDTA ethylenediaminetetraacetic acid
ELISA enzyme-linked immunosorbent assay
Fab monovalent antibody fragment
Fig figure
FITC fluorescein isothiocyanate
g gram
G acceleration due to gravity
Gly glycine
h hour
H and E haematoxylin and eosin
HEPES 4-(2-hydroxyethyl)-piperazine-ethane sulphonic acid
Hyp hydroxyproline
IgG immunoglobulin G
ABBREVIATIONS

IL-1  Interleukin-1
Ile  isoleucine
in  inch
l  litre
lb  pound
LBTI  lima bean trypsin inhibitor
Leu  leucine
Ln  natural logarithm
log  logarithm
m  minute
M  molar
mg  milligram
ml  millilitre
mm  millimetre
mM  millimolar
μ  micron
μCi  microCurie
μg  microgram
μl  microlitre
μM  micromolar
NEM  N-ethylmaleimide
nm  nanometre
NSS  non-immune sheep serum IgG
oPA  1,10-phenanthroline (o-phenanthroline)
ABBREVIATIONS

p    probability
PBS  phosphate buffered saline
PMSF phenylmethylsulphonyl fluoride
Pro  proline
s    second
SBTI soy bean trypsin inhibitor
SD   standard deviation
SDS  sodium dodecyl sulphate
SDS-PAGE  sodium dodecyl sulphate polyacrylamide gel electrophoresis
σ    standard error
TEMED NNN′N′-tetramethylenediamine
TIMP tissue inhibitor of metalloproteinases
Tris tris(hydroxymethyl)methylamine
TrisHCl tris(hydroxymethyl)methylammonium chloride
w/v  weight/volume
CLAIM TO ORIGINALITY

The studies presented in this thesis were devised and carried out by the author. There have been no previous specific measurements of collagenase activity in colonic healing, apart from those acknowledged and criticised within this thesis. There has been no previous immunolocalisation of collagenase and tissue inhibitor of metalloproteinases in colonic healing.

The biochemical assay methods used were not original and have been acknowledged accordingly. The establishment of a system of culture for rabbit colon, the adaptation of a radiochemical assay for the specific measurement of collagenase in the culture medium and the subsequent application of these methods were performed by the author, with the assistance of Dr F J Savage who also carried out the gel electrophoresis.

The immunohistochemical methods of localisation of collagenase and TIMP were devised by Ms R M Hembry who supplied all the necessary antibodies. Initial experiments were carried out under her supervision at Strangeways Research Laboratory, Cambridge. Subsequent experiments were performed by the author.

Data arising from the experiments were collated, analysed and prepared for this thesis by the author.
I am grateful to the Special Trustees and Consolidated Postgraduate Fund of University College Hospital who provided the financial support for this project.

This study could not have been undertaken and completed without the help of the following counsellors, friends and colleagues, whose contributions I would like to acknowledge, with my thanks.

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The late Professor Charles Clark allowed and encouraged me to work in his department. Paul Boulos initiated the project, established the funding, and supervised and supported both the work and the completion of the manuscript. Felicity Savage participated in every part of the project, whether rewarding, frustrating or sheer hard work. The late Mike Lewin gave advice and encouragement on the experiments, the manuscript and the statistical analysis and improved my artistic endeavours. Paul Jayaraj and Anna Sewerniak gave freely of help and advice. Peter Luther and Robin Williams produced photographic illustrations with expertise and effort. Domingos Lacombe collaborated in additional experimental work.
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Also: Phil Belsham and Gordon Henson helped to set up and keep my computer running, freeing me completely of the need for secretarial assistance. Mr. David Bailey advised on the manuscript and painstakingly corrected the grammar and spelling.
The enzyme collagenase has been implicated as a major factor in the dehiscence of large bowel anastomoses. This study re-investigates the role of collagenase in colonic healing in the light of considerable advances in the current knowledge of this specific enzyme.
On the nature of wound healing:

an uncomplicated incised wound falls within the definition of a 'self-limited disease...to which there is due a certain succession of processes, to be completed in a certain time; which time and processes may vary with the constitution and condition of the patient, and may tend to death, or to recovery, but are not known to be shortened, or greatly changed by medical treatment.'

Jacob Bigelow, 'Nature in Disease', p4, Boston 1854 (Howes et al 1929)

The ability to recover from injury and to repair wounded tissue, given the right circumstances, is a fundamental part of life itself and one of the attributes by which we can distinguish the living from the inanimate object. Wound healing 'being one of the most important topics in surgery requires to be attentively studied, and its effect carefully watched, before any one can practice in his profession with credit to himself or advantage to others' (Cooper 1835). Thus, long recognised as fundamental to the practice of
CHAPTER I.1 Historical Review

surgery, the events that occur during wound healing have been investigated in great depth. Perhaps the greatest advance in this direction took place in 1865, only 11 years after Bigelow's statement, when Joseph Lister introduced antisepsis into wound management and encouraged the belief that the majority of wounds should heal without infection (Ellis 1984). With subsequent knowledge and research, current surgical practices endeavour to create the most suitable environment for the uncomplicated healing of wounds by the use of antiseptic cleansing solutions, improved methods of closure and the application of sterilised dressing materials suited to the circumstances.

There have been considerable advances in the understanding of the assessment of a patient's general constitution and condition at the time of wounding and of the steps that can be taken to improve that condition, as far as possible, in order to encourage healthy, rapid healing. Even so, while comprehension of the processes involved has increased immeasurably and the range of available medical treatment has grown prodigiously, the essence of Jacob Bigelow's statement on self-limited disease remains as applicable to wound healing in 1988 as it was in 1854. Apart from a greater
knowledge of those detrimental factors which should be avoided, modern medical treatment has so far had minimal impact on the basic process of wound healing itself, which remains the province of nature (Zederfeldt 1980, Ellis 1984, Hunt 1984).

From earliest times it was obvious that wounds of the intestine gave rise to particular problems. The fatality of intestinal perforation was noted in Biblical times (Judges 3: 16–25) but occasional cases of recovery were reported, due to the spontaneous adhesion of parietal and visceral peritoneum preventing the flow of intestinal contents into the abdomen. Intervention by intestinal suturing was disparaged by Celsus in Ancient Rome (1938) but the technique was revived by Abulkasem in the eleventh century (Senn 1893). In 1396 Lanfrank advocated the use of sutures to encourage fistula formation (Kerr 1923) and over the next 400 years this was the most successful of the approaches which have been recorded. One alternative was closure of the intestinal wound, usually with the suture ends brought out through the abdominal wound to maintain close approximation of the bowel to the parietal peritoneum; another was closure of the bowel over a supporting cylinder made from a variety of materials, including
CHAPTER I.1 Historical Review

India rubber, cardboard, fish glue and goose trachea (Senn 1893). The overall success of these surgical procedures, up to the beginning of the 19th century, was probably little better than the incidence of spontaneous recovery. Indeed, in military circles, surgical intervention for intestinal wounds sustained in battle was considered unwise, even as late as 1915 (Dunphy 1970, Schilling 1985).

In 1812, however, Travers reported his animal experiments, which showed that intestinal wounds could be repaired without the support of the parietal peritoneum by approximating the two serosal surfaces and returning the gut to the abdomen. Fourteen years later Lembert described an inverting sero-muscular suture technique which is still the basis of current practice (Goligher 1984f, Kerr 1923, Dunphy 1970) and, in civilian practice, by 1885 the suture of all bowel wounds, however small, was strongly advocated (Gross 1843, Pilcher 1885, Dunphy 1970). Senn (1893) has elegantly described the many innovations in suture technique which were proposed over the course of the 19th century and also the great variety of ingenious mechanical aids invented during this period. Czerny introduced a second row of sutures in 1881 and Connell...
CHAPTER 1.1 Historical Review

(1892) described a method for uniting divided bowel ends which still has its place in modern surgery. It was Halsted (1887), later supported by Kerr (1923), who first recognised the importance of the submucosal layer of the intestine, which Lembert had carefully included in his stitches, as the fibrous coat which provided anchorage for secure sutures.

It is interesting to note that, before Lembert's innovations and Lister's introduction of antiseptic precautions and aseptic sutures, it was generally recognised that the outcome of surgery for intestinal wounds, very poor overall, was a little better when the large bowel was damaged rather than the small (Celsius 1938, Senn 1893). This presumably relates to the nature of the intestinal contents. At a time when operations had to be performed as rapidly as possible and the quality of the materials depended on what was to hand, liquid small bowel contents, with their digestive enzymes, would have been more difficult to isolate from the peritoneal cavity than solid faecal material, despite the dangers of a greater bacterial load. The advent of anaesthesia allowed the surgeon to operate more slowly and carefully and develop techniques for watertight anastomosis of the intestine. By the end of
the 19th Century, experience showed that resection and anastomosis in the colon, particularly on the left side, were much more hazardous than elsewhere in the intestine (Goligher 1984a). In recent times it has been the risk of leakage from large bowel suture lines which has been the stimulus for a great deal of experimental study and innovation in technique.

Balfour described a method of recto-sigmoid anastomosis in 1910 and, between 1898 and 1940, a variety of techniques was introduced to create an 'aseptic' anastomosis (Goligher 1984a). However, Grey Turner (1929) considered a proximal defunctioning colostomy essential and a surgeon as distinguished as Lahey would not make a primary anastomosis in large bowel (Dunphy 1971). Von Mikulicz was able to reduce the operative mortality from 43 per cent for primary anastomosis to 12.5 per cent with the use of an exteriorisation and resection procedure also developed independently by Paul. The 'standard' operation progressed from anastomosis with caecostomy to preliminary colostomy, followed by anastomosis at a second operation (Goligher 1984a).

The introduction of antibiotics and intestinal antiseptics for bowel preparation in the 1940's had a
considerable effect on colonic surgery. A mortality of 
15 per cent, with a 60 per cent incidence of septic 
complications, for primary anastomosis in 1930, was 
reduced to 2 per cent mortality and 6 per cent septic 
complications in the same hospital with the use of 
antibiotics (Dunphy 1971), although better understanding 
of fluid and electrolyte balance, the use of blood 
transfusion and improvements in anaesthetic techniques 
also contributed to this. By 1953, Lloyd-Davies was 
able to achieve an operative mortality as low as 3 per 
cent, which was not due to septic problems, for primary 
colonic anastomosis (Goligher 1984a).

The problem of colonic anastomotic breakdown

The recorded incidence of dehiscence of colonic 
suture lines in modern surgical practice depends partly 
on operative variables and partly on the means used to 
assess that dehiscence has occurred. Clinical estimates 
of the rate range from 4 to 8 per cent (Goligher 1984b 
and c) but when digital examination, sigmoidoscopy and 
radiology are employed the reported incidence changes to 
a figure between 0 and 30 per cent for the colon as a 
whole (Foster and Leaper 1984). In the case of low 
anterior resection of the rectum a radiological leak
rate of 20 per cent has been suggested (Foster and Leaper 1984) but others quote an incidence as high as 69 per cent (Goligher et al 1970a).

While the true frequency of anastomotic dehiscence is open to question, the consequences of such failure are clear. The higher incidence of dehiscence revealed by X-ray indicates that a proportion of these cases are subclinical and have little or no effect on the patient's overall recovery. The clinical consequences can range from local abscess formation to generalised peritonitis but clinically significant dehiscence produces a three-fold increase in the mortality of large bowel surgery and the average length of stay in hospital is doubled (Fielding et al 1980, Foster and Leaper 1984, Schrock et al 1973). In a few cases, anastomotic dehiscence occurs in apparently ideal circumstances with no indication of a precipitating factor and it is these cases which constitute the main justification for further investigation into the problem of healing in the large bowel.
CHAPTER 1.2 Wound Healing

WOUND HEALING

HISTOLOGICAL FEATURES OF WOUND HEALING

The essential features of wound healing are the same in all soft tissues. Since many of the histological events in the healing process have been determined from the study of skin wounds, it is appropriate to describe the general features first and then to discuss the way in which healing of the intestine differs from that of skin.

Primary wound healing

Primary wound healing, or healing by first intention, occurs in clean incised wounds of the skin when the edges are correctly apposed and is the most desirable result in surgical practice. The sequence of events can be split into phases in order to discuss the processes involved but each phase passes smoothly into the next and one event waxes as the previous one wanes. The following description is based largely on the reviews by Irvin in 1981 and 1984.

The first phase of healing is an acute inflammatory reaction; there is a brief period of vasoconstriction in the wound edges followed by vasodilatation, while blood
fills the defect in the tissue and clots to form a fibrin-rich haematoma. Blood flow slows in the capillaries and polymorphonuclear leucocytes begin to adhere to the capillary walls, which become more permeable, allowing the cells to enter the fibrin plug. Neutrophil polymorphs and a few lymphocytes are found in the wound within three hours and increasing numbers enter over the first 48 hours. They are surrounded by serum proteins and strands of fibrin forming a network.

Monocytes appear within 24 hours; their numbers increase and they become the predominant wound cell by the fifth day. While the neutrophils function mainly by releasing the contents of their cytoplasmic granules, the mononuclear cells become tissue macrophages, showing active phagocytosis of extracellular material.

Still in the first phase of healing, new blood vessel buds begin to sprout from capillaries in the wound edges. Lymphatic channels develop more slowly, so drainage of the wound does not take place in the early stages and oedema results from the increased capillary permeability. Undifferentiated mesenchymal cells from the perivascular connective tissue appear in the wound towards the end of the first phase and differentiate into fibroblasts.
CHAPTER 1.2 Wound Healing

The second phase of healing, the period of fibroplasia, begins by the fourth or fifth day. The macrophages gradually lose their predominance and give way to the fibroblasts which are synthesising components of connective tissue matrix. Granulation tissue fills the wound, composed of new capillaries, fibroblasts, macrophages and mast cells. The fibroblasts synthesise proteoglycans and new collagen, recognised histologically as reticulin, while the new vessels become canalised and some develop into arterioles and venules. Collagen production increases, although the fibres are still randomly arranged.

One to two weeks after wounding, in the clean sutured wound, the second phase gradually gives way to the third phase of healing. Cell numbers diminish and much of the new vascular tree disappears, leaving a dense network of collagen fibres in ground substance, which together constitute the relatively avascular scar tissue. Now a slow process of organisation and remodelling takes place to remove the excess collagen and, in skin, leave those fibres which are correctly orientated along lines of tension. The remaining few inflammatory cells work to dismantle buried suture material and any residual wound debris. This third
phase could be said never to end since it subsides gradually into the normal turnover and remodelling processes which occur in most tissues continuously. Remodelling restores the wound area as closely as possible to the state of the original tissue and the degree to which the process is successful is reflected in the size and appearance of the mature scar.

Secondary wound healing

In larger wounds with loss of tissue and no apposition of the edges, secondary healing takes place. Larger amounts of granulation tissue are formed to fill the defect and the inflammatory response may be more florid. A process of contraction to reduce the size of the wound takes place in the granulation tissue, brought about by the presence of myofibroblasts, but fibrous tissue formation is also greater and the resulting scar is correspondingly more pronounced.

Epithelial repair

In wounds of the skin, epithelial repair accompanies the processes described above. Within 24 hours the basal epidermal cells at the edge of the wound begin to migrate over the cut dermal surface, enlarging
CHAPTER 1.2 Wound Healing

and multiplying to produce a thickened advancing edge. The edges meet and bridge the defect and the thickened epidermis fills the surface of the wound as keratinization begins. Epithelial cells also migrate along suture tracks. In excised superficial skin wounds damaged hair follicles and sweat and sebaceous glands provide islands of epithelium which also spread out and help to cover the defect. Peritoneal wounds, on the other hand, are repaired by cells from the subperitoneal connective tissue, so that healing takes place over the whole area simultaneously (Raftery 1984).

Intestinal healing

Intestinal healing follows essentially the same sequence of events as in skin wounds. Cellular infiltration takes place over the first two to three days, creating a reparative framework of macrophages and fibroblasts with new vessel formation. The surface columnar epithelial cells do not migrate but mitosis in the glandular crypts provides cells for the restoration of epithelial continuity. The integrity of the wound in the early stages depends on fibrin adherence of the cut edges and the support provided by the suture material but, in the intestine, tensile strength builds up more
CHAPTER 1.2 Wound Healing

quickly than in skin so there is a shorter period of reliance on the sutures. It is more important that intestinal stitch lines are leak-proof over this initial period (Foster and Leaper 1984). Proper apposition of all the tissue layers in the intestinal wall is difficult to achieve and the cut edges are often deliberately inverted or everted to ensure a watertight seal. This interferes with primary healing because the folded edge undergoes necrosis and has to be removed. Even careful 'end-on' apposition of the edges, such as that described by Gambee (1951), produces necrosis of the mucosal margin (Stein and Barry 1983) but in uncomplicated cases there is healing of the mucosa by secondary intention with primary healing in the deeper layers (Foster and Leaper 1984, Zumtobel et al 1980). The end result, even with inversion or eversion, is complete regeneration of a smooth mucosal surface and minimal scarring through the underlying layers.

Hesp and colleagues (1985) have described the histological features of healing in inverted small and large bowel anastomoses in rabbits. Granulocytes were present in both types of wound within 3 hours of surgery and reached maximum numbers after 12 to 24 hours. Monocytes and fibroblasts both began to appear at 24
CHAPTER 1.2 Wound Healing

hours and were numerous in tissue examined after 3 and 7 days. In ileal anastomoses, granulocytes disappeared from the wound area between 3 and 7 days and necrotic tissue at the margins was cleared away rapidly. In the colon, necrosis remained widespread at 7 days, accompanied by granulocyte infiltration, and it took longer for the mucosal defect to be bridged. The microvascular changes were similar in both types of tissue and Shikata et al (1982) found no differences in local blood flow between colonic and ileal anastomoses in dogs.

Getzen and Holloway (1966) compared inverting and everting anastomoses histologically in the colon of dogs. Within 4 hours fibrin effectively sealed both types of suture line. Between 1 and 7 days progressive regression of the folded mucosal margin occurred in both inversion and eversion and a smooth join developed between 7 and 21 days. A greater inflammatory response was seen in the inverted edges, with more oedema and persistence of inflammatory cells over a longer period. Trueblood et al (1969), however, found that, in rats, everted suture lines took longer to re-organize and establish smooth continuity of the layers than inverted ones.
Herrmann et al (1964) studied end-to-end anastomoses in rat colon. Haemorrhage, diffuse oedema and the deposition of fibrin were found around the anastomotic site, with exudation of neutrophils and other inflammatory cells reaching a peak between 24 and 48 hours. Inflammation of the invaginated wall margin was very marked. By 4 days, granulation tissue occupied the whole wall but the oedema and inflammation were beginning to subside as fibroblast proliferation took over. Collagen deposition was prominent between 6 and 10 days and both epithelial and vascular proliferation were seen at 1 week. The rate at which orderly restoration of the layers occurred and the exuberance of the granulation tissue were very much dependent on the degree of necrosis and sloughing at the wound margins. Thus, in some places, epithelium covered a granulomatous nodule at three weeks; in others, smooth muscle proliferation was found at 10 days and re-establishment of the various wall layers could be seen to begin by two weeks. These two pictures could be found at different sites in the same anastomosis and this probably accounts for the disparity in findings in the studies discussed above. After 3 weeks, gradual remodelling produced thinning of the wall and progressively better...
orientation of the layers, although the anastomotic scar line could still be identified a year after operation.

**BIOCHEMICAL ASPECTS OF WOUND HEALING**

Several chemical mediators are involved in the orderly progression of the inflammatory response which accompanies wound healing. Platelets and mast cells release histamine, which may initiate vasodilatation. The same cells also secrete vasoactive serotonin while the kinins, bradykinin and kallidin, are released from plasma proteins by the action of kallikrein to produce vascular engorgement and increased capillary permeability. Prostaglandins may be derived from the tissues or from neutrophils; prostaglandins $E_1$ and $E_2$ cause vasodilatation and increased permeability and other prostaglandins may help to determine the duration of the inflammatory response (Irvin 1981, 1984).

Chemotactic agents encourage white cell accumulation at the site of injury. Platelets which have been activated by tissue injury release platelet-derived growth factors which are chemotactants for fibroblasts and vascular endothelial cells. Fibrinogen polymerisation and subsequent fibrinolysis produce chemotactant fibrinopeptides

Cytokines, notably Interleukin-1, are released by macrophages and have far-reaching effects on connective tissue formation, encouraging proliferation of fibroblasts and increasing collagen and fibronectin synthesis. Interleukin-1 also promotes collagenase, neutral protease and prostaglandin E\(_2\) production, increasing connective tissue destruction (Dayer 1987, Pasternak et al 1986). The situation is clearly complex since the same mediators contribute to both sides of the equation and intricate feedback mechanisms are involved (Krane 1987).

Collagen synthesis and lysis both occur continuously in connective tissue and there is evidence that both processes are much increased during tissue repair (Edwards and Dunphy 1958, Carrico et al 1984). Collagen synthesis has been measured by the uptake of radio-labelled proline or by prolyl hydroxylase activity (Hunt and Pai 1972, O'Hare et al 1983). Such studies indicate that collagen synthesis accelerates considerably in the early days of wound healing. Measurements of collagen concentration, however, show an
initial decrease even when synthesis is increased
(Dunphy and Udupa 1955). The balance between synthesis
and lysis is lost and lysis predominates in the first
few days; later, synthesis is the dominant event and the
collagen concentration increases (Adamsons et al 1966).

The changes which are taking place in the collagen
balance are reflected in the development of tensile
strength in skin wounds. During the first few days,
when lysis exceeds synthesis, there is a lag phase but
the tensile strength then begins to increase rapidly as
new collagen is laid down. Although collagen deposition
ceases after 10 to 14 days, tensile strength continues
to rise more slowly and this can be attributed to the
development of increasing numbers of cross-links as the
collagen matures (Van Winkle 1969, Dunphy and Udupa
1955, Dunphy and Jackson 1962).

In the colon, the delicate balance in collagen
metabolism in the early stages is particularly liable to
become upset (Foster and Leaper 1984, Hawley 1970). The
collagen dynamics of healing in the intestine have been
studied by many different groups and the changes are
similar to those in wounds at other sites; the findings
will be discussed more fully in a later section (Chapter
1.3.ii).
CHAPTER 1.3 Intestinal Healing - methods

INTESTINAL HEALING

(i) METHODS OF ASSESSMENT

Early knowledge of intestinal healing came from observation of the clinical course in patients who made a spontaneous or assisted recovery from an intestinal injury. Trial and error, and the highly uncertain outcome of intervention in the absence of antisepsis, led to the mixed opinions of the value of intestinal suturing in the early 19th century. Subsequently, improvements in technique and advances in knowledge have been based on experimental evidence, usually gathered in animals, but the final justification for any new development is still an assessment of the effects in clinical practice.

Clinical studies

From observations of single or multiple cases, clinical investigation of intestinal healing has developed along two lines - the analysis, often retrospective, of the clinical course in large numbers of patients to identify factors which are associated with a good or a bad result, and the planned prospective comparison of the outcome of an established technique
CHAPTER 1.3 Intestinal Healing - methods

with that of a modification in method or adjuvant therapy. The assessment in such studies is based on the clinical course of the patients, the incidence of complications and the results of post-operative tests, particularly the use of contrast radiology to assess anastomotic integrity in the healing period. Since radiology reveals suture-line leaks which may not be clinically apparent (Goligher et al 1970a), the criteria for failure must be assessed carefully before the results of different studies can be compared (Foster and Leaper 1984).

The more important of these clinical studies have established the risks of leakage from an anastomosis and identified factors which contribute to those risks. Schrock and colleagues (1973) reviewed the records of 1703 patients undergoing colonic anastomosis over a 20 year period. 4.5 per cent had clinically significant disruption of the suture line and the mortality associated with dehiscence was 33 per cent, compared with 2.6 per cent when the anastomosis was intact. Factors which contributed to an increased risk of dehiscence were increasing age of the patient, anaemia prior to surgery and irradiation before surgery. Emergency procedures carried higher failure rates, as
did operations which lasted more than 5 hours. The presence of infection at the time of surgery, intra-operative hypotension or transfusion and the presence of malignant disease were adverse factors. Extraperitoneal anastomoses leaked three times more often than intraperitoneal suture lines and the overall dehiscence rate for anastomosis to the rectum was 9.2 per cent.

Fielding et al (1980) reported a computerised multicentre study of 1466 anastomoses in patients with colorectal cancer. An overall clinical dehiscence rate of 13 per cent rose to 18.7 per cent after extraperitoneal suture of the rectum but the pathological stage of the tumour did not affect healing unless the operation was considered palliative. When anastomotic leakage occurred, the length of hospital stay was doubled and the mortality trebled: in this study, the most significant contributory factor was the role of the individual surgeon with overall responsibility for the care of the patient.

Debas and Thomson (1972) also established anastomotic dehiscence as the single most important cause of mortality in their series of 838 colectomy patients, with emergency operation the most significant
CHAPTER I.3 Intestinal Healing - methods

contributory factor. In 1970, Goligher et al (1970a) reported particularly on dehiscence after anterior resection of the rectum and sigmoid colon. The high rate of disruption (51 per cent) rose even higher, to 69 per cent, when the suture line was extraperitoneal and it was this work which established the role of post-operative contrast radiology as an objective assessment of the integrity of anastomoses in clinical studies. Irvin and Goligher (1973) recognised the importance of faecal loading as a risk factor, while a clinical study by Whitaker et al (1970) showed the relevance of blood loss during operation.

Clinical investigations have been used to show an improvement when blood viscosity is reduced before operation (Tagart 1981) and to compare the results with different types of suture material (Clark et al 1972, 1977). Goligher et al compared inverting and everting suture techniques (1970b) and one and two layer anastomoses (1977), as did Maurya et al (1984), while Heald and Leicester (1981) reported good results after stapled rectal anastomosis and Bailey et al (1984) advocated a single-layer polypropylene technique. More recent clinical studies have examined the need for defunctioning colostomy (Röher et al 1985, Shannon and
CHAPTER 1.3 Intestinal Healing – methods

Moore 1985) and investigated the use of a biofragmentable ring to maintain apposition of the bowel ends during healing (Hardy et al 1987). Irving and Scrimgeour (1987) have used clinical results to suggest that bowel preparation is unnecessary before anastomosis if a regime of systemic antibiotic prophylaxis is used.

Animal studies

The performance of intestinal anastomosis in animals has been used to develop surgical skills, to test the introduction of new techniques and to investigate more thoroughly the events which occur during healing. Animal experiments allowed Travers (1812) to show that sutured bowel could be returned to the peritoneal cavity and Halsted's operations on dogs (1887) showed very clearly the importance of the submucosal layer in the strength of a suture line.

The results of animal experiments were initially judged by observation and simple tests in the same way as clinical studies. More detailed observation in the form of histological examination, which is available only when the outcome is unsatisfactory in human cases, can be made at predetermined times in animals by sacrifice, post mortem examination and removal of the
CHAPTER 1.3 Intestinal Healing - methods

healing tissues. Such methods continue to provide useful information. Hesp et al (1985) and Stein and Barry (1983) have fully described the sequence of histological changes taking place in intestinal anastomoses in rabbits and rats, with the former report examining small and large bowel healing and the latter comparing methods of suturing. Matolo et al (1976) showed that satisfactory healing can take place in the presence of peritonitis while Jansen et al (1981) confirmed the importance of the submucosal layer and investigated the use of a magnetic ring device for apposition of the bowel ends in dogs. Ravitch, a pioneer in the investigation of stapled anastomoses, used observation and histological evidence in his review of intestinal healing (Ravitch et al 1981). His own findings and those of others showed that, histologically, stapled anastomoses healed in a similar way to some sutured sites and could remain secure in the presence of peritonitis, ischaemia or obstruction. Cohen et al (1985) found that the deleterious effects of ischaemia at an anastomosis could be considerably mitigated by the pre-operative administration of antibiotics. Meese and colleagues (1986) used observation, contrast radiology and post-mortem
CHAPTER 1.3 Intestinal Healing - methods

histology to assess the effects of chronic radiation
damage on colonic healing in dogs.

Mechanical methods

The use of animals allows investigation of the
healing anastomosis to be more detailed, even if the
healing tissue is destroyed in the process. Mechanical
methods can be applied to test the strength of the
suture line and were probably first applied to
intestinal anastomoses by Chlumsky in 1899. Using water
to distend the bowel, Chlumsky found that intestinal
suture lines were weaker after three or four days than
when first made and only returned to full strength after
10 to 14 days. Methods of testing the strength of skin
wounds by pulling them apart were developed by Paget in
1853 (Sandblom and Muren 1954) and by Howes et al
(1929), who recognised that the tensile strength across
the stitch line was a reflection of the quality of
healing. Skin wounds showed a similar lag period of
weakness before the tensile strength increased rapidly,
accompanied by a multiplication of fibroblasts within
the wound. Botsford (1941) used a tensiometer which
could test a skin wound without removal from the animal
and a similar device was used by Sandblom and Muren
(1954) to demonstrate that small changes in technique, such as whether a skin wound was repaired on the nearer or further side of an animal from the operator, could influence the healing process.

Although tensiometers could be used to assess intestinal wounds by pulling strips of tissue apart (Howes et al 1929), the equivalent measurement in hollow organs was initially better tested by inflating a segment of the gut with gas or liquid and recording the pressure at which it burst, in the same manner as used in Chlumsky's earlier experiments. This overcame the problems of trying to apply even tension to the circumference at the end of a floppy tubular structure. Nelsen and Anders (1966) discussed the physics of hollow tubes and realised that the tension in the wall could be calculated from measurement of the diameter of the intestine as well as the bursting pressure. They felt that this gave a better assessment of the strength of the wound, although the accuracy of this calculation must be dependent on the method used to record the diameter at the time of bursting.

Experiments to measure the bursting pressure or bursting wall tension have been carried out by many workers to assess intestinal healing. Herrmann et al
(1964) correlated both the bursting pressure and the breaking strength of colonic anastomoses in rats with the histological phases of healing up to a year after operation. Getzen and Holloway (1966), Trueblood and colleagues (1969), Loeb (1967), Hamilton (1967) and Ravitch et al (1981) carried out some of the many comparisons of inverting and evert ing suture techniques and similar experiments for one and two layer anastomoses, different suture materials and the use of staples were reported by Letwin and Williams (1967), Robbs (1977), Graffner et al (1984a and b), Penninckx et al (1984) and Ballantyne et al (1985). Aszodi and Ponsky used bursting pressure to determine the effects of corticosteroids (1984) and 5-fluorouracil (1985) on small bowel healing, while Smith et al assessed the influence of surgical drains (1982) and faecal loading (1983) in rat colon. Daly et al (1972) monitored the consequences of protein depletion and Ward (1985) showed that even minor degrees of malnutrition could impair colonic healing.

A detailed record of the development of strength in wounds of all regions of the rat intestine has been made in a series of studies (Högström and Haglund 1985a, Högström et al 1985b, Jönsson et al 1983, Jiborn et al
CHAPTER I.3 Intestinal Healing - methods

1978b and c); the findings will be discussed in relation to the changes in collagen in Chapter I.3.ii.

Mechanical methods continue to be used in the assessment of intestinal healing and Udén et al (1988) have recently investigated the effects of proximal colostomy on colonic anastomotic breaking strength.

Biochemical measurements

With increasing knowledge of the biochemistry of tissues, it became apparent that the strength of the submucosal layer of the intestine, stressed by Halsted and others, was related to the presence of collagen in this layer. Cronin et al, in 1968, began to measure the collagen concentration in the wall and led the way for many studies of collagen turnover during healing. This body of work is now extensive and will be discussed more fully in the next section (Chapter I.3.ii).

Other methods of investigation

Other methods of investigation have been applied to aspects of intestinal healing, most of which relate to assessment of the blood flow at the site of an anastomosis. Measurements of local blood flow have been made with electromagnetic flow probes (Whitaker 1968),
CHAPTER 1.3 Intestinal Healing - methods

(ii) COLLAGEN DYNAMICS

Since the 1960's, when collagen was recognised as the chief constituent of the important submucosal layer of the intestinal wall, measurements of collagen in animals have been made by many workers to assess the changes which take place in the healing period in a variety of circumstances. Studies were designed to assess the normal sequence of events in uncomplicated healing and to compare the healing when aspects of technique were altered or when complications were induced.

In 1968, Cronin et al (1968a) reported a study of anastomoses performed in the distal colon of rats. Three days after operation the hydroxyproline concentration in the wall of the colon 1.25 and 2.5 cm proximal to the suture line fell by 40 per cent. On the distal side the concentration remained almost unchanged. Over the period from 5 to 10 days after operation, the concentration proximally returned to normal and by 14 days it had reached a level higher than that in unoperated colon.

In a second study (1968b) the same workers investigated collagen synthesis in the colon wall by measuring the uptake of tritiated proline and found
CHAPTER 1.3 Intestinal Healing - collagen

that, at the same 3 day point when hydroxyproline was reduced, the synthesis of collagen was increased 15-fold proximal to the anastomosis and 5-fold distally. By giving the labelled proline earlier in the experiment they also attempted to look at collagen breakdown. They found greater quantities of label in those animals which had undergone anastomosis than in those which had not; they interpreted this as indicating that, during the processes of collagen degradation and synthesis after operation, it is the older, and therefore unlabelled collagen which is degraded first, leaving a relatively greater amount of label in the animals with an anastomosis than in the controls.

A similar decrease in hydroxyproline concentration was found by Hawley (1970) and Hawley et al (1970) after distal colonic anastomosis in rabbits. Cronin et al (1968b) and Hawley (1970) concluded that there was a considerable increase in both collagen synthesis and degradation after colonic anastomosis. At 3 days, degradation was dominant, resulting in a net fall in hydroxyproline concentration. After this time synthesis overtook lysis and the concentration not only returned to normal but 'overshot', to give a temporary level greater than in unoperated colon.

49
CHAPTER 1.3 Intestinal Healing - collagen

These changes in hydroxyproline corresponded with a decrease and subsequent rise in the bursting pressure or bursting wall tension of the anastomosis, indicating that the mechanical strength of the wall was closely linked to its collagen content. Since both bursting pressure and collagen were at their lowest 3 days after operation, Hawley extended his investigations to include a study of the collagenolytic activity of the wall in normal colon and after anastomosis. Using the method of Gross and Lapiere (1962), he measured the lysis of collagen gels when explants of colon wall were incubated for 72 hours.

Hawley's work demonstrated several important facts. In the normal rabbit, explants from the intestine lysed collagen gels. This lysis was greater when explants were taken from the distal colon than from the small intestine or caecum, while gastric wall had almost no activity. These variations in activity with site in the gastrointestinal tract appeared to correlate with the risk of anastomotic dehiscence at various sites in normal clinical practice. The colon has a higher rate of anastomotic breakdown than the small bowel and the distal colon is more susceptible to dehiscence than the proximal (Goligher 1984a).
CHAPTER 1.3 Intestinal Healing - collagen

Hawley investigated collagenolysis in the distal colon after anastomosis. Three days after operation the amount of lysis increased in the region of the suture line, correlating with the fall in collagen. Lytic activity also increased in other parts of the intestine when an anastomosis was made in the distal colon, and even after a laparotomy at which no other procedure was carried out; there seemed to be a generalised response by the intestine to the trauma of any operation but the changes were not as marked as at the site of an anastomosis.

Hawley went on to study the effects of infection on colonic anastomoses. In the presence of infection the region of the suture line showed a greater decrease in bursting wall tension, a greater fall in hydroxyproline concentration and a greater increase in collagenolytic activity than after an uncomplicated operation.

As a result of these experiments, Hawley concluded that collagenase was an important factor in anastomotic healing. He suggested that normal distal colon had more collagenase activity than the rest of gastrointestinal tract and that the trauma of resection and anastomosis stimulated an increase in activity; this led to a net fall in collagen and, consequently, a decrease in the
CHAPTER I.3 Intestinal Healing - collagen

strength of the wall in the early post-operative period. When an anastomosis was associated with infection all these changes were more marked.

Other reports supported Hawley's findings. Gries and Grasedyck (1969) used a synthetic substrate to assess the collagenolytic activity of homogenised tissue. The oesophagus in rabbits had higher activity than the large and small bowel while the stomach again showed almost no collagenolysis. Activity in large bowel was a little higher than small bowel. This work, however, indicated that the enzyme activity originated in the muscle layers of the bowel wall rather than in the mucosa and that a high level was also present in the wall of the aorta.

Further studies of hydroxyproline provided more information about collagen turnover after operation. Irvin and Hunt (1974a) confirmed the fall in collagen concentration at 3 days followed by a return towards normal levels. In a second study using $^3H$-labelled proline (1974c), a comparison of the salt-soluble, acid-soluble and insoluble fractions of collagen again indicated that there was more lysis of the older, more stable collagen than of the newly formed portion. Wise et al (1975) found a fall in hydroxyproline after
colonic anastomosis in dogs which reached a trough after 5 days; by 19 days this had returned to normal levels and it remained so up to 31 days. After ileal anastomosis, no fall occurred but a subsequent rise took the collagen concentration above normal after 19 days. Greenstein and colleagues’ work on dogs in 1978 indicated a 45 per cent decrease in collagen 4 days after colonic anastomosis, which was all from the mature collagen fraction, with the newly-synthesised portion unchanged. If the dogs were given enteral feeding immediately after operation, the loss of mature collagen was abolished and the newly-synthesised fraction showed an increase.

Jiborn and colleagues began a series of experiments in 1978 which carefully assessed the collagen changes in the region of colonic anastomoses in rats (1978a). Once more, the fall in hydroxyproline was most noticeable in the region of the suture line on the fourth day, returning to normal around the tenth day and then ‘overshooting’ to a level above that of unoperated colon at 28 days after operation. These changes were very marked proximal to the anastomosis and up as far as the equivalent of the transverse colon, while on the distal side the changes were more localised to the suture line.
Greater alterations in hydroxyproline occurred when the suture was continuous than when interrupted sutures were used. Continuous sutures were also accompanied by a higher incidence of anastomotic complication, supporting the hypothesis that infection and other problems enhance the increase in collagenase activity and exaggerate the collagen losses. Dilation of the colon caused by obstruction at the stitch line resulted in a 50 per cent reduction in hydroxyproline on the proximal side on day 4, with no change on the distal side.

In a study similar to that by Cronin et al (1968b), Jiborn et al (1980a) measured the uptake of tritiated proline after an inverting, continuous suture, left colonic anastomosis. Uptake, indicating collagen synthesis, increased 8-fold in the region proximal to the suture line on the fourth post-operative day and by a factor of 3.5 distally. Less marked increases were seen in other regions of the colon. One week after operation the uptake of label was still much increased. The overall picture was one of greatly increased collagen turnover for some time after operation, with lysis exceeding synthesis initially, particularly on the proximal side.

Many subsequent studies have measured
CHAPTER 1.3 Intestinal Healing - collagen

hydroxyproline levels in the intestine of various
laboratory animals to study the effects of changes in
anastomotic technique, complications or adjuvant
therapy. The overlap between such experiments is great
and the important conclusions, as they relate to
clinical practice, will be summarised in Chapter
I.3.iii.

The accumulated findings as they relate to
intestinal healing in rats, rabbits and dogs are as
follows:

Collagen changes in the rat intestine after
anastomosis have been studied more extensively than in
any other animal and results are available for the
period from the time of operation up to 28 days later,
with particular emphasis on the first 14 days. The main
findings after colonic anastomosis are as stated
earlier: hydroxyproline concentration in the colon wall
decreases rapidly from the time of operation to a trough
level approximately 40 per cent below normal after 3
days. After this time the level begins to rise,
reaching the normal concentration between 7 and 14 days
after operation and then rising above normal. These
findings are accompanied by an initial fall and
subsequent rise in bursting pressure and bursting wall
CHAPTER 1.3 Intestinal Healing – collagen
tension of the anastomotic segment, suggesting that the
collagen concentration in the wall is related to its
strength. Increased levels of hydroxyproline are still
measureable 21 and 28 days after anastomosis. These
findings have been shown by the work of Irvin (1976,
1978), Irvin and Hunt (1974a, b and c) Jönsson et al
(1985b), Blomquist et al (1984a–d, 1985a and b), Cronin
et al (1968a and b), Brennan et al (1984), Foster et al
(1985), Jiborn et al (1978a, 1980a and b), Högström et
and b), Winsey et al (1987), Stewart (1973), de Roy van
Zuidewijn et al (1987), Kirk and Irvin (1977) and

and Klein (1982a) and Colin et al (1979) investigated
small bowel anastomoses in rats, showing that similar
changes occurred but the fall in collagen concentration
was less marked and started later than in colon. The
changes were also more localised and the difference
between the proximal and distal sides of the suture line
did not occur in small bowel (Jönsson et al 1985a, 1986,
1987). After ileocolic anastomosis the changes in ileal
collagen concentration were closer to the changes in the
colon (Jönsson et al 1985c).
CHAPTER 1.3 Intestinal Healing - collagen

Fewer groups have studied rabbits but the findings in the first 10 days have been demonstrated by Hendricks et al (1985), Hesp et al (1984a and b), Hawley (1970), Hawley et al (1970), Irvin and Edwards (1973) and Shandall et al (1985, 1986). The changes in colon are similar to those in rats but the hydroxyproline level falls more rapidly to reach a trough 38 per cent below normal in the first 48 hours (Hendricks et al 1985, Hesp et al 1984a). Collagen concentration reaches normal after 7 days and rises above it by 14 days. Small bowel anastomoses again show changes which are less marked and which recover more rapidly (Hesp et al 1984a and b, Hendricks et al 1985). Wise et al (1975), Greenstein et al (1978), Templeton and McKelvey (1985) and Yamakawa et al (1971) have shown similar findings in dog colon, with the fall occurring between three and six days.

Impaired healing has been demonstrated in infection (Letwin and Williams 1967, Irvin 1976, Yamakawa et al 1971, Hawley 1970, Hesp et al 1984b), obstruction (Jiborn et al 1978a, 1980b), ischaemia (Kirk and Irvin 1977), malnutrition (Irvin 1978) and uraemia (Colin et al 1979). Radiation (Winsey et al 1987) and chemotherapy (deRoy van Zuidewijn et al 1987) are also detrimental. Resting the colon, either by low residue
CHAPTER 1.3 Intestinal Healing - collagen

diet (Blomquist et al 1984a-d) or by defunctioning colostomy (Blomquist et al 1985a and b, Udén et al 1988), also reduces the collagen concentration but not the breaking strength in intact bowel. Both measurements are reduced during the healing of defunctioned anastomoses.
(iii) FACTORS IN ANASTOMOTIC DEHISCENCE

A great many investigators, using combinations of the methods previously discussed (Chapter I.3.i), have examined the events which occur during the healing of intestinal anastomoses and identified several factors which are important in relation to suture line dehiscence.

Anatomical site

In modern surgical practice the risks of suture line break down are greater after large bowel anastomosis than in the small bowel (Hesp et al 1986). Goligher et al (1970a), Schrock et al (1973), Schrock and Christensen (1972) and Fielding et al (1980) have shown that distal colonic suture lines are more prone to dehiscence than proximal ones and low anterior resection of the rectum carries a much higher rate of leakage than operation above the peritoneal reflection. This has been related to the lack of serosal covering, since oesophageal anastomoses are also subject to a high risk of leakage. In the oesophagus, however, the consequences of failure are even more severe since the reported mortality ranges from 54 to 100 per cent (Foster and Leaper 1984).
CHAPTER 1.3 Intestinal Healing - factors

Bowel preparation

Smith et al (1983) correlated faecal loading in the colon of rats with impaired healing, confirming the findings of Irvin and Goligher (1973), Rosenberg et al (1971) and Barker et al (1971). A regime of oral sulphonamides and non-absorbed antibiotics was the first combination used to prepare the lumen of the bowel before surgery and produced a considerable improvement in the results of primary colonic anastomosis (Dunphy 1971). The antibiotics, however, introduced risks of post-operative enterocolitis because of alteration in the bacterial flora and the production of resistant organisms or overgrowth with yeasts (Muir 1968, Dunphy 1971, Keighley et al 1979, Goligher 1984d, Bucknall 1984). In the 1960's sulphonamides were combined with various laxatives and enemata to cleanse the bowel. Since complete sterilisation of the lumen cannot be achieved and mechanical clearance of the faecal contents effectively reduces the bacterial count, Tyson and Spaulding (1959) recommended cleansing methods of preparation only. In the 1970's elemental diets which produce minimal faecal residue were tried without much benefit (Goligher 1984d, Hares and Alexander-Williams 1982).
CHAPTER 1.3 Intestinal Healing - factors

Bowel preparation is currently directed at clearing as much solid and liquid material as possible. The combination of several days of laxatives and enemata has largely been replaced by other mechanical means such as total gut irrigation, introduced in a pilot study by Hewitt et al (1973) and in a trial involving 81 patients by Crapp et al (1975). Alternatives include osmotic laxatives such as mannitol and strong oral purgatives like sodium picosulphate with magnesium citrate (Picolax) or liquid senna (X-Prep). Systemic antibiotic regimes are usually included in the peri-operative period, with some evidence that they may improve anastomotic healing (LeVeen et al 1976, Cohen et al 1985); they certainly play an important role in the prevention of infection in the wound of the abdominal wall (Bucknall 1984, Hares and Alexander-Williams 1982, Keighley et al 1979). A recent study (Irving and Scrimgeour 1987) suggests that bowel preparation can be dispensed with altogether if systemic antibiotic prophylaxis is used. Despite the impressive results, this conclusion can be questioned (Johnston 1987) and more convincing evidence is necessary before this approach is advocated for general use.
Intestinal obstruction

Since faecal loading of the bowel affects anastomotic healing (Smith et al 1983), it is not surprising that suture lines break down more often when intestinal obstruction is present at the time of operation. Anderson and Lee (1981) found a 30 per cent incidence of faecal fistula formation when primary anastomosis was performed for sigmoid volvulus and Phillips et al (1985) reported a leak rate of 18 per cent when an anastomosis was created in the initial treatment of malignant large bowel obstruction. Obstruction alters the bacterial flora in the colon and reduces the blood supply to the wall, which delays angiogenesis and inhibits normal healing (Foster and Leaper 1984). In addition, the general condition of the patient is less favourable.

The risks can be avoided by postponing anastomosis, as with a preliminary Hartmann's procedure, or by defunctioning the suture line with a temporary proximal colostomy. The actual incidence of dehiscence is not reduced by colostomy, and there is experimental evidence for impairment in healing in the defunctioned bowel (Blomquist et al 1985a and b, Udén et al 1988), but the consequences are usually considerably mitigated and the
frequency of septic complications is lower (Goligher et al 1970a, Muir 1968, Foster and Leaper 1984). Colostomy closure itself, in experienced hands, carries a very small risk of faecal fistula formation (Thomson and Hawley 1972, Irvin 1987, Livingston et al 1989) but the study of the results of colostomy closure confirms several aspects of colonic healing in general. The routine use of prophylactic antibiotics has considerably reduced the overall complication rates, mainly because of the decreased incidence of wound infections (Foster et al 1985b, Irvin 1987, Demetriades et al 1988, Kyle and Isbister 1989). Younger patients suffer fewer complications and the experience of the surgeon is a major factor in reducing the risks (Irvin 1987, Demetriades et al 1988, Livingston et al 1989). Muir (1968) and, later, Dudley and colleagues (1980) described methods of colonic irrigation which can be used to empty an obstructed colon at the time of operation; these are gaining popularity, since temporary colostomy can then be avoided in many cases (Thomson and Carter 1986).
CHAPTER 1.3 Intestinal Healing - factors

Sepsis and peritonitis

The presence of sepsis at the time of colonic surgery considerably increases the mortality. Debas and Thomson (1972) found that 6 out of 10 deaths after emergency right hemicolec tomoy were due to established peritonitis and Goligher and Smiddy (1957) found a 74 per cent mortality in colonic carcinoma presenting with faecal peritonitis. Perforating injuries of the colon have been associated with a 15 per cent mortality and 58 per cent complication rate, even if they are not closed in the primary treatment (Schrock and Christensen 1972), although recent reports show an improvement in these figures (Shannon and Moore 1985). Distant sepsis also has an inhibitory effect on healing (Bucknall 1984).

As a complication of colonic anastomosis, it may be difficult to determine whether infection is the cause or the effect of dehiscence (Foster and Leaper 1984, Goligher 1984f), although bacteriological evidence suggests that suture-line leakage precedes the peri-anastomotic infection (Gallagher et al 1982). Histologically, infected skin wounds contain fewer fibroblasts and the collagen is disorganised. A decrease in collagen content extends for some distance from the wound and the softened, weakened tissue has
CHAPTER 1.3 Intestinal Healing - factors

reduced suture-holding capacity (Foster and Leaper 1984). In infected intestinal anastomoses, disturbed collagen synthesis and decreased collagen content have been demonstrated (Irvin and Hunt 1974b, Irvin 1976, Hawley et al 1970, Yamakawa et al 1971, Hesp et al 1984b) and Hawley's measurements of collagenolytic activity showed a marked increase when anastomoses were deliberately infected in rabbits (1970).

Forrest (1983) relates the increased risk to the fact that fibronectin, formed in the very earliest phases of healing, is susceptible to the action of proteases released by polymorphonuclear leucocytes. In the presence of infection, with its greater inflammatory reaction, the number of polymorphs increases and more protease activity probably occurs. Hunt (1984) feels that the balance between the synthesis of collagen by fibroblasts and the collagenolytic activity of the enzymes released by inflammatory cells is more important and this appears to be the majority view, since a great deal of investigation in intestinal healing has been directed at the imbalance in the collagen equation.
CHAPTER 1.3 Intestinal Healing - factors

Primary pathology

Since conditions such as infection, which increase the inflammatory reaction at an anastomosis, carry greater risks of dehiscence, it might be expected that operations for inflammatory conditions of the bowel also carry an increased risk. This is confirmed by the 3 per cent operative mortality in diverticular disease (Goligher 1984g). Resections for Crohn's disease, even in the small bowel, carry a 4.1 per cent mortality for a first procedure and 8.3 per cent for later ones, while large bowel anastomoses show 2 per cent mortality and 6 per cent clinically significant leakage (Goligher 1984h). Hesp et al (1986) have also shown a higher incidence of small bowel anastomotic leakage in inflammatory bowel disease.

Irvin and Goligher (1973) showed that there is a higher incidence of anastomotic dehiscence in the rectum when surgery is carried out for palliation of a malignant tumour or for removal of a Dukes' C lesion but they suggested that this might be due to the increased operative trauma involved in removing a larger or more fixed tumour. Stewart (1973) also suggested that the implantation of malignant cells at the time of anastomosis might impair healing.
CHAPTER 1.3 Intestinal Healing - factors

One particular factor which may be associated with surgery for malignant disease is pre-operative irradiation therapy. Irradiated tissue shows a poorer response to wounding because of the induction of obliterative endarteritis and a deficiency in stem cells along the small vessels (Bucknall 1984, Carrico et al 1984, Winsey et al 1987). This microangiopathy increases the risk of anastomotic dehiscence three-fold (Schrock et al 1973, Ormiston 1985), whether the tissue was irradiated for the current disease or for an unrelated lesion in the past.

Aspects of operative technique

There has been much controversy in the past about the ideal means of joining two bowel ends and many workers have compared inverting and everting techniques, single and two layer anastomoses, interrupted and continuous sutures, different suture materials and various mechanical devices to avoid hand suturing (Ballantyne 1984). No single technique is ideal for all situations and different reports concerning the advantages of any one variation are often contradictory. It should be remembered that the production of a leak-proof suture line is more important in the bowel
CHAPTER 1.3 Intestinal Healing - factors

than the provision of great tensile strength (Foster and Leaper 1984).

Travers (1812) and Lembert (Goligher 1984f) both described suture techniques which produced inversion of the bowel ends and Gambee's end-to-end type of suture (1951) still encouraged serosa-to-serosa apposition. Although this principle was questioned by Smith in 1895, it was generally followed until Getzen and Holloway (1966) published evidence that eversion produced sounder healing. After several reports in favour of each option, Goligher et al (1970b) ended the arguments by showing that the increased incidence of faecal fistula with eversion was sufficiently great to terminate their clinical study prematurely. Although eversion has since been shown to be satisfactory when stapling devices are used (Ravitch et al 1981), the development of circular staplers has resulted in a general return to inverted anastomoses (Goligher 1984f).

Comparisons of one and two layer anastomoses generally show little difference (Goligher 1984f, Irvin et al 1973) although Everett (1975) produced evidence that one layer of sutures is better for low rectal anastomoses. Goligher et al (1977) found a slight advantage when two layers were used and this aspect is
probably a matter of individual experience. The choice of suture materials is wide, and increasing, and this also is a question of personal preference since other details of surgical technique are generally more important (Khoury and Waxman 1983, Capperauld and Bucknall 1984). Polyglycolic acid and polyglactin may, however, have advantages over catgut for the inner layer of a colonic anastomosis (Clark et al 1972, 1977, Deveney and Way 1977). Stainless steel wire produces less inflammatory reaction than other non-absorbable materials but was previously unpopular because it is difficult to handle; stapling devices now allow it to be used extensively (Goligher 1984f, Heald and Leicester 1981, Ravitch et al 1981, Khoury and Waxman 1983, Graffner et al 1984a and b, Everett et al 1986).

Particular attention should be paid to ensuring an adequate blood supply to the cut ends of the bowel and to avoiding tension on the suture line. Sufficient local oxygenation is essential for collagen synthesis (Hunt and Pai 1972, Niinikoski et al 1972) and hypoxia retards angiogenesis and epithelialisation in skin (Foster and Leaper 1984), although increasing the inspired oxygen tension does not improve colonic healing or reduce the dangers of ischaemic anastomoses (Kirk and
CHAPTER 1.3 Intestinal Healing - factors

Irvin 1977). Sheridan et al (1987) have shown that lower oxygen tension at the site of an anastomosis correlates with clinical leakage. Intra-operative blood transfusion is related to anastomotic leakage (Whitaker et al 1970, Schrock et al 1973) but should not be avoided because a 10 per cent fall in circulating blood volume reduces colonic perfusion by 30 per cent (Whitaker et al 1970, Gilmour et al 1980) and decreases the collagen concentration in the wall (Foster et al 1985); it is the need for transfusion which increases the risk.

Smith et al (1982) showed that the presence of latex drains interfered with anastomotic healing. The use of any drain introduces a foreign body which will increase the inflammatory reaction and, although a drain may be expected to guide the passage of any leakage along a chosen path, it may also increase the risk of such leakage.

Other aspects of technique are less the subject of dispute or experiment but belong to the realm of common sense. The care taken by the operator is clearly important and the level of experience of the surgeon has been discussed by Fielding et al (1978). Gentle handling and careful haemostasis will reduce the amount...
CHAPTER 1.3 Intestinal Healing - factors

of devitalised tissue in the wound while an extensive or
difficult dissection will understandably increase the
risk of operative complications. As discussed above,
the formation of a defunctioning colostomy does not
prevent anastomotic dehiscence but usually reduces the
dangers and the final decision on this point must be
made by assessment of the precise situation in the
particular patient at the time of operation.

Patient status

The general and nutritional status of the patient
can strongly influence the healing of an intestinal
anastomosis. Evidence has been collected to show that
anaemia (Foster and Leaper 1984, Schrock et al 1973) and
protein depletion (Daly et al 1972) both increase the
risk of dehiscence, while Irvin and Goligher (1973)
found an association between dehiscence and reduced
pre-operative levels of serum protein and albumen. Even
subclinical malnutrition may prejudice healing (Ward et
al 1982, Ward 1985) and the presence of an intestinal
fistula may be an important source of nutritional
losses. Specific deficiencies may also be important;
vitamin C deficiency may be associated with poor healing
(Dunphy and Udupa 1955, Zederfeldt 1980, Irvin 1981,
CHAPTER 1.3 Intestinal Healing - factors

Pinnell 1983) and zinc has been shown to be relevant to
wound repair (Zederfeldt 1980, Khoury and Waxman 1983). Jaundice (Bucknall 1984) and uraemia (Colin et al 1979)
are both associated with defective collagen synthesis
and the presence of malignant disease in the patient, whether the reason for the operation or unrelated, may
impair the healing process (Irvin et al 1978, Schrock et
al 1973, Stewart 1973). Increasing age may be
detrimental in itself (Schrock et al 1973, Irvin and
Goligher 1973, Zederfeldt 1980) or may represent the
increased incidence of other factors with advancing age
(Khoury and Waxman 1983, Bucknall 1984). Diabetes
mellitus is associated with vascular changes, a higher
risk of septic complications and poor wound healing
(Bucknall 1984, Carrico et al 1984). Treatment with
certain drugs, such as corticosteroids, cytotoxics and
anti-metabolites, may similarly interfere with healing
and predispose to infection (Carrico et al 1984, deRoy
van Zuidewijn et al 1987, Aszodi and Ponsky 1984, 1985,
Ehrlich et al 1973). Unrelated trauma can also
prejudice healing in an intestinal anastomosis (Irvin
and Hunt 1974a and b, Schrock and Christensen 1972,
Zederfeldt 1980). In most patients it is probably the
combination of several factors working together that
CHAPTER I.3 Intestinal Healing - factors

determines the overall risk of anastomotic leakage.

Collagen turnover

The importance of collagen as the major component of the submucosal layer of the intestinal wall has already been stressed (Chapter I.3.ii). The turnover of collagen in the wall after anastomosis has been considered a reflection of the increase in strength at the suture line and the overall progress of the healing process. Factors which were associated with a lower collagen concentration in the wall, such as infection, were also associated with increased dehiscence rates and Hawley's work (1970) indicated that reductions in collagen are due to increased collagenolysis, overriding the increased synthesis which also takes place. Although greater collagenolytic activity was present in normal colon than in other parts of the intestine, it is not clear whether an increase in collagenolysis is itself the cause of dehiscence in some situations or whether this increase is the mechanism by which other factors impair intestinal healing.

Recognition of the importance of collagenolysis in colonic healing has led to the use of agents to inhibit collagenase activity during the period when an
CHAPTER 1.3 Intestinal Healing - factors

anastomosis is vulnerable. No direct inhibitor of collagenase is currently available for therapeutic use, although the natural inhibitor, tissue inhibitor of metalloproteinases (TIMP), has been purified and may be an appropriate agent (Reynolds 1986, Kerwar et al 1986). Collagenase can be inhibited indirectly by blocking the action of activators such as plasmin and trypsin.

Collagenolysis, demonstrated by the method of Gross and Lapiere (1962), has been inhibited in rabbit colon by administration of aprotinin, soy bean trypsin inhibitor (SBTI) and lima bean trypsin inhibitor (LBTI) (Jayaraj et al 1983a, Lewin et al 1986). Studies of anastomotic healing have been reported in which aprotinin improved the post-operative bursting pressure in rabbits (Delaney and Lalor 1976) and rats (vonBary et al 1976). The breaking strength (Young and Wheeler 1982) and collagen concentration, as measured by a Sirius Red dye technique, were also increased in rabbits (Young and Wheeler 1983). Högström et al (1985d) found aprotinin had no effect on anastomotic breaking strength in rats but some benefit seemed to be gained with S-2411, a synthetic kinin-kallikrein inhibitor. Breaking strength 24 hours after operation was also improved by tranexamic acid, an inhibitor of plasminogen activator, and by
CHAPTER 1.3 Intestinal Healing - factors

Tiopronin, another metalloproteinase inhibitor (Högström and Haglund 1985b). SBTI diminished the fall in breaking strength for a longer period than tiopronin (Högström et al 1985c).

Despite these advantages shown in animals, a clinical trial of aprotinin in colonic anastomotic healing was inconclusive (Young and Wheeler 1984). This prospective, randomised, double-blind trial included patients undergoing right hemicolecction with ileocolic anastomosis as well as left hemicolecction and anterior resection and these options were not equally distributed between the control and treatment groups. The use of staples for low anterior resection introduced a further variable and both benign and malignant conditions were included in the study, so it is not surprising that the results did not demonstrate any significant difference in the first 100 patients.

All these experiments on healing, both animal and human, have assumed that any benefit from the agents is achieved by inhibition of collagenase or collagenolysis but no measurements of the enzyme have been available for this to be properly assessed. Collagenolytic activity has been demonstrated in human colon by Sturzaker and Hawley (1962), Riley and Peacock (1967)
and Jayaraj et al (1983b), using the method of Gross and Lapiere (1962). Increased knowledge of the biochemistry of collagen degradation has shown that it is necessary to distinguish the specific enzyme, collagenase, from other non-specific proteolytic enzymes. Since collagenase activity appears to be the key to extracellular collagen breakdown (Murphy and Reynolds 1985), the present study has attempted to establish the appropriate methodology to measure this enzyme specifically and to reassess the role of collagenase in colonic anastomotic healing.

Before considering how this was carried out, it is necessary to review the current state of knowledge about collagen and collagenase.
COLLAGEN

Collagen is the most abundant protein in the animal kingdom. The term covers a family of related proteins with characteristic chemical and morphological structures which together constitute approximately 25 per cent of the dry body weight in mammals (Montes and Junqueira 1982). Collagen is the major component of extracellular connective tissue matrix and is found primarily in the form of insoluble fibres, in association with proteoglycans, glycoproteins and elastin. It is one of the few constituents of higher organisms which has appreciable tensile strength (Irvin 1981) and it is therefore responsible for the varying mechanical properties and structural natures of different tissues. Thus, it forms 72 per cent of the dry weight of skin, with its protective flexibility, and 86 per cent of Achilles tendon, where tensile strength is paramount (Uitto et al 1986). It forms 4 per cent of liver, providing the structural framework to hold the specialised cells together as an organ and to keep the different elements in the correct relationship to each other. It is mineralised to produce a rigid yet light skeleton in bone, while in the intestine, where it forms
CHAPTER 1.4 Collagen

15 per cent of the dry weight (Gottrup 1981), and in blood vessels, collagen forms a flexible supporting sleeve which allows expansion of the lumen and movement of the contents without loss of integrity or elasticity in the wall (Jackson 1980).

Molecular structure

The collagen molecule comprises three long polypeptide alpha chains wound together into a characteristic triple helix. The helical nature of the molecule is possible because of the presence of glycine at every third residue in the chains, since only the small side group of glycine will fit into the centre of the helix. The α chains have long sections with the repeating sequence Gly-X-Y, where X and Y are frequently proline and hydroxyproline and these latter two imino acids account for about 20 per cent of the total amino acids in collagen. Hydroxyproline and hydroxylysine, both found almost exclusively in collagen, participate in intra- and intermolecular cross-links, stabilising the helix and fusing the molecules into fibrils (Uitto et al 1986, Burgeson 1982, Pinnell 1983, Forrest 1983). The length of the helical section determines the nature of fibril formation in the various collagen types;
combinations of different types of collagen perform a wide range of functions in a variety of tissues and sites. Fibrils collect into fibres and bundles of parallel fibres form the rope-like collagen of tendon, known for its tensile strength. In cornea, the fibres are orientated at right angles to allow transparency and, in skin, a more random pattern gives flexibility (Pinnell 1983). In intestinal wall the larger fibres are orientated in a diagonal matrix, at +30° and -30° to the longitudinal axis, to provide an expandible elastic sleeve with an enmeshing network of smaller, randomly orientated fibres to maintain structural continuity (Orberg et al 1982).

Collagen types

At least twelve types of collagen, identified by Roman numerals, have been purified and described. They are found in different proportions in different tissues, have different functional properties and are composed of more than 25 different collagenous polypeptides. An individual alpha chain is identified by an Arabic number followed by the Roman numeral of the collagen type from which it comes and a collagen molecule may be composed of three identical chains, as in type II: \([\alpha 1(II)]_3\), or
CHAPTER 1.4 Collagen

A combination of different chains, as in type I: [α1(I)]2 α2(I) (Uitto et al 1986, Jackson 1980). A summary of the structure and function of the first four collagen types and their distribution is provided in Table 1.4.1.

The interstitial collagens, types I, II and III, are the major connective tissue collagens. They have a basic molecular weight of 300 000 (Uitto et al 1986), with each polypeptide chain being just over 1000 amino acids long (Murphy and Reynolds 1985); the triple helices aggregate to form fibrils and bundles of fibrils make up the collagen fibres. Intermolecular cross-linking produces precise overlapping with a regular stagger, one quarter the length of the molecule, which is responsible for the 67 nm banded appearance of these types of collagen under the electron microscope (Uitto et al 1986, Jackson 1980). Type I, made by fibroblasts, osteoblasts and odontoblasts, is the most common, found particularly in skin, bone, dentine and tendon (Montes and Junqueira 1982) and is also a major component of intestinal wall, chiefly in the submucosa (Orberg et al 1982, Fogdestam and Gottrup 1980). Type II, synthesised by chondroblasts, is the major collagen in cartilage with its fibrils embedded in abundant
## CHAPTER 1.4 Collagen

### Table 1.4.1

<table>
<thead>
<tr>
<th>Collagen type</th>
<th>Formula</th>
<th>Function</th>
<th>Tissue sites</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>$[\alpha_1(I)]_2\alpha_2(I)$</td>
<td>Resistance to tension</td>
<td>Dermis, bone, tendon, dentine, fascia, organ capsule, fibrous cartilage, sclera, intestine</td>
</tr>
<tr>
<td></td>
<td>$[\alpha_1(I)]_3$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>$[\alpha_1(II)]_3$</td>
<td>Resistance to pressure</td>
<td>Hyaline and elastic cartilage</td>
</tr>
<tr>
<td>III</td>
<td>$[\alpha_1(III)]_3$</td>
<td>Structural maintenance in expansible organs</td>
<td>Smooth muscle, nerve sheath, artery, lung, uterus, intestine, spleen, liver, kidney</td>
</tr>
<tr>
<td>IV</td>
<td>$[\alpha_1(IV)]_3$</td>
<td>Support and filtration</td>
<td>Epithelial and endothelial basement membranes</td>
</tr>
</tbody>
</table>
CHAPTER 1.4 Collagen

ground substance (Montes and Junqueira 1982). Type III is usually associated with type I and in many organs is related to the smooth muscle component, contributing to the mobility of the scaffolding (Montes and Junqueira 1982). In intestine it is present in the muscle layers and lamina propria and forms 20 per cent of the collagen content (Epstein and Munderloh 1975, Orberg et al 1982). Type III collagen is the first to be synthesised after injury (Zanaboni et al 1987, Page et al 1986) and, with fibronectin, forms part of the fibres recognised histologically as reticulin (Forrest 1983).

Type IV collagen is made by epithelial and endothelial cells and found in basement membranes. The helical region is much interrupted, so fibril formation does not take place and the molecule is susceptible to non-specific proteolytic enzymes, unlike the other main types (Uitto et al 1986, Burgeson 1982). The molecular structure also relates to its filtering capacity, studied extensively in glomerular basement membrane (Montes and Junqueira 1982). Type V collagen is intermediate in structure between the interstitial collagens and other types and is widely distributed, except in cartilage (Uitto et al 1986). Intimal collagen, type VI, has a high cysteine content and large
globular regions at the ends of a short helix. It is found in aortic intima, placenta, skin and kidney and in muscle. Type VII occurs in anchoring fibrils and type VIII in endothelium (Uitto et al 1986). IX, X, XI and XII are all minor components of cartilage (Martin et al 1985, Grant et al 1987, Olsen 1987).

Collagen types I, II and III, being the most abundant, have been the most widely studied and will be the types usually referred to in this work. Type IV is also widely distributed and has some major differences from the interstitial forms which will be discussed where relevant. Types I and III are the main collagen components of intestinal wall and therefore form the basis for this investigation.

Interaction with ground substance

Connective tissue is composed of collagen and elastin in an amorphous matrix called ground substance. This complex medium contains water, electrolytes, proteoglycans and glycoproteins in a heterogeneous combination (Jackson 1980, Irvin 1981). Proteoglycans, formerly known as mucopolysaccharides, contain glycosaminoglycan chains attached to a protein core (Hardingham 1987) and are largely responsible for the
physical characteristics of the ground substance, which can vary from a thin gel to a firm, elastic structure. Proteoglycans influence the formation and maturation of collagen fibrils (Irvin 1984) and their interaction with different collagen types determines the nature of the connective tissue in any one site (Jackson 1980). Interference in the reaction of proteoglycans with collagen can significantly alter the nature of a tissue as was shown in a classical experiment by Thomas (1956); papain injected into a rabbit ear hydrolyses the proteoglycans and decreases the chondroitin sulphate with consequent collapse of the ear. The nature of the ground substance and its ratio to collagen in connective tissue produce a rope-like material with resistance to distraction in tendon, a shock-absorber with resistance to compression in cartilage, a rigid supporting skeleton in bone and an expansile enveloping sheath for vessel walls and intestine (Jackson 1980).

Elastin

Connective tissue contains another fibrous protein, elastin, which is also synthesised by fibroblasts and has cross-links similar to collagen, but which develops its particular properties when swollen with water (Irvin
It is found where extensibility is important, in ligaments, arterial and intestinal walls and skin. Elastin is gradually lost with advancing age and little replacement occurs during wound repair, explaining the relative inflexibility of scar tissue (Irvin 1981, Forrest 1983).

**Fibronectin**

Fibronectin is a glycoprotein found in both plasma and extracellular matrix. The molecule has binding sites for other matrix elements, including collagen and fibrin, and also for cell surfaces; it is thought to act as a tissue adhesive, mediating the attachment of cells to the extracellular components of connective tissue and regulating cellular migration across surfaces (Forrest 1983, Yamada 1983). The binding site for fibronectin in the collagen molecule is in the only region susceptible to the action of vertebrate collagenase, which suggests that fibronectin also has a protective function against collagen degradation (Yamada 1983, Woolley and Evanson 1980).
Synthesis of collagen

Collagen synthesis can be considered as two phases. A series of intracellular steps lead to the synthesis and assembly of procollagen molecules; procollagen is transformed extracellularly to tropocollagen and incorporated into a stable cross-linked fibrillar structure (Montes and Junqueira 1982, Laurent 1987).

Collagen genes contain coding sequences (exons) separated by intervening sequences (introns) which are excised during processing in the nucleus before the transcription of messenger ribonucleic acid (mRNA) (Laurent 1987). Translation follows the usual steps for protein synthesis but takes an unusually long time, which may be related to the abnormal demand for prolyl and glycyl tRNAs or to the unfolding of the secondary structure of the procollagen mRNA (Pinnell 1983).

The preprocollagen polypeptide is composed of a central helical portion, two non-helical terminal regions and an initial signal sequence. In the rough endoplasmic reticulum proline hydroxylation and lysine hydroxylation take place and in the Golgi apparatus glycosylation and other enzymic steps. The signal sequence is removed at the cell membrane as the last step before secretion. Three \( \alpha \) chains twist around each
other to form the procollagen molecule (Montes and Junqueira 1982, Laurent 1987).

Procollagen is then secreted from the cell. Large parts of the globular terminal regions are removed by procollagen peptidases, leaving the long triple helix region with short non-helical ends characteristic of the collagen molecule, known at this stage as tropocollagen. Spontaneous self-assembly into fibrils occurs due to the formation of a series of covalent bonds, creating the cross-links which are responsible for the stability and strength of collagen (Montes and Junqueira 1982, Laurent 1987). Interacting with ground substance, the fibrils collect into the fibres and bundles of fibres characteristic of mature collagen.

The number of post-transcription steps and post-translational modifications involved in collagen synthesis allows a great variety of errors to occur, resulting in a considerable number of connective tissue disorders (Montes and Junqueira 1982). These range from osteogenesis imperfecta to some types of Ehlers-Danlos syndrome and keloid scar formation to achondrogenesis and scleroderma (Uitto et al 1986). Studies of defective collagen synthesis have contributed greatly to the understanding of normal collagen biochemistry.
Regulation of collagen synthesis

The multiple steps of collagen synthesis provide many stages at which regulation of the process can take place. Increased synthesis is required for growth and in response to injury but overproduction can be damaging. Culture conditions can alter the type of collagen secreted by the same cells: gingival fibroblasts increase the ratio of type III to type I at high cell density and a hyperoxic environment increases type III production in lung (Pinnell 1983).

Hormone involvement in regulation has been shown. Parathormone reduced collagen synthesis by 50 per cent in foetal rat calvaria and reduction was also seen with growth hormone, progesterone, corticosteroids and prostaglandins. Somatomedins increase production. Certain factors are essential for the enzymic stages of transformation, including zinc, calcium, ferrous ions and ascorbic acid; Vitamin C deficiency produces scurvy in which there is decreased production of procollagen and defective wound healing (Pinnell 1983). This is not a common problem in current practice, although relative deficiencies have been shown to develop in patients requiring large blood transfusions (McGinn and Hamilton 1976).
Collagen degradation

The multiple cross-linking within and between collagen molecules produces a very stable structure which is highly resistant to degradation (Sellers and Murphy 1981). A continuous slow process of turnover does take place, with a half-life between 50 and 300 days (Murphy and Reynolds 1985), but this is necessarily strictly controlled to avoid any deterioration in the function of the tissue as it is renewed. Collagen turnover is significantly increased in special circumstances, such as growth, involution of the post-partum uterus and wound healing, and excessive degradation occurs in some diseases, particularly arthritis, so that study of these conditions has provided much of the information about mechanisms of collagen breakdown (Sellers and Murphy 1981).

The first stage in breakdown of insoluble collagen fibres may be depolymerisation by an extracellular proteolytic process to loosen up the cross-linked matrix (Harris and Krane 1974b, Sellers and Murphy 1981, Pinnell 1983, Murphy and Reynolds 1985). Three possible pathways for further degradation exist, two extracellular and one intracellular.

Degradation of connective tissue during normal
turnover is probably dependent on the action of neutral metalloproteinases secreted by connective tissue cells. One of these is a specific collagenase which can cleave the three interstitial collagens, types I, II, and III, and this is believed to be a rate-limiting step since the individual intact molecules are resistant to non-specific proteolytic enzymes (Murphy and Reynolds 1985, Woolley and Evanson 1980, Peacock 1980, Harper 1980). Type I collagen in bone must be demineralised before becoming susceptible to collagenase (Harris and Krane 1974b, Krane 1982). A second enzyme, stromelysin, also known as proteoglycanase, acts on proteoglycans but can also degrade type IV basement membrane collagen, while a third, gelatinase, attacks denatured collagen (Sellers et al 1978, Werb and Gordon 1975, Sellers and Murphy 1981, Murphy and Reynolds 1985).

Intracellular degradation of collagen may be the means by which defective molecules are destroyed before they are secreted (Bienkowski 1984). Phagocytosis of damaged fibrils and fragments produced by the extracellular pathways is also known to occur. Once taken up, the fragments can be digested by lysosomal cathepsins which act at acid pH (Sellers and Murphy 1981, Harris and Krane 1974b, Murphy and Reynolds 1985).
CHAPTER 1.4 Collagen

The second extracellular pathway of collagen breakdown also involves enzymes which work at an acid pH. Serine and cysteine proteases, secreted by polymorphonuclear leucocytes and other inflammatory cells, may be able to degrade collagen directly in circumstances in which the pericellular environment becomes acidic. Elastase, a serine proteinase, can attack the non-helical terminal regions and interfere with intermolecular cross-links to produce free $\alpha$ chains which could be taken up by phagocytes. Cathepsins B, L and N are cysteine proteinases which may also act on the cross-links in the right conditions (Sellers and Murphy 1981, Murphy and Reynolds 1985). However, collagenase is generally felt to be the key enzyme in collagen degradation under normal circumstances and this enzyme will be fully discussed in Chapter I.5.
CHAPTER 1.5 Collagenase

COLLAGENASE

Knowledge about the nature and function of collagenases has increased steadily since collagenolytic enzymes were first demonstrated in tadpole tissues by Gross and Lapiere (1962). Much of the information has been gathered in the study of abnormal collagen degradation, such as occurs in rheumatoid arthritis and epidermolysis bullosa (Harris and Krane 1974c), and some studies have been carried out because of the significance of connective tissue breakdown in tumour invasion (Liotta et al 1982). Collagenase activity is relevant to aspects of rheumatology, oncology, dermatology, ophthalmology, dentistry and respiratory medicine, as well as being a subject for basic biochemical investigation. A bibliography which was complete only part-way into 1978, produced by Woessner (1980b), stretched to 33 pages of references and a great deal more evidence has accumulated since then!

It is not surprising, therefore, that the work of Hawley and others (1970), which implicated collagenase in colonic anastomotic dehiscence in the late 1960's, should need to be reconsidered as knowledge advances. While this work measured collagenolysis by the means
available at the time, no specific assay has since been applied to healing colonic tissue although changes in collagen levels have been interpreted in terms of collagenase activity. Perhaps more important, several workers have administered agents to inhibit the enzyme (Delaney and Lalor 1976, vonBary et al 1976, Young and Wheeler 1982, 1983, 1984, Högström et al 1985c and d, Högström and Haglund 1985b) without reference to the precise regulation of collagenase at various stages in its action and to the presence of naturally-occurring inhibitors.

Mode of action of collagenase

Specific collagenases have been identified in a wide range of tissues and animals but, in general, these enzymes have a number of properties in common. They are neutral metalloproteinases: proteolytic enzymes which work best at neutral pH and are dependent on a metal ion for their action. Collagenase is not stored ready for use; the enzyme is synthesised on demand and secreted as required, in a latent form. Activation is brought about in the extracellular space by another agent before collagenase can attack native collagen at a single specific site. The active form has a molecular weight
around 45 000, binds tightly to its substrate and can be inhibited by chelating agents, such as ethylene diamine tetraacetic acid (EDTA), but not by inhibitors of serine or sulphydryl proteases (Gross et al 1980, Peacock 1980, Harper 1980, Murphy and Reynolds 1985, Vaes 1972, Sellers and Murphy 1981, Kerwar et al 1986).

Mammalian collagenase has a very precise action on intact collagen: it cleaves the triple helix of type I collagen at a Gly-Ile bond in the α1(I) chains and a Gly-Leu bond in the α2(I) chain. The cleavage site lies in a region of low helix stability three quarters of the way from the amino to the carboxy terminus, between residues 775 and 776, and the same bonds elsewhere in the molecule are not affected (Murphy and Reynolds 1985, Harper 1980, Kerwar et al 1986). This region is also the binding site for fibronectin, which suggests that the cleavage site is normally protected 'in vivo' (Woolley and Evanson 1980, Harper 1980). The enzyme divides tropocollagen into a long fragment, known as TC^*, and a short TCB (Gross et al 1980, Peacock 1980, Harris and Krane 1974a, Eisen et al 1970). This action can be used to define a true collagenase; other enzymes which can attack intact collagen are termed collagenolytic (Woolley and Evanson 1980, Gross et al
CHAPTER 1.5 Collagenase

1980, Sellers and Murphy 1981). This is also quite distinct from the action of bacterial collagenase, an enzyme released by Clostridium histolytica and some other proteolytic bacteria, which degrades collagen directly into small peptides (Harris and Krane 1974a, Eisen et al 1970, Peacock 1980).

The three-quarter and one-quarter fragments of collagen which are produced by the action of collagenase denature spontaneously at 37°C and lose their helical structure. They are then susceptible to degradation by less specific proteases which were unable to attack the intact molecule, particularly gelatinase which can also accelerate the action of collagenase (Murphy and Reynolds 1985, Sellers and Murphy 1981, Harris and Krane 1974b).

Activated collagenase binds tightly to its substrate but different collagens have been shown to have different susceptibilities to the enzyme (Gross et al 1980, Welgus et al 1981, Mainardi et al 1986). 'Classic' or interstitial collagenase splits collagen types I, II, and III. Type IV is not affected but its short interrupted helical region can be degraded by other proteases, notably stromelysin (previously known as proteoglycanase) and a type IV collagenase identified
CHAPTER I.5 Collagenase

by Liotta et al (1982, Harper 1980, Sellers and Murphy 1981). The forms of interstitial collagenase secreted by connective tissue cells and by macrophages are similar (Werb and Gordon 1975, Welgus et al 1985a) but there are some differences in the enzyme produced by polymorphonuclear leucocytes (Murphy and Reynolds 1985, Weiss and Peppin 1986, Mainardi et al 1986, 1987). Neutrophil collagenase has the same site of action as the connective tissue form but this enzyme is stored in specific granules (Ohlsson 1980, Murphy et al 1980) and is less effective at degrading type III collagen. The details of activation are different (Weiss and Peppin 1986) and molecules of two different sizes appear to be involved (Mainardi et al 1987). Immunological differences also exist (Murphy and Sellers 1980, Weiss and Peppin 1986, Mainardi et al 1987). Neutrophil collagenase is the only form which is stored before use; tissue collagenases are synthesised on demand.

Regulation of collagenase activity

The lack of storage and need for activation allow several opportunities for the close control of collagenase activity. Various factors affect the synthesis of the enzyme, others control activation and a
third group rapidly inhibit the active form to keep a tight rein on the destructive process. A summary of this situation is given in Figure I.5.1.

Collagenase is generally synthesised by connective tissue cells, such as fibroblasts, osteoblasts, chondrocytes and synovial cells, the same cells which secrete its collagen substrate (Harris and Krane 1974a, Welgus et al 1985a and b, Woolley and Evanson 1980, Murphy and Reynolds 1985). A great deal of knowledge about collagenase production comes from the study of cells and tissues in culture, although this gives indirect information about the mechanisms 'in vivo'. This is related to the lack of collagenase storage; since the enzyme is synthesised on demand and tightly bound to its substrate it cannot usually be extracted from uncultured tissue in sufficient quantity for study (Harris and Krane 1974a, Murphy and Reynolds 1985).

Many proteases induce the synthesis of collagenase in culture, including plasmin, trypsin, chymotrypsin, papain and elastase (Werb and Aggeler 1978). Stimulation for synthesis 'in vivo' is thought to come from mediators, known as cytokines, which induce collagenase synthesis in the appropriate cells and, under certain conditions, can make it the major gene
Figure I.5.1

Summary of regulation of collagenase activity.

- **NH<sub>2</sub>** stimulator eg Interleukin 1
- **3/4** synthesising cell eg fibroblast
- **LATENT COLLAGENASE**
- **1/4** activator eg plasmin
- **ACTIVE COLLAGENASE**
- rapid inactivation by **TIMP**

**COLLAGEN**
CHAPTER 1.5 Collagenase

product. A variety of such inducing factors has been found in different situations (Mainardi et al 1986) but most are believed to be forms of Interleukin-1, which is effective in minute amounts for several cell types (Murphy and Reynolds 1985, Pasternak et al 1986). Interleukin-1 has many other actions, as mentioned earlier (Chapter 1.2); it can cause proliferation of fibroblasts and increase collagen synthesis but it is unlikely that all its properties are called into play in any one biological situation (Krane 1987). Heparin and histamine from mast cells also have some ability to induce collagenase secretion and parathyroid hormone is a stimulator for osteoblasts. Progesterone and oestrogen both have effects on collagen degradation in the uterus and corticosteroids are potent inhibitors of collagenase synthesis, which may account for their therapeutic assistance in some conditions (Woolley and Evanson 1980, Peacock 1980, Harris et al 1984, Murphy and Reynolds 1985, Harris and Krane 1974c).

Activation

A wide range of agents has been shown to activate latent collagenase. "In vitro", several activators are commonly used to demonstrate the presence of latent
enzyme, including proteinases, such as trypsin and plasmin, organomercurials, like 4-aminophenylmercuric acetate, and sodium thiocyanate (Biswas 1982, Mainardi et al 1986, Oyamada et al 1983, Eeckhout and Vaes 1977). Cathepsin B and kallikrein are also activators (Murphy and Sellers 1980, Eeckhout and Vaes 1977). For several years, there was contradictory evidence about whether latent collagenase might be an enzyme-inhibitor complex (Sellers et al 1977, Murphy et al 1977, Simpson and Mailman 1981, Sellers and Murphy 1981, Reynolds et al 1977, Vaes 1972, Welgus et al 1979, Pettigrew et al 1980, Kishi et al 1984) but it is now thought to be a precursor form which cannot be restored after activation (Reynolds 1986). There is no conclusive evidence that any peptide material is lost during activation (Murphy and Reynolds 1985). The activating system involved in any particular 'in vivo' situation is difficult to demonstrate but it is likely that plasmin, derived from the action of plasminogen activator on plasminogen, is the collagenase activator in some circumstances (Werb et al 1977, Eeckhout and Vaes 1977).
Inhibition

Since collagenase is a metallo-enzyme containing zinc, which requires ionic calcium for its activity, it is inhibited by chelating agents such as EDTA and 1,10-phenanthroline (oPA) but this is a non-specific effect (Kerwar et al 1986). α2-Macroglobulin is a general proteinase inhibitor found in serum, which is a potent inhibitor of collagenase, forming an irreversible enzyme-inhibitor complex (Werb et al 1974, Peacock 1980, Sellers and Murphy 1981).

Specific inhibitors of metalloproteinases have been identified in various tissue and cell preparations and body fluids (Reynolds et al 1977, Murphy and Sellers 1980, Welgus and Stricklin 1983, Bunning et al 1984, Welgus et al 1979, Vater et al 1979, Simpson and Mailman 1981, Murphy et al 1977, Pettigrew et al 1981). These potent inhibitors have been shown to be closely related and were named tissue inhibitor of metalloproteinases: TIMP (Cawston et al 1981, Murphy et al 1981). TIMP is a glycoprotein with a molecular weight of about 28 000 which combines rapidly and irreversibly with active collagenase in a 1:1 molar relationship (Welgus et al 1979, Cawston and Mercer 1986).

The same connective tissue cells secrete both TIMP
and collagenase (Murphy and Sellers 1980, Welgus et al 1985a and b, Heath et al 1982), which ensures that any latent collagenase which becomes activated is inhibited almost immediately. Additionally, TIMP inhibits the metalloproteinases which degrade the other elements of connective tissue, gelatinase and proteoglycanase so that any breakdown of connective tissue is tightly regulated (Cawston et al 1981, Sellers et al 1979, Welgus et al 1985b).

A further circulating inhibitor, \( \beta_1 \)-anticollagenase, has been identified in serum (Woolley et al 1976); this has many properties in common with the tissue inhibitors and may be another variety of TIMP (Welgus and Stricklin 1983, Reynolds 1986). In general, the different preparations of TIMP have less inhibitory activity against polymorphonuclear collagenase than against the tissue enzyme and they do not inhibit bacterial collagenase. \( \alpha_2 \)-macroglobulin inhibits bacterial collagenase as well as the mammalian enzyme; collagenase binds preferentially to \( \alpha_2 \)M in the presence of TIMP (Cawston and Mercer 1986) but this activity is unlikely to be important in the normal regulation of collagenolysis since \( \alpha_2 \)M is a very large molecule (MW 725 000) which cannot move far from blood.
vessels (Sellers and Murphy 1981). TIMP appears to be produced by connective tissue cells to control specifically the local extracellular activity of metalloproteinases while $\alpha_2$M remains confined to the bloodstream (Murphy and Reynolds 1985, Cawston and Mercer 1986).

An imbalance between enzyme and inhibitor seems to be required for any significant collagenolysis to occur and therefore investigation of TIMP activity is becoming an important part of research into collagen degradation (Sellers and Murphy 1981, Woolley and Evanson 1980, Murphy and Reynolds 1985, Reynolds 1986, Cawston and Mercer 1986, Welgus et al 1985b).
CHAPTER II

DETAILED STATEMENT OF THE PURPOSE OF THE INVESTIGATION

Review of the literature shows that collagen makes a major contribution to the strength of the intestinal wall and that the dynamics of collagen turnover are directly relevant to the healing of colonic anastomoses. The work of Hawley implicated the enzyme collagenase as an important factor in dehiscence of large bowel suture lines. Much subsequent work on anastomotic breakdown has depended on Hawley's conclusions and has interpreted collagen changes in terms of collagenase activity but few attempts to measure the enzyme have been reported. Several workers have investigated the effects of proteinase inhibition during colonic healing, with the assumption that a reduction in collagenase activity would be beneficial although the evidence from this group of studies is inconclusive.

Over the same period of time the volume of knowledge related to the action and control of collagenase has increased enormously. The specificity of the mammalian enzyme has been determined and the precise regulation of its synthesis, activation and inhibition indicate a tightly controlled system which may hold a key position in the breakdown of collagen.
CHAPTER II

This knowledge has advanced the understanding of various aspects of rheumatology, dermatology, dental pathology and ophthalmology but has not been related to the field of colonic healing.

It will become clear, however, that there are difficulties associated with the measurement of collagenase (Chapter III.3) which may account for the lack of direct assessment of the enzyme in colonic healing. It therefore becomes apparent that reappraisal of the biochemical literature and adaptation of the methodology for the assay of collagenase in the colon must be carried out before further information can be obtained about the role of the enzyme in anastomotic dehiscence. This thesis sets out to establish the appropriate methods for the investigation of collagenase activity in colon and to carry out the first application of these methods in order to define the role of this specific enzyme in normal colonic healing.
Reasons for use of a rabbit model

Although aetiological studies can be undertaken to identify risk factors in colonic anastomotic dehiscence, it is ethically improper and practically not possible in man to investigate fully the early events during healing because this would require tissue samples to be taken in the first post-operative days. Animal experiments are therefore essential in this field, both to evaluate improvements in technique and to extend the understanding of colonic healing. Many such studies have been carried out, as detailed in Chapter I.3, using investigative methods which could not be employed in human work because they depend on removal of an anastomosis from an animal at different times in the early post-operative period.

Studies in the dog or pig tend to be preferred for work on improvements in technique because of the closer similarity in size of these animals to man but there is little justification for the use of larger animals when investigating the basic processes which occur in colonic
healing. The most popular animals for such basic research have been the rabbit and the rat. The rat is smaller and cheaper, allowing for larger numbers to be studied and has been the choice of many groups in the past, notably Cronin et al (1968a and b), Irvin (1976, 1978), Irvin and Hunt (1974a and b) and investigators from Malmö in Sweden (Jiborn et al 1978a–c, 1980a and b, Jönsson et al 1983, 1985a–c, 1986, 1987, Blomquist et al 1984a–d, 1985a and b, Högström and Haglund 1985a and b, Högström et al 1985a–d, Udén et al 1988). The rat has an additional advantage as a model of colonic healing because, like man, the rat is an omnivore and consequently the anatomy of the rat colon resembles that of man, with a small caecum, a single major flexure rather than hepatic and splenic, and a left colon passing down to the rectum.

The rabbit, on the other hand, is a herbivore, with a much enlarged caecum and appendix, a proximal colon which can be divided into two parts by the formation of the taeniae, a fusiform region and a distal colon with a complete coat of longitudinal muscle (Snipes et al 1982). The lower end of this distal part of the colon merges into a rectal region, similar to man's, which passes below the level of the peritoneal reflection to
CHAPTER III.1 Methods - animal model

reach the anus (Craigie 1966). In neither animal would it be practical to form an anastomosis in the extra-peritoneal part of the rectum - a site which is particularly susceptible to breakdown in man. It is feasible, however, to create an anastomosis in the lower part of the distal colon of the rabbit and to extrapolate at least some of the characteristics of the subsequent healing process to the situation in man. The rabbit was the animal chosen for study by Hawley in his investigations into the relevance of collagenolysis to anastomotic dehiscence (1970). The method of Gross and Lapiere (1962), used by Hawley to measure collagenolytic activity in colon, proved suitable for rabbit (Hawley 1970, Jayaraj et al 1983a, Lewin et al 1986) and human tissue (Sturzaker and Hawley 1962, Riley and Peacock 1967, Jayaraj et al 1983b) but explants of rat colon have been found to produce no measureable lysis (A P Jayaraj, personal communication). More recently, the rabbit has been used by workers in Nijmegen in Holland (Hendricks et al 1985, Hesp et al 1984a and b, 1985) for studies of the collagen dynamics of intestinal healing. It was also the animal chosen by Gries and Grasedyck (1969) and Lunstedt et al (1984) for investigations into colonic collagenolysis and by Delaney and Lalor (1976)
CHAPTER III.1 Methods - animal model

and Young and Wheeler (1982, 1983) for their studies into the potential benefits of proteinase inhibition. There is some justification, therefore, for continuing to work with the animal in which the initial studies in this field were carried out, particularly with the intention of directly comparing the findings using new methodology with those of previous investigators.

Choice of operative variables

1) Site of colonic resection

In previous studies colonic resection and anastomosis have been performed at a site identified by the distance above the peritoneal reflection measured in centimetres. Personal observation has shown that, five to eight centimetres above this landmark, a site frequently chosen for 'distal' operations, the arterial supply to the rabbit colon undergoes a change which may be relevant to healing in this region. Above 8 cm the equivalent of the inferior mesenteric artery (Craigie 1966) runs in the mesentery at a distance of approximately 1 cm from the bowel, giving infrequent branches to it. Over the next three or four centimetres, this main vessel approaches closer to the colon wall and then, quite abruptly, begins to give more
CHAPTER III.1 Methods - animal model

branches, only two or three millimetres apart, so that the colon below this point has a more abundant blood supply (Fig III.1.1). Since inadequate blood supply is clearly recognised as a major risk factor in anastomotic breakdown (Foster and Leaper 1984, Kirk and Irvin 1977), it was felt important in this study to standardise the site of operation in relation to this pattern of vasculature. Consequently, the first branch of the inferior mesenteric artery proximal to the point of abundant branching was divided and ligated and the colon lying between the next branches in each direction was resected (Fig III.1.1). This gave a resected segment which was approximately 2 cm long and an anastomosis approximately 5 cm above the peritoneal reflection.

2) Suture material and technique

Earlier studies compared different suture materials to assess their effect on colonic healing but, in the multitude of studies performed, the results are frequently contradictory (Khoury and Waxman 1983, Capperauld and Bucknall 1984). Similarly, in the confines of small animal work, there are conflicting reports about the advantages of single or double layer anastomoses and continuous or interrupted sutures (Irvin
CHAPTER III.1 Methods - animal model

Figure III.1.1

Diagram of blood supply to distal colon in rabbit showing site of vessel ligation and resection.
and Edwards 1973, Templeton and McKelvey 1985, Goligher 1984f). For the purposes of this investigation, which was designed to study collagenase activity in uncomplicated healing, a rapid reproducible anastomosis, using readily available materials, was considered more important than the possible minor advantages of one technique over another. Anastomoses were therefore made using interrupted 6/0 silk sutures in a single layer, employing a standard pattern of twelve evenly-spaced sutures.

Investigators have shown a difference in healing when the bowel wall is inverted or evverted at the suture line. Although some reports indicate an advantage in eversion (Ravitch et al 1981, Getzen and Holloway 1966), the majority show that inverted anastomoses, with serosa to serosa approximation, heal with fewer complications than evverted ones (Goligher et al 1970b, Goligher 1984f). In performing rabbit colonic anastomoses, during the preliminary studies to determine the details of the standard operation for this investigation, it was observed that a variable degree of intestinal obstruction occurred at the stitch line when the bowel wall was inverted, due to the reduction in calibre of the lumen. No obstruction occurred at evverted
anastomoses unless an additional complication arose. Previously, in the study of collagenase activity and collagen changes at anastomoses, it has been shown that obstruction at the site of an anastomosis produces a greater degree of collagen loss than an unobstructed bowel and normal healing is delayed (Jiborn et al 1978a, 1980b, Foster and Leaper 1984). The variability of the obstruction produced by inversion was considered as undesirable as the obstruction itself and, therefore, anastomoses performed for this study of collagen changes and collagenase activity at the healing site were made initially with eversion of the bowel ends. Although this increased the risk of anastomatic dehiscence to a small degree, the study was carried out only on uncomplicated anastomoses and any which showed evidence of dehiscence were rejected from further investigation. A subsequent set of experiments compared these everted anastomoses with the results of an 'end-on' suture technique. This method aligns the layers of the bowel wall more accurately while still producing inversion of the mucosa; a minimal degree of serosa to serosa approximation is created without markedly increasing the risk of obstruction of the lumen.
CHAPTER III.1 Methods - animal model

3) Blood loss at operation

Whitaker et al (1970) have demonstrated that blood loss greater than 10 per cent of the total blood volume at operation has a detrimental effect on colonic anastomotic healing and this is supported by the findings of Gilmour et al (1980). The average blood loss occurring in the standard operation developed for this study was therefore determined in a pilot group of animals to ensure that it was well below this critical figure. The total blood volume in rabbits can be calculated from their weight using the figure 70 ml/kg (Green 1979). In 5 rabbits, the blood loss at operation was estimated by weighing all swabs before operation, mopping up all spilt blood as carefully as possible and weighing the swabs again at the end of the operation. By this means, the average blood loss at a standard operation was found to be 2.6 per cent of the total blood volume of the rabbit (range 0.7 to 5.6 per cent), safely below the critical 10 per cent mark. Blood loss was not measured at all operations because this value was found to be consistently low, while the method of measurement precluded the use of saline to moisten the exposed intestine during the procedure.
CHAPTER III.1 Methods - animal model

Standard operation

The standard distal colonic resection and everted anastomosis was performed on healthy female New Zealand White rabbits, ranging in weight from 1.49 to 2.52 kg (mean 2.04 kg). Animals were housed in individual cages in an animal house with free access to water and a standard rabbit pellet diet. No bowel preparation was carried out and there was no pre-operative restriction of food or water.

The rabbits were anaesthetised by slow intravenous injection of pentobarbitone (Saggatal, 60mg/ml) into the marginal ear vein, at a dose of 0.7 to 0.75 ml/kg. Occasional rabbits, approximately 1 in 15 (6.5 per cent), were particularly susceptible to pentobarbitone and succumbed immediately to this dose; a few were particularly hardy and required a small supplemental injection to achieve operative anaesthesia.

The abdominal wall was cleaned with liquid detergent and the operation was carried out with clean, but not sterile, instruments. A lower midline incision, 5 cm long, gave access to the distal colon and the site for resection was determined by the vasculature as described above. Faecal pellets were gently milked away from the proposed sites of division and, after ligature
of the appropriate branch of the mesenteric artery, a 2 cm segment of colon was removed. An end-to-end everted anastomosis was made with a single layer of full-thickness interrupted 6/0 silk sutures, evenly spaced using twelve stitches for each anastomosis. During the operation the exposed intestine was kept moist with 0.9 per cent saline. The abdomen was closed in two layers with continuous 2/0 silk sutures and the animal allowed to recover from the anaesthetic.

A later set of anastomoses was carried out using an 'end-on' suture technique. Sutures were inserted through the serosal surface and brought out in the submucosal layer at the cut edge rather than through the full thickness of the bowel wall (Fig III.1.2). When these sutures were tied the muscle layers of the wall were approximated edge-to-edge, while the mucosa, which was not included in the stitch, became inverted. Twelve sutures were spaced evenly as before and all other aspects of the operation were unchanged.

The rabbits were allowed free access to food and water after operation. The mean loss of body weight in the first twenty-four hours was 8.3 per cent (range 2.9 to 10.7 per cent) and most passed some faecal material within one or two days; weight loss after three days was
CHAPTER III.1 Methods - animal model

Figure III.1.2
Diagram of everted and 'end-on' suture techniques.

a) Everted sutures.

Sutures are passed through the full thickness of the bowel wall to produce eversion of all layers.

b) 'End-on' sutures.

Sutures brought out in the submucosal layer at the cut edge of the wall to produce mucosal inversion only.
similar (mean 7.3 per cent, range 2.1 to 12.2 per cent). At the appropriate time rabbits were killed by an overdose of pentobarbitone (Expiral 200 mg/ml, 0.75 ml/kg) given intravenously as before. The abdomen was opened and the site of operation inspected. Adhesions were separated gently to allow access to the anastomosis and removal of the required segments of colon. Any anastomosis which showed macroscopic evidence of any complication in its healing, such as obstruction, abscess formation, partial or complete dehiscence of the suture line, was excluded from further study to ensure that these experiments defined the situation in normal, uncomplicated colonic healing. No dehiscence was encountered but 10 out of 63 anastomoses (15.9 per cent) showed signs of abscess formation or of obstruction due to adhesions and these animals are not included in the analysis of results in later chapters.
CHAPTER III.2 Methods - collagen

MEASUREMENT OF COLLAGEN

An important feature of the molecular structure of collagen is the occurrence of glycine as every third amino acid residue (Pinnell 1983, Burgeson 1982, Uitto et al 1986). The repeated sequence Gly-X-Y, where X and Y are frequently proline and hydroxyproline, is the factor which allows the peptide chain to coil tightly and form one strand of the triple helix, giving collagen its ability to form fibrils and fibres and, consequently, its strength. Hydroxyproline is an amino acid which is very uncommon in other proteins but forms approximately 10 per cent of the residues in collagen, so the hydroxyproline content of a tissue is often used as a measure of its collagen content.

Several methods are available for the measurement of hydroxyproline in tissue homogenates. Woessner (1961) described an assay which was suitable for preparations in which hydroxyproline formed only a small proportion of the total amino acids and this has been applied to colon by several workers (Cronin et al 1968a and b, Hawley 1970, Yamakawa et al 1971, Stewart 1973, Wise et al 1975, Colin et al 1979, Gottrup 1981, Bark et al 1984, Brennan et al 1984, Savage 1985, Templeton and
CHAPTER III.2 Methods - collagen

McKelvey 1985, Foster et al 1985, Winsey et al 1987). This method can be applied to homogenates of wet or dried tissue by hydrolysing the sample with hydrochloric acid, then neutralising and diluting. Chloramine-T is added to initiate oxidation of the hydroxyproline to pyrrole, then perchloric acid to destroy the chloramine-T and acidify the solution and, finally, 4-dimethylaminobenzaldehyde (DAB) to form a chromagen which reflects the hydroxyproline content and can be measured spectrophotometrically.

The disadvantages of relying on hydroxyproline content as a measurement of collagen lie in the risk that the tissue may contain hydroxyproline in other proteins and in the variability of hydroxylation of collagen between one tissue and another. Since the collagen must be degraded for hydroxyproline to be measured, the method cannot distinguish collagen from gelatin. Attempts have therefore been made to measure collagen more directly. Sweat et al (1964) described the affinity of the dye, Sirius Supra Red F3B, for collagen and Junqueira et al (1979) published an assay using this approach. The collagen preparation is dried to a film, fixed with Bouin's fluid, and stained with the Sirius Red dye. After excess dye has been washed

117
CHAPTER III.2 Methods - collagen

off the film, the Sirius Red is eluted to be measured spectrophotometrically.

A modification of this method was employed by Young and Wheeler (1982, 1983) in their work on the use of aprotinin to improve colonic anastomotic healing but Sirius Red assays have not subsequently been used by other workers. Junqueira et al (1979) noted that their assay was unsuitable when the film of collagen was too thick and that the dye also stained a component of the complement system; they described the assay of pure preparations of collagen but did not test the method on tissue samples. Kuttan and DiFerrante (1980) modified the assay to allow larger numbers of samples to be tested in the wells of a multiwell plate and compared the results with those from a hydroxyproline assay. However, although they demonstrated interference by 0.5 per cent sodium chloride and sodium dodecyl sulphate and partial interference by urea and guanidium chloride, they also assayed only purified preparations of collagen. Subsequent modifications, using the Sirius Red binding as an assay for bacterial collagenase (Hassanein et al 1984), have again estimated samples of collagen prepared from different sources but have not assayed the collagen content of the tissues themselves.
Comparison of Hydroxyproline and Sirius Red Assays

A preliminary study was therefore carried out to compare these methods for the measurement of collagen in rabbit colon. Samples of normal colon resected at the standard operation, and post-operative tissue taken at the time of sacrifice, were divided in two to be assayed by both methods.

Hydroxyproline Assay

The method of Woessner (1961) was followed. Tissue was washed in 0.9 per cent saline, minced, homogenised in water and then assayed directly, or after freeze-drying and resuspension of the dried tissue in a small volume of water. Five 4 ml aliquots of each sample were autoclaved at 121°C (at a pressure of 15 lb/in²) for 24 hours in 2 M hydrochloric acid, to break down the proteins to their constituent amino acids. The samples were then neutralised (to bring the pH between 6.5 and 7.0) and diluted for assay. Two ml of the dilution, in duplicate, was mixed with 1 ml chloramine-T (0.05 M in water: propan-2-ol: citrate buffer, 10:15:25, prepared fresh daily; citrate buffer comprised 50 g citric acid monohydrate, 120 g sodium acetate trihydrate, 34 g sodium hydroxide and 12 ml glacial

119
acetic acid made up to 1 l at pH 6.0 and stored at 4°C to initiate oxidation of the hydroxyproline, and allowed to stand at room temperature for 20 minutes. 1 ml of 1.43 M perchloric acid was added, mixed and left for 5 minutes to destroy the chloramine-T and acidify the solution, before the addition of 1 ml 4-dimethylaminobenzaldehyde (10 per cent solution in propan-2-ol at 60°C, prepared daily). Fully mixed, the tubes were kept at 60°C for 20 minutes and then cooled with tap water for 5 minutes before measurement of the absorbence at 556 nm in a Cecil CE 2272 spectrophotometer. Hydroxyproline content was calculated by comparison with the standard curve of absorbence of hydroxyproline, diluted daily from a stock solution and run at the same time as the samples. The average reading from the two duplicates gave the value of hydroxyproline concentration for each autoclaved aliquot of sample and the mean value from 5 aliquots of each homogenate gave a result for any one piece of colon.
Sirius Red Assay

The method of Junqueira et al (1979) as adapted by Kuttan and DiFerrante (1980) was used, forming the collagen films in the wells of a multiwell plate. Samples of colon were washed, minced and homogenised as for the hydroxyproline assay and 200 μl aliquots of an appropriate dilution were placed into the wells of the plate. The plate was left to dry in air at room temperature so that the homogenate formed a thin film lining the well. This film was fixed by the application of Bouin's fluid (20 ml neutral formalin and 5 ml glacial acetic acid in 75 ml saturated picric acid solution) for one hour, washed with running tap water and then stained with a picrosirius solution (0.1 g Sirius Red F3BA, 'Gurr' BDH Ltd., in 100 ml saturated picric acid solution) for a further hour at room temperature. Unbound dye was removed by rinsing the plates in 0.01 M HCl and soaking in 0.01 M HCl for one hour. After drying, 1 ml of 0.2 M NaOH was added to each well to elute the dye which had been taken up by the tissue film. The resulting solution was collected from the well and its absorbance at 540 nm measured on a Cecil CE 2272 spectrophotometer. Comparison was made with the readings from standard solutions of
CHAPTER III.2 Methods - collagen

acid-soluble type I collagen (catalogue heading 'Type III', Sigma UK, Ltd) dissolved in acetic acid and placed in different wells on the same plate.

Results of Comparison

Tables A.1.1 to 5 in the Appendix show the individual measurements of hydroxyproline concentration for 35 pre- and post-operative segments of colon from 5 animals. The results between duplicates in the same assay run were consistent, as were measurements of the same sample in different runs. The mean coefficient of variation for 5 aliquots of the same homogenate was 35.83 per cent (standard error, σ 19.4), when hydroxyproline was measured in homogenates of wet tissue. The reproduction of results improved when the tissue was freeze-dried before measurement, with the mean coefficient of variation for 5 aliquots falling to 25.29 per cent (σ 10.2). The hydroxyproline assay therefore gave measurements for wet and dried colonic tissue which showed an acceptable degree of variation. These hydroxyproline concentrations are in agreement with the findings of Irvin and Edwards (1973), Hendricks et al (1985) and Hesp et al (1984a and b) in rabbit colon. The improvement in consistency of results for

122
CHAPTER III.2 Methods - collagen

dry colon reflects a more uniform distribution of the tissue when it was resuspended after freeze-drying than when it was homogenised initially.

The Sirius Red assay proved to be inconsistent in its measurement of the uptake of dye by tissue films, with poor reproducibility even in the readings for standard solutions of collagen. The use of a stream of hot air to dry the films onto the plates more rapidly did not improve the repeatability of the measurements. Pepsin digestion of the tissue was used to increase the accessibility of the dye to the collagen, as suggested by Kuttan and DiFerrante (1980), but the application of pepsin, either at room temperature or at 4°C, for a range of times up to 72 hours, also failed to improve the consistency of the findings. Incubation of samples with 3 per cent mercaptoethanol for one hour at 37°C, to reduce the Type III collagen and improve its binding to Sirius Red (Kuttan and DiFerrante 1980), did not affect the results. The poor reproducibility may be related to interference by other substances in the homogenate, as shown for 0.5 per cent sodium chloride and for tissue culture medium by Kuttan and DiFerrante (1980) or to the inaccessibility of the collagen to the dye without further extraction from the tissue. The Sirius Red
assay was therefore considered unsuitable for the measurement of the collagen content of rabbit colon and subsequent experiments used the hydroxyproline assay on freeze-dried samples of tissue.

**Amino acid assay**

Woessner's method for the measurement of hydroxyproline (1961) must be modified if the amino acid forms less than 2 per cent of the total amino acids in the sample. If this is so, the chromagen formed can be extracted with benzene and the sample decolourized by hydrogen peroxide, which removes only the colour contributed by hydroxyproline. If the absorbence is then measured again, the hydroxyproline content can be calculated by subtraction. The method of Rosen (1957) to measure total amino acid content was applied to a selection of pre- and post-operative colon samples in order to determine whether this additional step in the hydroxyproline assay was required.

1 ml of diluted and neutralised sample was mixed with 0.5 ml acetate cyanide buffer (36 g sodium acetate trihydrate, 1 mg sodium cyanide and 6.6 ml glacial acetic acid made to 100 ml) and 0.5 ml ninhydrin reagent (3 per cent w/v ninhydrin in 99 per cent ethanol). The
tubes were placed in a boiling water bath for 15 minutes and then diluted with 5 ml propan-2-ol:water (1:1). After mixing and cooling, the absorbance at 570 nm was measured with a Cecil CE 2272 spectrophotometer. A stock solution of glycine was diluted between 0 and 10 μg/ml to give a standard curve.

For 12 segments of colon, 6 assayed wet and 6 freeze-dried, the hydroxyproline content ranged from 3.43 to 8.39 per cent (mean 5.41 per cent) of the total amino acids (Table III.2.1). Since this percentage was always well above 2 per cent, the modification step in Woessner’s method was considered unnecessary for this study.

Protein Assay

As shown in Tables A.1.1 to 5 in the Appendix, the freeze-drying of both pre- and post-operative samples gave better reproducibility of readings than wet tissue; subsequent results were therefore expressed as the concentration of hydroxyproline per mg of dry tissue. This avoided a potential problem in the study of inflammatory reactions, since the water content of oedematous inflamed tissue may contribute so significantly to the weight that the measured
Table III.2.1

Concentrations of Hydroxyproline and Total Amino Acids measured in 12 segments of colon to confirm that hydroxyproline represents more than 2% of the total amino acid concentration in rabbit colon.

<table>
<thead>
<tr>
<th>Hydroxyproline</th>
<th>Amino Acids</th>
<th>% Hydroxyproline</th>
</tr>
</thead>
<tbody>
<tr>
<td>µg/mg tissue</td>
<td>µg/mg tissue</td>
<td></td>
</tr>
<tr>
<td>----------------</td>
<td>-------------</td>
<td>------------------</td>
</tr>
<tr>
<td>Measurements in wet tissue</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.71</td>
<td>40.49</td>
<td>4.22</td>
</tr>
<tr>
<td>2.49</td>
<td>52.20</td>
<td>4.77</td>
</tr>
<tr>
<td>1.80</td>
<td>36.20</td>
<td>4.97</td>
</tr>
<tr>
<td>1.90</td>
<td>31.35</td>
<td>6.06</td>
</tr>
<tr>
<td>1.82</td>
<td>37.45</td>
<td>4.86</td>
</tr>
<tr>
<td>1.33</td>
<td>38.78</td>
<td>3.43</td>
</tr>
<tr>
<td>Mean</td>
<td>1.84</td>
<td>39.41</td>
</tr>
<tr>
<td>± SD</td>
<td>0.38</td>
<td>6.98</td>
</tr>
<tr>
<td>Measurements in dry tissue</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19.73</td>
<td>276.36</td>
<td>7.14</td>
</tr>
<tr>
<td>19.39</td>
<td>348.30</td>
<td>5.57</td>
</tr>
<tr>
<td>19.61</td>
<td>306.71</td>
<td>6.39</td>
</tr>
<tr>
<td>16.34</td>
<td>372.70</td>
<td>4.38</td>
</tr>
<tr>
<td>32.61</td>
<td>388.63</td>
<td>8.39</td>
</tr>
<tr>
<td>16.94</td>
<td>361.60</td>
<td>4.68</td>
</tr>
<tr>
<td>Mean</td>
<td>20.77</td>
<td>342.38</td>
</tr>
<tr>
<td>± SD</td>
<td>5.89</td>
<td>42.60</td>
</tr>
</tbody>
</table>

Mean percentage in 12 segments 5.41
± SD 1.39

126
concentration of hydroxyproline in wet tissue is altered by dilution alone. For a similar reason, it may be preferable to express the hydroxyproline content in relation to the total protein content, since the inflammatory infiltrate is protein-rich. The protein concentration of the tissue was therefore also measured.

The protein assay described by Lowry et al (1951) uses the Folin-Ciocalteu reagent and is considered particularly suitable for the assay of mixed tissue proteins. The following reagents were used:

Reagent A: 2 per cent sodium carbonate in 0.1 M sodium hydroxide
Reagent B: 2 per cent sodium potassium tartrate
Reagent C: 1 per cent copper sulphate pentahydrate
Reagent D: Folin reagent diluted 1:1 to 1 M in acid
Reagent E: B:C:A in the proportions 1:1:100

Tissue samples were prepared by freeze-drying and hydrolyzing in 2 M HCl as for hydroxyproline assay. After neutralisation, aliquots of the same diluted preparation were assayed for hydroxyproline and for protein. 0.5 ml of sample, in duplicate, was mixed with 2.5 ml of reagent E and left at room temperature for 10
CHAPTER III.2 Methods - collagen

minutes. 0.25 ml of reagent D was then added and the mixture left at room temperature for 30 minutes while the colour developed. The absorbence at 750 nm was read using a Cecil CE 2272 spectrophotometer and the protein content calculated from the standard curve produced by a solution of bovine serum albumen. As for hydroxyproline, the protein concentration for any one segment of colon was derived from the mean concentration in 5 aliquots of homogenate, each assayed in duplicate, and was expressed in μg/mg dry weight of tissue.
CHAPTER III.3 Methods - collagenase

MEASUREMENT OF COLLAGENASE ACTIVITY

(i) INTRODUCTION

The first demonstration of animal collagenolytic activity was made by Gross and Lapiere in 1962, using amphibian tissues. Explants from tadpole tails were cultured on gels made from collagen in Tyrode's solution. During incubation the tissue secreted proteolytic enzymes into the gel and these degraded the collagen, producing a clear area of lysis around the explant in an otherwise opaque gel. After 72 hours incubation, the size of the area of lysis was recorded as a measurement of the collagenolytic activity of the explant.

The assay developed by Gross and Lapiere (1962) was initially considered to measure collagenase activity and was the method available to Hawley for his studies on the relevance of collagenase to colonic anastomotic breakdown (1970, Hawley et al 1970). Subsequent work on the breakdown of colonic anastomoses has depended on Hawley's conclusions but it can now be recognised that the Gross and Lapiere assay provides only a non-specific measurement of collagenolytic activity. The collagen in the gel is susceptible to degradation by proteases other
than collagenase and, since the tissue is applied
directly to the substrate gel, it is probable that
non-specific enzymes produced by the explant contribute
to the observed collagen lysis. The method does not
allow manipulations to activate latent enzyme and
complete failure to show any lysis from some explants,
which is a recognised hazard of the assay (Riley and
communication), may indicate the absence of activators
or the presence of inhibitors, rather than an absence of
collagenase (Biswas 1982). Alternatively, the tissue
may fail to establish itself in the culture conditions
(Grillo and Gross 1967, Riley and Peacock 1967). A
tissue culture system introduces the possibility that
the measurement reflects the synthesis of collagenase
`in vitro` rather than the enzyme activity `in vivo`. The
products of the lytic activity of an explant on a
collagen gel cannot be examined directly to establish
the specificity of the assay for collagenase and the
proportion of latent enzyme cannot be determined without
the facility for activation. To measure `in vivo`
collagenase activity, therefore, it is preferable to
avoid culture and extract the enzyme from fresh tissue.
(ii) EXTRACTION OF COLLAGENASE FROM FRESH TISSUE

The fastest rate of collagen degradation identified in nature takes place in the involuting uterus after parturition (Woessner 1980a) and Ryan and Woessner (1971) used this organ to develop a procedure to detect collagenase in uncultured tissue. Since they suspected that the enzyme was bound closely to its substrate, they centrifuged homogenates of post-partum rat uterus at a speed which would precipitate the collagen fibrils with the enzyme bound to them. The resulting pellet was incubated at 37°C for up to 48 hours, in the presence of antibiotics to prevent bacterial growth, and calcium, which is a requirement for collagenase activity. The enzyme cleaved the endogenous collagen and produced soluble peptides containing hydroxyproline, so the collagenase activity could be determined by measuring the amount of hydroxyproline released from the pellet. Ryan and Woessner (1971) measured an enzyme that was calcium-dependent and totally inhibited by ethylenediamine tetraacetic acid (EDTA), with an optimum pH of 7.5, optimum temperature of 37°C and measurable activity at 30°C. By recombination of the pellet with the initial supernatant, they showed that centrifugation had not only precipitated the collagenase but also
CHAPTER III.3 Methods — collagenase, extraction

separated it from inhibitors.

This pellet assay was used by other workers to demonstrate collagenase activity in various tissues, including tadpole tail, bone matrix implants, rheumatoid synovium and human skin (Woessner 1980a). The specificity is likely to be better than the method of Gross and Lapiere (1962), since the substrate is the native tissue collagen, but Woessner accepted that other proteolytic enzymes might give positive results (Woessner 1980a) and, while the enzyme remains bound to the collagen, the nature of the reaction products cannot be fully assessed. This endogenous substrate contains non-helical portions and other elements that are susceptible to proteases besides collagenase. The enzyme/substrate ratio cannot be altered, so measurements of enzyme activity may be affected by a limiting amount of available substrate, the presence of inhibitors, or other factors which interfere with the optimum conditions for collagenase activity. It is not possible to characterise the enzyme completely and prove that it is a specific collagenase without a procedure which will separate it from the endogenous collagen.

Weeks et al (1976) developed a system for extracting collagenase from involuting uterus, using a
high concentration of calcium, and heating. Since collagenase cannot act without calcium, they suggested that the ion may be involved in the linkage of collagenase to collagen, so excess calcium might displace the enzyme from its binding sites. When collagen is heated to a temperature between 58 and 62°C, it shrinks, and this change in configuration of the molecule may help to release any collagenase. By resuspending the pellet from the Ryan and Woessner assay in a high concentration of calcium and heating to 60°C for four minutes, Weeks et al produced a soluble fraction which showed collagenase activity when applied to a substrate of exogenous collagen, previously labelled with ¹⁴C-glycine. With a higher temperature or longer period of heating the yield was reduced because the enzyme began to denature along with the collagen. They were able to extract up to 70 per cent of the collagenase in involuting rat uterus by this method. The action of the enzyme was dependent on calcium and completely inhibited by EDTA, with a broad pH tolerance centred around 7.5. Disc electrophoresis demonstrated that the products of collagenase activity were three-quarter and one-quarter fragments of the collagen molecule. Morales et al (1978) showed that the same
CHAPTER III.3 Methods - collagenase, extraction

extraction procedure was effective for the small quantities of enzyme present in ovarian Graafian follicles and collagenase was also demonstrated in inflamed gingiva (Woessner 1980a).

Weeks and colleagues (1976) used trypsin to inactivate \( \alpha_2 \)-Macroglobulin, which was thought to be the main inhibitor of collagenase in the initial supernatant. In this process, they found that the trypsin increased the collagenase activity by a greater amount than a simple removal of inhibition and so demonstrated activation of latent enzyme. Subsequent work has shown that latent collagenase can also be activated by plasmin (Werb et al 1977) and by organomercurials such as aminophenylmercuric acetate (APMA) (Sellers et al 1977).

The characterisation of collagenase was now established and confirmation of the enzyme includes the following criteria. Latent collagenase can be activated by trypsin or APMA and the enzyme activity is dependent on calcium, inhibited by EDTA, has an optimum pH of 7.5 but a wide tolerance, and an optimum temperature of 37°C but with reduced activity still detectable at 30°C. The demonstration of three-quarter and one-quarter cleavage products is now considered to be the final proof of the
specificity of an assay for collagenase, since the mammalian enzyme has such a precise action on intact collagen (Vaes 1972, Sellers and Murphy 1981).

Experiments to extract collagenase from colon

In preliminary experiments in the present study, the method of Weeks et al (1976) for extracting collagenase from fresh tissue was applied to resected and post-operative colon from 12 rabbits. Colon was collected from the animal, opened to remove any faecal material and washed in ice-cold saline. After blotting dry for weighing, the tissue was homogenised with a Silverson Laboratory mixer/emulsifier in a small volume of cold 10 mM CaCl₂ with 0.25 per cent Triton X-100. The homogenate was centrifuged at 6000 G for 20 minutes at 2°C, to precipitate the collagen fibrils with collagenase, and the supernatant, containing inhibitors, was discarded. The pellet was resuspended in 50 mM Tris/HCl buffer, pH 7.4, containing 100 mM CaCl₂ and 150 mM NaCl, and heated to 60°C for 4 minutes to release the enzyme from its substrate. After centrifugation at 10 000 G for 20 minutes, the supernatant was retained and dialysed against 9 volumes of 50 mM Tris/HCl with 150 mM NaCl, pH 7.4, to restore the calcium
concentration to 10 mM. The solution was spun once more at 6000 G for 20 minutes and the supernatant then assayed for collagenase activity by the \(^{14}C\)-collagen assay of Cawston and Barrett (1979, see Chapter III.3.iv for details).

No detectable collagenase activity was extracted from rabbit colon by this procedure. Modifications were therefore made to the procedure to try to improve the extraction of enzyme. The initial collecting solution was changed to the Triton/calcium solution or to the Tris/HCl buffer. The calcium concentration in the extracting solution was varied between 10 mM and 100 mM and the concentration of Tris/HCl in the buffer between 40 mM and 50 mM. The temperature to which the pellet was heated to release collagenase from collagen was altered between 40°C and 60°C without producing any activity and the results were no different when the heating step was excluded completely. The concentration of tissue was increased to improve the detection of any collagenase activity and several different homogenisers, including a Braun Mikro-dismembrator II and a hand-driven Teflon pestle homogeniser, were tried. The speeds of centrifugation were increased to improve the separation of fractions, although this ran a risk of
precipitating inhibitors into the enzyme fraction, and
the fractions which were usually discarded after
spinning were retained, resuspended as required, and
dialysed against the Tris/HCl, NaCl buffer to be run in
the collagenase assay.

At no time did any fraction of fresh colon produced
by these extraction experiments show any collagenase
activity within the radiochemical assay, whether
activated with trypsin or APMA or run alone. Nor was it
possible to demonstrate any collagenase by its action on
endogenous collagen using the pellet assay of Ryan and
Woessner (1971) on fresh or frozen colon (stored in
liquid nitrogen) from a further 11 rabbits. In total,
63 pieces of pre- and post-operative colon were
collected and assayed in one or other of these ways
without demonstrating any detectable collagenase
activity.

Previously, Kortmann and von Bary (1977) and von
Bary et al (1978) have reported the extraction of
collagenase from human colon by slow stirring in cold
salt solution followed by ammonium sulphate
precipitation. The tissue was considered acceptable up
to 24 hours post-mortem and then frozen in liquid
nitrogen, while sodium azide and the high salt
concentration of the extraction mixture acted as anti-bacterial agents. While the counts reported from a $^{14}$C-collagen assay appear reasonable, the nature of the products of enzyme activity was not proven by electrophoresis. This method of extraction, originally described by Wirl (1975), was applied to involuting uterus by Weeks et al (1976), who found only 50 per cent extraction compared with their method described above, despite the high level of collagenase activity in this tissue. No follow-up study has confirmed the validity of the Wirl extraction for colonic collagenase and the specificity of the system when applied to this tissue must remain open to question.

Brennan and colleagues (1984), in a study of prostaglandins in anastomotic healing in rat colon, homogenised post-operative tissue in 100 mM Tris buffer, pH 7.6 containing 15 mM CaCl$_2$. Little detail of their experimental methods is provided but they state that they were able to measure collagenase activity by the method of Cawston and Barrett (1979). These findings could not be reproduced in the present study. Gries and Grasedyck (1969) and Lunstedt et al (1984) have used synthetic substrates to demonstrate collagenolytic activity in rabbit colon but the specificity of these
methods for collagenase can be questioned, as discussed in Chapter III.3.iv.

Oyamada and colleagues, who first reported the demonstration of a specific collagenase from the culture of rabbit colon (1983), stated that they were unable to extract enzyme from uncultured tissue. It was therefore concluded from the present investigations that it was necessary to culture rabbit colon tissue in order to assess collagenase activity.
Oyamada and colleagues (1983) characterised collagenase from the culture of rabbit colon. They grew strips of tissue in Tyrode's solution and confirmed the enzyme specificity by electrophoresis. Normal rabbit colon secreted collagenase after a lag period of 12 hours, with activity reaching a plateau by 3 or 4 days. Explants showed histological signs of deterioration and decreased secretion of enzyme after 5 days in culture.

The collagenase activity of other tissues has also been studied in culture. Reports of the secretion of the enzyme into culture medium followed the first demonstration of collagenolytic activity by Gross and Lapiere (1962). Collagenase was secreted by rheumatoid synovium (Harris and Krane 1974b), bone and skin explants (Vaes 1972) and synovial fibroblasts (Werb and Burleigh 1974). Skin fibroblasts, foetal bone, uterus (Sellers et al 1978, Sellers and Murphy 1981), alveolar and peritoneal macrophages (Werb and Gordon 1975, Horwitz and Crystal 1976), dental pulp (Kishi and Hayakawa 1982), gingiva (Heath et al 1982), articular chondrocytes (Hunter et al 1984) and vulval tissue (Barnes and Douglas 1985) have all been cultured for collagenase activity.
Woolley and Evanson (1980) comment that normal tissues usually exhibit little or no extractable collagenase activity but almost always secrete the enzyme into culture medium from explants and for most of these tissues it is possible to measure enzyme after culture but not before (Kishi and Hayakawa 1982, Vaes 1972, Oyamada et al 1983, Horwitz and Crystal 1976). This may relate to the minimal amount of activity in normal tissues, in which collagen turnover is relatively slow, or to the binding of collagenase to its substrate. It may also be due to the presence of inhibitors in the tissue (Harris and Krane 1974a), or to the presence of α2-Macroglobulin in the serum within an explant (Werb et al 1974). Activated enzyme is rapidly complexed by tissue inhibitor of metalloproteinases (TIMP), in an irreversible process (Welgus et al 1985b), and the extraction used by Weeks et al (1976) may only be suitable for tissues with minimal TIMP activity (which may be the case temporarily in involuting uterus).

Culture introduces several potential drawbacks in the assessment of the collagenase activity of a tissue, since the conditions are inevitably altered from the 'in vivo' state. Dissection of the tissue to provide explants adds an element of trauma which may itself
stimulate collagenase synthesis. Factors in the culture medium, particularly in added serum, may either increase or inhibit enzyme secretion or activity. However, culture methods have been shown to differentiate the collagenase activity in some tissues in different pathological states. Heath et al (1982) found increased collagenase secretion in explants of diseased human gingiva compared with normal. The normal tissue produced latent enzyme only, while inflamed gingiva secreted active and latent collagenase with peak activity on the third day in culture. Barnes and Douglas (1985) found complete inhibition of secretion in explants of vulval tissue from patients with lichen sclerosus, while vulval explants in other conditions secreted latent collagenase; active enzyme was measured in tissue from cases of Paget's disease of the vulva. It therefore seemed probable that, although direct extraction of the enzyme from fresh colon would have been preferable, culture of rabbit colon explants before and after operation would show collagenase activity which at least partially reflected the changes induced by resection and anastomosis, either in terms of total activity or in the proportions of active and latent enzyme.
Establishment of culture conditions

The most immediate difficulty faced in the culture of colonic tissue is the elimination of faecal organisms in order to allow the colonic cells to grow unhindered. Bacteria which are viable at the time of incubation of the tissue could be expected to establish themselves more effectively than the colon cells, preferentially utilising the nutrients in the culture medium as well as consuming the nutrients provided by the explant itself. Oyamada et al (1983) demonstrated a significant decrease in the measured collagenase activity of medium when there was bacterial contamination of their explants. Additionally, even a low-grade bacterial infection of the culture may add bacterial proteolytic enzymes to the medium which will invalidate later assay results.

Elimination of bacteria requires a method of mechanical and pharmacological washing of the tissue before culture which will not affect the viability of the cells in the process.

This was accomplished in this study by removing each segment of colon from the animal, opening it along the anti-mesenteric border and carefully removing any solid faecal material with forceps. The segment was then allowed to soak, with frequent intermittent
agitation, in five separate changes of Hank's balanced salts solution (Table A.2 in Appendix). The first three changes contained ten times the 'standard' concentration of antibiotics for tissue culture medium: 600 mg/l benzyl penicillin, 1 g/l streptomycin and 25 mg/l amphotericin B. The final two changes contained the standard concentrations of the same antibiotics: 60 mg/l penicillin, 100 mg/l streptomycin and 2.5 mg/l amphotericin. Using this regime, and including the same antibiotics in the subsequent culture medium, infection rarely occurred in cultures of rabbit colon.

While in the fifth change of Hank's solution, segments of colon for culture were transferred to a tissue culture hood (Bassaire dust-free assembly cabinet, PB Mk 7) and, from that point on, were handled with sterile technique. The colon was dissected to produce six explants of full wall thickness from each area under study. Explants were transferred to pre-weighed sterile tubes, containing sterile Hank's solution, to be weighed and the four pieces closest in weight, between 30 and 50 mg wet weight, were then cultured.

The optimum weight for explants was established in a set of preliminary experiments. Pieces of colon less
than 25 mg in weight frequently failed to show any
secretion of collagenase into the medium, probably
because of failure of the tissue to establish itself in
culture. Occasionally, small explants exhibited
excessive collagenase activity, which may relate to the
large surface area : weight ratio but may also be due to
the decreased accuracy of measurement when a small piece
of tissue is weighed in a larger volume of fluid.
Explants larger than 60 mg produced less activity per
milligram than smaller pieces and the tissue sometimes
showed earlier signs of deterioration, due either to
there being insufficient medium for their size or to the
relatively small surface area exposed to the medium.
Explants were therefore subsequently kept between 30 and
50 mg wet weight and collagenase activity was expressed
per mg wet tissue.

Culture was carried out in 6-well multiwell tissue
culture dishes (Gibco) with each explant occupying one
3.5 cm diameter well. The explant was placed on a
stainless steel culture grid, so that the tissue was
supported on the surface of 4 ml of culture medium.
This ensures adequate exposure of the tissue to
atmospheric oxygen; it may be deprived of oxygen if it
is fully immersed in medium. Additionally, the
CHAPTER III.3 Methods - collagenase, culture

supporting platform may reduce the tendency of the tissue to remodel, which is particularly relevant in collagenase studies. The culture plates were incubated in humidified air at 37°C and the medium was collected and replaced every 24 hours.

The culture medium used for colon was Dulbecco’s modification of Eagle’s medium (DMEM) (Flow Laboratories, Table A.3 in Appendix). Supplements were added to the medium to ensure the correct conditions, both for tissue maintenance and for secretion of collagenase into the medium in a state amenable to assay. The following supplements (recommended by J Wright, personal communication) were used:

- 20 mM HEPES (4-(2-hydroxyethyl)-1-piperazine-ethane sulphonic acid)
- 2 mM L-glutamine
- 0.5 g/l sodium bicarbonate
- 1 per cent foetal calf serum - this was acid-treated by addition of 5 M HCl, to pH 3.0 for 1 hour at room temperature, and returned to pH 7.6 with 5 M NaOH.
- 60 mg/l benzyl penicillin
- 100 mg/l streptomycin
- 2.5 mg/l amphotericin B
CHAPTER III.3 Methods - collagenase, culture

All supplements were added to the medium through millipore filters (Flowpore D, Sartorius Ltd) to ensure sterility, using a combination of filters with 0.45 and 0.20 μ pore sizes to prevent the passage of bacteria. Samples from each preparation of medium were incubated for 72 hours under the usual culture conditions to confirm sterility before use.

Penicillin, streptomycin and amphotericin B were added to the medium to prevent the growth of any remaining or contaminant micro-organisms. Foetal calf serum contains factors which enhance cell growth in culture but was acid-treated to destroy α2-Macroglobulin, a major inhibitor of collagenase found in serum (Werb et al 1974, Harris and Vater 1980, Harris et al 1984, Cawston and Mercer 1986). This acid-treatment, however, interferes with the frequently-used culture system of incubation in an atmosphere enriched with 5 per cent CO₂ (Oyamada et al 1983, Fell et al 1986, Heath et al 1982, Barnes and Douglas 1985, Werb and Burleigh 1974, Hunter et al 1984, Cawston and Tyler 1979), which prevents the release of carbon dioxide from medium containing a high concentration of sodium bicarbonate. The cultures were consequently incubated in air and the concentration of
sodium bicarbonate was suitably reduced to maintain the
pH at 7.6, using the additional buffering capacity of
HEPES.

Collection of medium from culture plates was
carried out daily, under sterile conditions. The medium
was examined for any sign of infection, indicated by
cloudiness of the fluid or an abnormal change in colour
of the phenol red indicator. Samples showing such
suspicious features (which were uncommon) and random
samples of medium from every six-well plate, were
checked for the presence of micro-organisms by
examination under a phase-contrast microscope (Olympus
BHA). Both medium and explant from any infected well
were discarded. Medium was collected into tubes
containing sodium azide to give a final concentration of
0.2 per cent. The contents of each well were divided in
two and stored frozen at -20°C so that, for subsequent
assaying, one half could be thawed and diluted leaving a
duplicate sample untouched. The azide acted as an
antibacterial agent to prevent later contamination of
the samples, ensuring that no bacterial collagenase or
other bacterial proteolytic enzyme interfered with assay
results.
Determination of time in culture

Studies of collagenase activity secreted by tissues in culture frequently show that little enzyme is detectable during the first 24 hours of culture, while activity often reaches a plateau after 3 or 4 days (Murphy et al 1977, Sellers et al 1978, 1979, Harris and Vater 1980, Vaes 1972, Fell et al 1986, Heath et al 1982, Werb et al 1977, Hunter et al 1984, Kishi et al 1984, Welgus et al 1985a). Oyamada and colleagues (1983), maintaining rabbit colon explants in Tyrode’s solution, also found reduced activity on the first day of culture. Their levels reached a peak by 3 days and the explants showed histological signs of deterioration after 5 days.

A pilot set of explants was cultured to confirm these reports and to determine a suitable period of culture to measure peak enzyme secretion. Twenty-eight explants, 7 from resected and 21 from post-operative colon segments, were placed in culture for 96 hours and the collagenase activity in the medium from each 24 hours was assayed by the method of Cawston and Barrett (1979), as described later (Chapter III.3.iv). With each explant the secretion of collagenase into the medium seemed to reach a plateau by 72 h of culture.
CHAPTER III.3 Methods - collagenase, culture

since the measured activity never rose significantly after 96 h and often began to fall (Tables III.3.1 and III.3.2, Figures III.3.3 and III.3.4). In subsequent experiments, therefore, explants were cultured for 72 h and the collagenase activity in the medium from each 24 h was determined.
CHAPTER III.3 Methods - collagenase, culture

Table III.3.1

Total Collagenase Activity in 7 individual explants of resected colon over 96 h in culture in U x 10^-3/mg wet weight of tissue

<table>
<thead>
<tr>
<th>Explant</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>96 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>C2Ra</td>
<td>34.00</td>
<td>247.80</td>
<td>334.77</td>
<td>306.04</td>
</tr>
<tr>
<td>C2Rb</td>
<td>0.00</td>
<td>350.68</td>
<td>361.55</td>
<td>281.97</td>
</tr>
<tr>
<td>C2Rc</td>
<td>14.47</td>
<td>374.49</td>
<td>503.97</td>
<td>546.46</td>
</tr>
<tr>
<td>C3Ra</td>
<td>59.63</td>
<td>433.75</td>
<td>490.73</td>
<td>440.03</td>
</tr>
<tr>
<td>C3Rc</td>
<td>6.87</td>
<td>58.91</td>
<td>215.09</td>
<td>200.62</td>
</tr>
<tr>
<td>C4Ra</td>
<td>34.88</td>
<td>313.72</td>
<td>382.59</td>
<td>197.78</td>
</tr>
<tr>
<td>C4Rc</td>
<td>23.74</td>
<td>319.96</td>
<td>309.28</td>
<td>229.09</td>
</tr>
<tr>
<td>Mean</td>
<td>24.80</td>
<td>299.90</td>
<td>371.14</td>
<td>314.57</td>
</tr>
<tr>
<td>± SD</td>
<td>20.18</td>
<td>120.72</td>
<td>101.38</td>
<td>132.02</td>
</tr>
</tbody>
</table>
Table III.3.2

Total Collagenase Activity in 21 individual explants of post-operative colon over 96 h in culture in U x 10^-3/mg wet weight of tissue

<table>
<thead>
<tr>
<th>Time in culture</th>
<th>Explant</th>
<th>24 h</th>
<th>48 h</th>
<th>72 h</th>
<th>96 h</th>
</tr>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Pr2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C3Pf</td>
<td>67.64</td>
<td>373.76</td>
<td>731.66</td>
<td>686.57</td>
<td></td>
</tr>
<tr>
<td>Pr</td>
<td>C4Pe</td>
<td>47.83</td>
<td>626.09</td>
<td>514.13</td>
<td>664.85</td>
</tr>
<tr>
<td></td>
<td>C4Pg</td>
<td>27.69</td>
<td>324.99</td>
<td>270.34</td>
<td>211.84</td>
</tr>
<tr>
<td>Pr</td>
<td>C2Pa</td>
<td>19.40</td>
<td>530.08</td>
<td>463.88</td>
<td>382.71</td>
</tr>
<tr>
<td></td>
<td>C2Pc</td>
<td>14.47</td>
<td>449.32</td>
<td>424.61</td>
<td>179.88</td>
</tr>
<tr>
<td>Pr</td>
<td>C2Pd</td>
<td>0.32</td>
<td>562.56</td>
<td>489.71</td>
<td>279.29</td>
</tr>
<tr>
<td></td>
<td>C3Pb</td>
<td>13.90</td>
<td>347.27</td>
<td>846.09</td>
<td>744.48</td>
</tr>
<tr>
<td></td>
<td>C4Pb</td>
<td>5.83</td>
<td>331.03</td>
<td>183.64</td>
<td>199.49</td>
</tr>
<tr>
<td></td>
<td>C2Da</td>
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<td>267.07</td>
<td>343.54</td>
<td>251.02</td>
</tr>
<tr>
<td></td>
<td>C2Db</td>
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<td>116.10</td>
<td>270.92</td>
<td>154.79</td>
</tr>
<tr>
<td>Di0</td>
<td>C3Da</td>
<td>25.29</td>
<td>404.68</td>
<td>435.15</td>
<td>416.30</td>
</tr>
<tr>
<td></td>
<td>C3Db</td>
<td>31.76</td>
<td>347.27</td>
<td>529.04</td>
<td>419.37</td>
</tr>
<tr>
<td></td>
<td>C4Da</td>
<td>67.75</td>
<td>419.24</td>
<td>803.57</td>
<td>571.82</td>
</tr>
<tr>
<td></td>
<td>C4Db</td>
<td>33.31</td>
<td>316.35</td>
<td>163.69</td>
<td>155.23</td>
</tr>
<tr>
<td></td>
<td>C2De</td>
<td>50.08</td>
<td>156.14</td>
<td>141.41</td>
<td>182.75</td>
</tr>
<tr>
<td></td>
<td>C2Df</td>
<td>21.87</td>
<td>215.89</td>
<td>192.73</td>
<td>154.79</td>
</tr>
<tr>
<td></td>
<td>C2Dj</td>
<td>73.19</td>
<td>252.44</td>
<td>185.52</td>
<td>302.73</td>
</tr>
<tr>
<td>Di2</td>
<td>C3De</td>
<td>57.81</td>
<td>345.23</td>
<td>348.61</td>
<td>363.65</td>
</tr>
<tr>
<td></td>
<td>C3Df</td>
<td>19.41</td>
<td>126.43</td>
<td>190.43</td>
<td>167.97</td>
</tr>
<tr>
<td></td>
<td>C4De</td>
<td>64.40</td>
<td>349.75</td>
<td>475.52</td>
<td>300.17</td>
</tr>
<tr>
<td></td>
<td>C4Df</td>
<td>42.57</td>
<td>328.98</td>
<td>534.93</td>
<td>438.92</td>
</tr>
<tr>
<td>Mean Activity</td>
<td>33.87</td>
<td>342.41</td>
<td>406.62</td>
<td>344.22</td>
<td></td>
</tr>
<tr>
<td>± SD</td>
<td>22.64</td>
<td>132.31</td>
<td>209.79</td>
<td>187.13</td>
<td></td>
</tr>
</tbody>
</table>

152
Figure III.3.3
Graph of collagenase activity against time in culture for 7 explants of resected colon.
CHAPTER III.3 Methods - collagenase, assay

Figure III.3.4

Graph of collagenase activity against time in culture for 21 explants of post-operative colon.
ADAPTATION OF A RADIOCHEMICAL ASSAY FOR COLLAGENASE

Current methods of quantifying collagenase activity use acid-soluble collagen as the substrate, often labelled with $^{14}$C or $^{3}$H to produce fragments which can be measured by scintillation counting (Cawston and Barrett 1979, Gisslow and McBride 1975, Johnson-Wint 1980, Terato et al 1976, Werb and Burleigh 1974, Hu et al 1978, Dean and Woessner 1985). The collagen may be allowed to form fibrils and deposited as a thin film on a suitable surface so that the fragments produced by enzyme activity are released into buffer for counting (Johnson-Wint 1980). Alternatively, the intact collagen is retained in its soluble form and separated from the products by precipitation (Cawston and Barrett 1979, Hu et al 1978, Terato et al 1976). In principle, these assays are similar to the original method of Gross and Lapiere (1962), in which the fragments produced by collagenase activity could be separated from the undegraded collagen, previously labelled 'in vivo', to measure how much activity had occurred. However the secretion of enzyme by the tissue, usually into culture medium, now takes place as a separate stage and the solution containing the enzyme is assayed by comparison with bacterial collagenase and trypsin controls.
Labelling of the purified substrate with $^{14}C$ can now be carried out 'in vitro'. These methods can be checked by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) to confirm that they are specific for the first step in collagen degradation, with the enzyme releasing the characteristic three-quarter and one-quarter fragments of collagen.

A second group of assays has been developed which employ synthetic substrates to determine collagenase activity. Masui et al (1977) synthesised a 2,4-dinitrophenyl octapeptide containing the glycine-isoleucine bond found in the $\alpha 1$ chain of type I collagen, which is the site of mammalian collagenase action. They used amino acid analysis to confirm that this was the bond which was being cleaved (Morales et al 1978) and labelled the substrate with a fluorescent tag to measure the fragments released. Gisslow and McBride (1975) and Woessner (1979) have found that these methods are not suitable for impure preparations of collagenase since the substrate is also susceptible to other proteases which may be present. The octapeptide is not a substrate for some true collagenases (Gisslow and McBride 1975) and Woessner (1979) has suggested that the methods measure the activity of another
chapter III.3 Methods – collagenase, assay

Metalloproteinase one step further along the pathway of collagen degradation than collagenase itself. Since mammalian collagenase cleaves only a single bond in each chain of the collagen molecule and has been shown to have only a limited ability to cleave the same bond in other proteins, Murphy and Reynolds (1985) concluded that the precise orientation of the bond within the collagen triple helix is essential for the efficient action of the enzyme. This view is supported by others (Harris and Krane 1974b, Eisen et al 1970, Gross et al 1980, Harper 1980). Assays employing a synthetic substrate, therefore, are less likely to reflect collagenase activity accurately than those using intact collagen.

Greater sensitivity and very precise specificity are promised in the development of methods of radioimmunoassay for collagenase (Takahashi and Koda 1984, J J Reynolds personal communication) but these are not yet fully established. For these reasons it was decided to adapt a radiochemical method which uses intact collagen as a substrate and the assay described by Cawston and Barrett in 1979 was considered suitable for colonic tissue. This assay uses acid-soluble type I collagen as its substrate, acetylated with $^{14}$C-acetic
anhydride, which attaches a radio-label to the lysine residues. The collagen is incubated with the enzyme in the presence of calcium for up to twenty-four hours. Complete breakdown is measured by the addition of bacterial collagenase to some tubes. Activator, such as trypsin or aminophenylmercuric acetate, may be added to the samples so that the active and total amounts of enzyme can be measured and latent activity calculated. In the modification of Terato et al (1976), the reaction is stopped by the addition of o-phenanthroline (oPA) in dioxane and the intact collagen is allowed to form fibrils by incubation for a further hour. The addition of a dioxane/methanol mixture improves the separation of the reaction products from the undegraded substrate by centrifugation. The proportion of radioactivity in the supernatant is counted to allow calculation of the percentage breakdown that has taken place, which is a measure of collagenase activity. The reaction products can be analysed by SDS-PAGE (Cawston and Tyler 1979) to show that the collagen has been split into the three-quarter and one-quarter 'clips', but not into smaller fragments, confirming the specificity of the assay for collagenase.
Labelling of collagen

The procedure for acetylating collagen with $^{14}$C-acetic anhydride, described by Cawston and Barrett (1979), was applied to acid-soluble type I collagen (prepared from calf skin, catalogue heading 'Type III', Sigma UK, Ltd). 250 mg of collagen, dissolved in 50 ml 0.2 M acetic acid, was dialysed at 4°C against 10 mM disodium tetraborate containing 200 mM CaCl$_2$ (pH 9.0). 250 μCi of [1-$^{14}$C] acetic anhydride (Amersham International plc) in dry dioxane was added to the collagen and stirred slowly at 4°C for 2 to 3 hours. The labelled collagen was dialysed at 4°C against several changes of 50 mM Tris/HCl, pH 7.6, containing 200 mM NaCl, 5 mM calcium acetate and 0.03 per cent toluene, until all unbound radiolabel had been removed (approx 80 per cent of the original amount). Labelled collagen was diluted to 1 mg/ml with 0.2 M acetic acid and then mixed with unlabelled collagen (also 1 mg/ml) until there were approximately 60 000 disintegrations per minute per milligram of collagen. The labelled collagen was stored in acetic acid at -20°C and, before use, was thawed and dialysed against 50 mM Tris/HCl, pH 7.6, containing 200 mM NaCl and 0.5 per cent Brij-35.
CHAPTER III.3 Methods - collagenase, assay

Assay conditions

To run an assay, the appropriate sample dilutions and control solutions were prepared in triplicate in microcentrifuge tubes and made up to 200 µl with 100 mM Tris/HCl buffer, pH 7.6, containing 15 mM CaCl₂ and 3 mM phenylmethylsulphonyl fluoride (PMSF), so that every tube contained at least 100 µl of this buffer. Calcium is the metal ion required for the action of collagenase (Woessner 1980a, Mookhtiar et al 1986a), while PMSF inhibits any serine proteases which may be present in the sample (Liotta et al 1982). Samples were assayed directly to measure active collagenase. Total enzyme activity was also measured by activation of a second set of samples with 0.67 mM aminophenylmercuric acetate (APMA). APMA was dissolved initially in 200 µl of dimethyl sulphoxide (DMSO) and then diluted with 100 mM Tris buffer, pH 8.2, to reach a concentration of 10 mM. 40 µl of this solution in each microcentrifuge tube gave a final concentration of 0.67 mM, established by other workers as the optimum activating concentration for collagenase (Sellars et al 1977, Cawston and Tyler 1979, Heath et al 1982, Fell et al 1986).

100 µg of labelled collagen (100 µl of 1mg/ml) was added to each tube as substrate. The tubes were mixed
and incubated in a water-bath at 35°C for 15 hours. Preliminary experiments, detailed below, determined the optimum temperature for this system and the optimum time for incubation.

After incubation, the reaction was stopped by the addition of 20 μl 1,10-phenanthroline (oPA), 80 mM in 50 per cent dioxane, and the tubes were returned to the water-bath for a further hour to allow any undegraded collagen to form fibrils. The contents were then mixed with 0.4 ml dioxane/methanol 4:1, to aid the precipitation of undegraded collagen, and centrifuged for 5 minutes at 11,000 G in a Hawksley Microhaematocrit centrifuge.

To measure breakdown, 0.4 ml of each supernatant was mixed with 3 ml emulsifier scintillant (ES 299, Packard) and counted in a Packard Tri-Carb 2000CA liquid scintillation counter.
Determination of precise conditions of assay

1. Temperature

Collagenase is an enzyme which remains active, albeit with reduced efficiency, at temperatures below 37°C while other proteases are almost inactive below body temperature; therefore running an assay at less than 37°C will help to increase its specificity for collagenase. However, assays for collagenase demand precise temperature control, since the rate at which the enzyme activity varies with temperature is high. The activity of human skin fibroblast collagenase has been shown to decrease three-fold for every 2°C decrease in temperature between 37 and 30°C (Harris et al 1984), so a small difference in temperature between one assay run and the next, or even during the course of a 15 hour incubation, will considerably alter the amount of enzyme activity measured and invalidate any comparison of results between runs. For this reason, the definition of a unit of collagenase activity should include the temperature at which that activity was measured.

In addition, collagen denatures to gelatin at 60°C and even purified preparations show a small proportion of spontaneous breakdown at lower temperatures (Woessner 1980a, Harris and Vater 1980). The process of labelling
with $^{14}$C was also found to render the collagen more susceptible to spontaneous degradation, as noted by Dean and Woessner (1985). It may, therefore, be advantageous to run a collagenase assay at a temperature which is below the optimum for the enzyme, in order to reduce this 'background' level of breakdown and also to reduce interference by the action of other proteases (Harris and Vater 1980).

A series of assays, using medium samples from normal colon explants, was run at a range of temperatures between 31 and 35°C to determine the optimum temperature for the system to be used in this investigation. Table III.3.5 shows the readings for buffer only tubes and bacterial collagenase tubes when incubated between 31 and 35°C and the increase in measured collagenase activity in medium from normal colon over this temperature range. Above 35°C, the level of spontaneous breakdown of labelled collagen became unacceptably high. At 35°C, the level of spontaneous breakdown was sufficiently low and the levels of activity in the samples were sufficiently high for the assay to give a useful range of measurement of collagenase activity (Fig III.3.6). Many other investigators (Terato et al 1976, Eisenberg et al 1984,
CHAPTER III.3 Methods - collagenase, assay

Table III.3.5
Effect of Temperature of Incubation on Control Readings and Total Collagenase Activity of Normal Colon Explants. Figures are means from three assay runs.

<table>
<thead>
<tr>
<th>Temp of Buffer incubation in °C</th>
<th>Total in dpm</th>
<th>Blank in dpm</th>
<th>% Blank</th>
<th>Mean total collagenase breakdown of total normal colon, 72 h U x 10^{-3}/mg wet weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>31</td>
<td>881</td>
<td>4970</td>
<td>17.72</td>
<td>4.63</td>
</tr>
<tr>
<td>32</td>
<td>564</td>
<td>5088</td>
<td>11.08</td>
<td>18.99</td>
</tr>
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<td>33</td>
<td>827</td>
<td>5530</td>
<td>14.95</td>
<td>68.74</td>
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<td>34</td>
<td>939</td>
<td>5435</td>
<td>17.28</td>
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</tr>
<tr>
<td>35</td>
<td>1466</td>
<td>5393</td>
<td>27.18</td>
<td>356.87</td>
</tr>
</tbody>
</table>

164
CHAPTER III.3 Methods - collagenase assay

Figure III.3.6

Effect of temperature of incubation on collagenase assay

![Bar graph showing the effect of temperature on collagenase activity. The x-axis represents incubation temperature in °C (31 to 35), and the y-axis represents collagenase activity in dpm. The graph shows a peak in collagenase activity at 35 °C.]
CHAPTER III.3 Methods - collagenase, assay

Heath et al 1982, Kishi and Hayakawa 1982, Kishi et al 1984, Fell et al 1986, Sellers et al 1977, Golub et al 1984, Hunter et al 1984, Werb and Burleigh 1974, Pettigrew et al 1980) have also used 36°C as a working temperature for collagenase assays. A water-bath temperature of 35°C was therefore established as the most appropriate for the present system and precise control to within 0.5°C was maintained for all subsequent assays, using a Grant waterbath with thermostatic control checked by independent thermometer. A subsequent study by Mookhtiar et al (1986) has demonstrated that radiolabelled preparations of collagen, used as assay substrates, have different susceptibilities to spontaneous degradation as the temperature increases. This is dependent on the source of collagen. Collagen derived from calf skin, as used in the present assay, is more labile above 35°C than collagen derived from other sources.

2. Time of incubation

As with most enzymes, the activity of collagenase increases with time of incubation at a fixed temperature. However, while many enzymes show a rapid rate of increase in activity and reach a plateau after
30 or 60 minutes (bacterial collagenase produced 100 per cent breakdown of collagen within one hour), mammalian collagenase activity increases gradually over a much longer period of time. In addition, the background level of spontaneous collagen degradation was found to increase gradually over several hours. Assays were therefore set up with matching samples and run for a range of incubation times at 35°C to establish the time period over which the rate of increase in collagenase activity was linear, while keeping the background level of degradation reasonably low. Collagenase activity at 35°C was calculated after different incubation times between 4 and 8 hours and between 14 and 20 hours (at 2 hour intervals). Table III.3.7 shows the calculated active and total collagenase activity for two of these samples over these incubation times. The calculated activity (which takes account of time of incubation) shows a plateau between 8 and 16 h and then begins to fall (Fig III.3.8). For practical reasons, an incubation time of 15 hours was established as a suitable time for routine assay of large numbers of samples.
CHAPTER III.3 Methods - collagenase, assay

Table III.3.7

Effect of Time of Incubation on Collagenase Activity.
Each result derived from four dilutions of medium.

<table>
<thead>
<tr>
<th>Time of incubation in hours</th>
<th>Blank as % breakdown</th>
<th>Total Active</th>
<th>Total Collagenase U x 10^-3/mg wet tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>11</td>
<td>57.9</td>
<td>49.6</td>
</tr>
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<td>6</td>
<td>17</td>
<td>80.4</td>
<td>33.6</td>
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<td>104.0</td>
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<td>16</td>
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<td>97.1</td>
<td>61.2</td>
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<td>18</td>
<td>27</td>
<td>71.6</td>
<td>40.3</td>
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<tr>
<td>20</td>
<td>26</td>
<td>68.9</td>
<td>54.5</td>
</tr>
</tbody>
</table>

168
CHAPTER III.3 Methods - collagenase, assay

Figure III.3.8

Effect of time of incubation on collagenase assay.

![Graph showing effect of time of incubation on collagenase assay.](image-url)
3. Dilution of samples of medium

Standard preparations of mammalian collagenase are not commercially available for calibration of an assay system. Moreover, when samples of culture medium from colonic explants were first assayed the concentration of enzyme present was unknown, so a series of dilutions was tested to find a concentration within the range of the assay system. Accurate determination of collagenase activity was only possible when the collagen breakdown by the enzyme (after subtraction of the buffer blank) was between 15 and 70 per cent of the total collagen breakdown produced by bacterial collagenase. In addition, the level of activity varied between one sample and another, so a range of dilutions was established which ensured that at least one dilution of a sample lay within the range of activity of the assay.

Incubating for 15 hours at a temperature of 35°C, up to 0.96 Unit of collagenase activity per milligram of tissue could be measured satisfactorily, where 1 unit is that activity which will degrade 1 µg of collagen per minute at 35°C. Dilutions of culture medium which contained 40, 20, 10 and 5 µl of each sample were found to give levels of activity such that at least two, and usually three or four, of the dilutions showed a linear
increase in the measured amount of collagen breakdown with increased volume of sample at this temperature and time of incubation. These four dilutions were therefore assayed routinely for each sample of medium and this gave a further check on the assay system since most samples confirmed the linearity of activity with enzyme concentration in the absence of a standard solution of collagenase.

Control tubes

A set of control tubes was included in every assay run to check on the conditions and to allow calculation of the collagenase activity in the samples. Background levels of spontaneous collagen degradation were measured in tubes containing buffer and labelled collagen only. Background levels greater than 30 per cent of total breakdown suggested a defect in the run, such as a rise in temperature. Total degradation of the collagen was measured by the addition of 100 μg of bacterial collagenase from Clostridium histolyticum (Type IA, Sigma UK, Ltd). In addition, every run of the assay included a set of tubes containing collagen, buffer and 10 μg trypsin. This demonstrated the susceptibility of the preparation of labelled collagen to non-specific
proteases and was used as 'quality-control' to confirm that the batch of collagen had not denatured at any stage. Since preparations of mammalian collagenase are not available and it was not possible to plot a standard curve of activity, background and trypsin controls were used to assess whether the precise assay conditions for any one run were sufficiently altered to invalidate the calculation of collagenase activity in the samples.

**Calculation of collagenase activity**

In each assay run, the buffer control tubes provided a count of disintegrations per minute (dpm) for background collagen breakdown. This level was subtracted from all other counts before any further calculations were carried out. The bacterial collagenase tubes gave the level for 100 per cent breakdown and enzyme activity was calculated proportionately from this figure. Since each tube contained 100 μg of collagen this could be converted to a figure expressing how much collagen had been degraded in each tube in a specific time. One unit of collagenase activity is defined as that required to degrade 1 μg of collagen per minute at the temperature of incubation. The number of units of collagenase
activity per tube at 35°C could therefore be calculated and adjusted to give units per ml of sample. Samples consisted of culture medium, with tissue explants each cultured in 4 ml medium and the medium changed every 24 hours, so one 4 ml collection contained all the enzyme secreted by the explant in that 24 hours. By taking into account the dilution of the medium, the units of activity could then be expressed per mg wet weight of tissue, for each 24 h in culture.

The following formula was derived for calculation of collagenase activity in a sample of culture medium:

\[ a = \text{dpm for sample (mean of 3 tubes, background subtracted)} \]
\[ b = \text{dpm for total breakdown from bacterial collagenase tubes (mean of 3 tubes, background subtracted)} \]
\[ w = \text{mg wet weight of tissue explant} \]
\[ t = \text{hours of incubation, at 35°C} \]
\[ v = \mu l \text{ volume of medium assayed} \]

Since \( b = \text{dpm from breakdown of 100 } \mu g \text{ collagen} \)
\[ a \text{ dpm represents breakdown of } 100 \times \frac{a}{b} \mu g \text{ collagen by the sample in } t \text{ hours} \]
CHAPTER III.3 Methods - collagenase, assay

In 1 minute:

\[
\frac{100 \times a}{60 \times t \times b} \, \mu g \, \text{collagen degraded}
\]

For 1 ml medium:

\[
\frac{1000 \times 100 \times a}{60 \times t \times b \times v} \, \mu g \, \text{collagen degraded per minute} = \text{units of collagenase activity at } 35^\circ C
\]

For 4 ml medium from w mg tissue:

\[
4 \times 1000 \times 100 \times a \, \text{units collagenase activity per} \\
60 \times t \times b \times v \times w \, \text{mg tissue per 24 hours culture}
\]

Assayed over 15 h:

\[
444.4 \times a \, \text{units collagenase activity per mg tissue} \\
b \times v \times w
\]

Electrophoretic check of assay specificity

When the assay conditions had been fully defined, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out, using the method of Laemmli (1970, Laemmli and Favre 1973), to confirm that the enzyme activity being measured was specific to mammalian collagenase. Concentrated samples incubated at a reduced temperature of 25°C were run in 7.5 per cent gels to separate the reaction products and to compare them with undegraded collagen and with the products of bacterial collagenase tubes. The reaction
products of true mammalian collagenase activity are
three-quarter and one-quarter fragments of the collagen
triple helix. These should be seen as bands below the
intact collagen on the gel as shown in Fig III.3.9,
drawn from Cawston and Tyler (1979).

Preparation of plates:

Stock acrylamide solution consisted of 29.2 g
acrylamide and 0.8 g bisacrylamide in 100 ml H₂O. 7.5
per cent slab gels were made from a degassed mixture of
10 ml stock acrylamide, 10 ml 1.5 M Tris buffer, pH 8.8
with 0.4 g/100 ml sodium dodecyl sulphate (SDS), 18 ml
H₂O, 2 ml ammonium sulphate (15 mg/ml) and 30 µl
NNN’N’-tetramethylenediamine (TEMED). 3 per cent
stacking gels used 1 ml stock acrylamide, 1.25 ml 0.5 M
Tris buffer, pH 6.8 with 0.4 g/100 ml SDS, 6 ml H₂O, 0.5
ml ammonium sulphate and 10 µl TEMED. Plates were
prepared in advance and stored at 4°C but stacking gels
were added immediately before use.

Preparation of samples:

Assay tubes were set up with activated samples and
twice the normal buffer concentration to allow a more
concentrated solution of reaction products to be applied
Demonstration of collagen breakdown products by SDS-PAGE (reproduced from Cawston and Tyler 1979).

1 Purified pig synovial collagen + type I collagen

2 Type I collagen alone
to the gel. The tubes were incubated for 15 hours at 25°C so that the products of collagenase activity would not spontaneously degrade any further. At the end of incubation, 50 µl of 50 mM Tris/HCl buffer, pH 7.6, containing 10 per cent glycerol, 2.5 per cent SDS, 4 per cent mercaptoethanol and 0.01 per cent bromphenol blue, was added to each tube. This mixture was heated to 100°C for 10 minutes so that the mercaptoethanol could break down any collagen cross-links (which are not split by collagenase). The addition of glycerol helps to retain the sample in its channel when it is applied to the gel and bromphenol blue is a tracker dye so that progress through the gel can be monitored.

**Electrophoresis:**

Gels were run in 25 mM Tris buffer, pH 8.4, containing 192 mM glycine and 0.1 per cent SDS, at 15°C. 60 µl samples were run into the gel at 25 volts for 45 minutes and then at 60 volts until the tracker dye approached the end of the plate (3 to 4 hours). Gels were fixed in a solution of 136 mM 5-sulphosalicylic acid and 734 mM trichloroacetic acid and then equilibrated with destain solution (H₂O / methanol / glacial acetic acid, 6:3:1) before staining for 2 hours.
CHAPTER III.3 Methods - collagenase, assay

with PAGE blue (G 90, 'Electran', BDH Ltd.), 0.2 per cent in H₂O / methanol / glacial acetic acid, 45:45:10. Multiple changes of destain, with 10 per cent glycerol in the last change, were applied until the background was almost clear and then the gels were stored in 10 per cent acetic acid, which removed the remaining colour from the background without affecting the stained bands.

Electrophoretic gels were run at intervals, with random samples of culture medium, to confirm the specificity of the collagenase assay. On each gel the bands produced by enzyme activity were compared with those from undegraded radiolabelled collagen substrate and with a molecular weight marker ('Electran' molecular weight marker, range 12 300 - 78 000, BDH Ltd). Figure III.3.10 shows a typical gel. The molecular weight marker, in channel 1, shows bands for ovotransferrin, albumen, ovalbumen and carbonic anhydrase, as marked. Channel 3 shows the α and β bands produced by intact collagen, while in channel 4 bacterial collagenase activity has broken the collagen into fragments too small to be retained by the gel. Culture medium samples have been applied to channels 2, 5 and 6. Each shows a band which corresponds to the albumen in the molecular
CHAPTER III.3 Methods - collagenase assay

Figure III.3.10

Results of SDS-PAGE experiment to demonstrate the specificity of the collagenase assay.

1. Molecular weight marker.  
2, 5, and 6. Collagen + samples:  2 and 6. show activity; 5. very little activity.

3. Collagen only.  
4. Bacterial collagenase + collagen.
weight marker and is due to the presence of albumen in the foetal calf serum added to the culture medium. Sample Cl3Pa2, in channel 5, had little collagenase activity when measured by the assay and produced only the bands of intact collagen on the gel. Samples Cl3Pb2 and Cl3Ph2, in channels 2 and 6, both had sufficient activity to degrade some collagen, producing α^a and β^a bands alongside the α and β bands. Faint α^a bands are also seen further down the gel in these channels.

The radiochemical assay measured an enzyme which was dependent on calcium, inhibited by EDTA and oPA, active at pH 7.6 and at temperatures from 25° to 37°C. Random samples run on SDS-PAGE showed that the products of degradation of the collagen were the characteristic three-quarter and one-quarter fragments and that the radiochemical assay was therefore specific for mammalian interstitial collagenase.

For subsequent experiments medium samples were incubated with labelled collagen as described for 15 hours at 35°C. Each assay run included a set of control tubes. 40, 20, 10 and 5 µl volumes of each sample were assayed in triplicate to measure active collagenase and a second set of tubes was activated with APMA to measure
CHAPTER III.3 Methods - collagenase, assay

total enzyme activity. The linear portion of its own
dilution curve was used to calculate the activity in any
one sample and the mean result from the medium collected
from three separate explants gave the value for
collagenase activity in each segment of colon for each
24 hours in culture. The coefficient of variation in
medium samples from sets of three explants was
calculated for this assay (Table III.3.11). In ten
segments of colon the mean coefficient of variation was
11.37 for the measurement of active collagenase and
16.07 for total collagenase activity.
CHAPTER III.3 Methods - collagenase assay

Table III.3.11

Coefficient of variation for collagenase assay between 3 explants from each segment of colon.

Collagenase activity in U x 10^-3/mg wet tissue

| Explant Explant Explant Mean of explants Coefficient of variation |
|------|------|------|------------------|-----------------|
| a    | b    | c    | ± SD of variation |
| 140.93 | 168.56 | 142.87 | 150.79 ± 15.42 | 10.23 |
| 300.56 | 308.54 | 278.81 | 295.97 ± 15.39 | 5.20 |
| 341.16 | 368.14 | 474.02 | 394.44 ± 70.23 | 17.80 |
| 464.36 | 357.16 | 369.02 | 396.85 ± 58.77 | 14.81 |
| 301.83 | 244.34 | 273.35 | 273.17 ± 28.75 | 10.52 |
| 262.83 | 347.65 | 307.64 | 306.04 ± 42.43 | 13.86 |
| 313.43 | 342.53 | 335.38 | 330.45 ± 15.16 | 4.59 |
| 124.09 | 131.50 | 135.35 | 130.31 ± 5.72 | 4.39 |
| 173.98 | 218.26 | 246.21 | 212.82 ± 36.42 | 17.11 |
| 86.19  | 114.72 | 93.08  | 98.00 ± 14.89 | 15.19 |

Mean coeff of variation 11.37
standard error 4.92

Total activity in 10 segments

| Explant Explant Explant Mean of explants Coefficient of variation |
|------|------|------|------------------|-----------------|
| a    | b    | c    | ± SD of variation |
| 461.26 | 490.44 | 669.87 | 540.52 ± 112.96 | 20.90 |
| 693.33 | 425.56 | 596.51 | 571.80 ± 135.58 | 23.71 |
| 329.60 | 298.44 | 398.44 | 342.16 ± 51.17 | 14.95 |
| 280.18 | 229.07 | 336.84 | 282.03 ± 53.91 | 19.11 |
| 439.32 | 376.66 | 438.47 | 418.15 ± 35.93 | 8.59 |
| 265.66 | 186.94 | 279.40 | 244.00 ± 49.89 | 20.45 |
| 487.46 | 410.27 | 304.90 | 400.88 ± 91.64 | 22.86 |
| 547.54 | 598.83 | 446.57 | 530.98 ± 77.47 | 14.59 |
| 915.42 | 837.62 | 911.59 | 888.21 ± 43.85 | 4.94 |
| 127.63 | 103.00 | 117.63 | 116.09 ± 12.39 | 10.67 |

Mean coeff of variation 16.08
standard error 6.07
CHAPTER III.4 Methods - immunohistochemistry

IMMUNOLOCALISATION OF COLLAGENASE AND TIMP

An alternative approach to the investigation of collagenase in colonic anastomotic healing is by the use of specific antibodies to both rabbit collagenase and its inhibitor, tissue inhibitor of metalloproteinases (TIMP). A reliable method of radioimmunoassay for collagenase is not yet available (J J Reynolds, personal communication) but antibody localisation of the enzyme in tissue can be carried out. Histochemical techniques are employed to apply the antibody to tissue sections and to visualise its distribution by the application of a second antibody labelled with fluorescein isothiocyanate (FITC) (Hembry et al 1986).

Immunolocalisation of the enzyme provides information of a different character from that available by assay: it gives an assessment of the distribution of collagenase through the tissue layers and can be used to identify the synthesising cells which are the source of any increase in secretion. In addition, this approach requires only limited culture of the tissue, overcoming many of the criticisms of the radiochemical assay employed in this investigation (Chapter III.3.iv), since the results will reflect more closely the situation in
the animal at the time of sacrifice than measurements from tissue cultured for up to 72 hours.

The accuracy of information obtained from such a technique is clearly dependent on the specificity of the antibodies employed (Woolley et al 1980). Previous immunolocalisation studies of collagenase have indicated a wide distribution of the enzyme in normal tissues (Montfort and Pérez-Tamayo 1975a and b). This does not correlate with biochemical findings and raises doubts about the specificity of the antibodies employed (Hembry et al 1986). The antibodies used in the present study were developed at Strangeways Research Laboratory, Cambridge. Anti-collagenase IgG was produced by the immunisation of sheep with a purified preparation of rabbit collagenase and the specificity of the antibody was determined by double immunodiffusion, electrophoretic immunoblotting and immunoprecipitation (Hembry et al 1986). This antibody was characterised in experiments with collagen fibrils, cultured rabbit chondrocytes and post-partum rabbit uterus. It was shown to react with both active and latent collagenase and can be used to identify extracellular enzyme, enzyme bound to collagen and secretory granules within cells. A study of the distribution of collagenase in rheumatoid
CHAPTER III.4 Methods - immunohistochemistry

synovium by Woolley et al (1977) used an antibody which reacted primarily with active enzyme and therefore limited the scope of the investigation.

The sensitivity of histochemical methods is greater than that of the radiochemical assay, since a specific antibody can localise collagenase synthesis within a single cell, but these methods cannot be considered truly quantitative and caution must be applied in drawing conclusions about the relative amounts of activity seen in one tissue section compared with another.

Antibody to tissue inhibitor of metalloproteinases was developed by immunising sheep with a preparation of purified rabbit TIMP (Gavrilovic et al 1987). The specificity of this antibody was demonstrated by similar methods to those used for the sheep anti-rabbit-collagenase and characterised in experiments using cultured chondrocytes and endothelial cells. This antibody adds another dimension to the investigation of collagenase in the colon, since the localisation and distribution of the inhibitor can be compared directly with those of the enzyme when the two antibodies are applied to adjacent sections cut from the same block of tissue. Localisation of the inhibitor alongside the
enzyme would suggest a limitation of enzyme activity in those areas, since collagenase is rapidly inhibited by TIMP.

Non-immune IgG was prepared from pooled normal sheep serum in the same way as anti-collagenase and anti-TIMP IgG (Hembry et al 1985) and used alongside these antibodies as a control for the non-specific uptake of second antibody.

The second antibody was a pig anti-sheep monovalent antibody fragment, prepared from the serum of pigs immunised against antibody monomer from pooled normal sheep serum; this was then labelled with fluorescein isothiocyanate (Hembry et al 1985). This pig-FITC binds to whichever sheep IgG has been applied to the tissue previously and allows it to be visualised with a fluorescent microscope.

Preparation of tissue

Segments of rabbit colon were removed at operation or post-mortem as required and opened along the anti-mesenteric border. Any faecal pellets were removed with forceps and the segment was immersed in Hank’s balanced salts solution (Table A.2 in Appendix). Each piece of colon was washed by soaking and intermittent
agitation in five changes of Hank's solution, the first three containing 10 times the usual concentration of antibiotics, in the same way as tissue was prepared for culture (Chapter III.3.iii). The segment was transferred to a tissue culture hood whilst in the fifth wash and dissected with sterile instruments. Each segment of colon was cut longitudinally into strips, 3 mm wide, and adjacent strips were either processed immediately or placed into short-term tissue culture. One strip from each segment of colon was fixed in formal saline to be processed for routine histology.

The strips of tissue for short-term culture were placed in separate 3.5 cm diameter wells of a six-well tissue culture plate, each containing 4 ml of Dulbecco's modification of Eagle's Medium (Table A.3 in Appendix). The medium was prepared in the same way as for longer-term culture for the collagenase assay and contained the same supplements and antibiotics with the addition of 5 μM monensin. Monensin is a monovalent ionophore which prevents the translocation of secretory proteins (Ledger et al 1980, Nagase et al 1983); this causes the accumulation of collagenase and TIMP in the Golgi apparatus and secretory granules of cells which are actively synthesising enzyme or inhibitor, making
them easier to identify microscopically (Hembry et al 1985, 1986). Culture plates were incubated in a humidified atmosphere at 37°C for three, six or twenty-four hours. The tissue was then removed from culture and processed in the same way as uncultured tissue.

Each strip of colon, cultured or not, was orientated on a millipore filter and embedded in 7 per cent gelatin in 0.9 per cent saline, containing 0.2 per cent sodium azide as an antibacterial agent. Filters were indelibly marked, so that the proximal end of the tissue could be identified when cutting sections, and the strip was orientated so that sections would be cut at 90° to the plane of the bowel wall to show a longitudinal section of the segment. Tubes containing tissue and gelatin were frozen for 90 seconds in liquid nitrogen and stored at -20°C until required.

**Histocherstry**

Gelatin blocks were transported in liquid nitrogen, as necessary, for cutting. Frozen sections, 4 to 7 μ thick, were cut on a Slee cryostat and fixed for 30 minutes in 4 per cent paraformaldehyde in phosphate buffered saline (PBS, composed of 8.5 g NaCl, 2.56 g
anhydrous Na$_2$HPO$_4$ and 0.16 g NaH$_2$PO$_4$.2H$_2$O per litre, brought to pH 7.35 with 6 M HCl). After fixing, sections were washed in PBS. All washing stages comprised three changes of PBS at intervals of 5 minutes. The tissue was then permeabilised in 0.1 per cent Triton X-100 for 5 minutes to allow subsequent penetration of antibody into cells. After further washing, sections were treated for 10 minutes with 4-chloronaphthol, 2.8 mM in methanol/PBS (1:5) containing 0.01 per cent hydrogen peroxide, to prevent the non-specific binding of FITC-antibody by inflammatory cells (Johnston and Bienenstock 1974, Kingston and Pearson 1981). Sections were washed again and incubated at room temperature in a moist atmosphere for 30 minutes with 5 µg of either anti-collagenase IgG, anti-TIMP IgG or non-immune sheep serum IgG (NSS) (100 µl of a dilution containing 50 µg/ml in PBS). Slides were washed to remove excess first antibody and incubated for 30 minutes with 2.75 µg of second antibody, pig anti-sheep Fab (11.0 mg/ml preparation diluted 1:400 in PBS and 100 µl used). The sections were washed once more, stained for 2 minutes with methyl green (1 mg/ml) as nuclear counter-stain, and washed again. Dried slides were mounted in glycerol/PBS.
CHAPTER III.4 Methods - immunohistochemistry

mounting fluid containing additives to reduce fading of fluorescence (Citifluor Ltd., London).

Sections were examined on the same day by epifluorescence, using either a Leitz Dialux 20 photomicroscope or a Zeiss Photomicroscope III, with standard wide and narrow-band filters. Sections were examined under low power with a rhodamine filter to map the layout of the tissue and then under higher powers with the FITC filter to determine the localisation of fluorescent antibody within it. Slides incubated with NSS served as controls for the non-specific uptake of FITC-antibody. Fluorescent photomicrographs were taken on Kodak Ektachrome 400 ASA Daylight colour film uprated to 1600 ASA during processing (Colour Processing Laboratories Ltd., London).

Routine histology

Segments of colon which had been fixed in formal saline after removal from the animal were embedded in paraffin wax for staining with haematoxylin and eosin (H and E), so that the findings in frozen sections from gelatin blocks could be compared with standard histopathology. Dried sections were cleared in xylene and dehydrated in alcohol; they were stained with
haematoxylin for 10 minutes, washed and counter-stained with eosin for 15 seconds. Slides were mounted in DPX and examined on a Leitz Dialux 20 microscope using a tungsten lamp. Photomicrographs were taken on Kodak Ektachrome 50 ASA colour film for tungsten light.

Selected sections from gelatin blocks were also restained with haematoxylin and eosin for more direct comparison of the findings, since areas and cells which had shown collagenase or TIMP activity could then be identified specifically by H and E. For restaining of frozen sections, which had been stored at 4°C, the cover slips were removed by soaking in PBS and the slides washed in tap water. Sections were stained for 1 minute in Harris' haematoxylin, washed in water and counter-stained for 15 seconds with eosin. After further washing, slides were dehydrated in alcohol, cleared in CNP and mounted in DPX. Examination and photography were as for formalin-fixed tissue.

Experiments to determine methodological details

Neither collagenase nor TIMP could be localised in sections from normal rabbit colon resected at operation. This accords with the expectation that insufficient enzyme and inhibitor are present in unstimulated tissue.
CHAPTER III.4 Methods - immunohistochemistry
to be identifiable by this method and is in agreement
with the findings in non-gravid rabbit uterus, normal
rabbit cartilage (Hembry et al 1986) and inactive scar
tissue (Hembry and Erlich 1986). The stimulation of six
hours in culture was insufficient to alter this finding.
Both collagenase and TIMP could, however, be identified
easily in post-operative colon, with and without
culture. All further details of methodology were
therefore determined on post-operative colon, while
carrying out the earliest of the experiments in
application of this technique.

Culture with monensin

Adjacent strips of tissue were cultured for three,
six or twenty-four hours with monensin to assess the
time period required for complete penetration of the
tissue by the ionophore. There was no difference in the
distribution of collagenase or TIMP secreting cells seen
after three or six hours culture, indicating that the
penetration of monensin into the tissue was complete
within three hours. At some post-operative times
secreting cells were found in uncultured tissue while at
others cells were seen after culture only, confirming
the need for short-term culture to map completely the
CHAPTER III.4 Methods - immunohistochemistry

changes at different post-operative stages. Restaining of sections from cultured tissue with haematoxylin and eosin, however, showed marked deterioration compared with tissue which had been embedded in gelatin immediately; such destruction was considerably greater after six hours than after three (Fig III.4.1). Tissue which had been cultured for twenty-four hours underwent so much deterioration that sections were difficult to cut and orientation of the tissue was sometimes impossible. In all further studies, therefore, culture with monensin was confined to 3 hours to minimise tissue damage and retain sufficient structure to appreciate fully the locations of enzyme and inhibitor.
CHAPTER III.4 Methods - immunohistochemistry

Figure III.4.1

Effect of short-term culture on colonic tissue.

Gelatin-embedded frozen sections restained with H and E.

Bar = 50 \mu m

Similar regions of colon prepared a) fresh and b) after 6 hours culture showing marked deterioration and cell loss in culture, particularly in the mucosal layers.
CHAPTER IV.1 Investigation - biochemistry

INVESTIGATION OF COLLAGENASE IN COLONIC HEALING

BIOCHEMICAL MEASUREMENTS

(i) EXPERIMENTS

The methods for measurement of collagen and protein content of tissue, established as most suitable in Chapter III.2, and the specific assay for collagenase activity of culture medium developed in Chapter III.3 were applied to rabbit colon to investigate the quantitative changes in collagen and collagenase in the early post-operative period after anastomosis.

Previous studies have shown that the collagen concentration of rabbit colon falls after resection and anastomosis, showing some decrease within 3 hours and reaching a trough level 48 hours after operation (Hawley et al 1970, Hawley 1970, Irvin and Edwards 1973, Hesp et al 1984a, Hendricks et al 1985). If increased collagenase activity is responsible for the decrease in collagen, the enzyme level might be expected to reach a peak before the trough in collagen occurs, probably within 24 hours of surgery, and decline thereafter. The timing for investigation was therefore chosen to coincide with this anticipated peak of collagenase
activity and to cover the period when activity should be decreasing. Tissue was collected on the first and third post-operative days, in different animals, and the segments resected at operation gave values for normal tissue, with each rabbit providing its own control.

Validation of control segment

Resection of a 2 cm segment at operation meant that any tissue removed post-operatively was taken from colon which was initially proximal or distal to the control segment. A preliminary set of measurements was therefore made to establish whether the hydroxyproline concentration of colon varied anatomically over this region. Six healthy rabbits were killed without any operation being performed. From each, the 2 cm 'resected' segment, described in Chapter III.1, was removed along with 2 cm segments proximal and distal to this, which would have become the 'proximal' and 'distal' segments had an anastomosis been made. This tissue was homogenised and freeze-dried for hydroxyproline assay, as described in Chapter III.2. Table IV.1.1 shows the results for each segment in each animal; as before, each value is the mean concentration derived from duplicate assays of 5 aliquots of
### Table IV.1.1

Hydroxyproline Concentration of Normal Rabbit Colon

in µg/mg dry weight of tissue

<table>
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<th></th>
<th>&quot;Proximal&quot;</th>
<th>&quot;Resected&quot;</th>
<th>&quot;Distal&quot;</th>
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<td>12.75</td>
</tr>
<tr>
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</tr>
<tr>
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<tr>
<td>AC</td>
<td>13.93</td>
<td>14.17</td>
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</table>

Mean ± SD 10.61 ± 1.96 12.23 ± 3.96 12.03 ± 1.17

Log transformed figures

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</table>

Mean ± SD 2.35 ± 0.17 2.45 ± 0.38 2.48 ± 0.10
CHAPTER IV.1 Investigation - biochemistry

homogenate from each segment of tissue. The normal hydroxyproline concentration of rabbit colon did not vary significantly over this distance at this site, confirming that the resected segment in each animal was a valid control for the subsequent post-operative measurements of hydroxyproline (see page 202 for statistical methods).

Biochemical measurements before and after anastomosis

The standard colonic resection and anastomosis, described in Chapter III.1, was carried out on 10 rabbits. The resected tissue from each was retained, washed in Hank's solution and four explants were removed and placed in culture for collagenase assay as described in Chapter III.3. The remaining portion was homogenised and freeze-dried for hydroxyproline and protein assay. Five animals with uncomplicated anastomotic healing were killed twenty-four hours after operation and five after three days. Any adhesions were divided carefully and a 5 cm segment of colon containing the anastomosis at its centre was removed. Washing in five changes of Hank's solution was carried out in preparation for tissue culture, as described in Chapter III.3, and then the anastomosis was divided at the stitch line so that
CHAPTER IV.1 Investigation - biochemistry

proximal and distal tissue could be processed separately (Fig IV.1.2). Strips of colon were taken adjacent to the suture line on each side (Pr0 and Di0) and 2 cm away, proximally and distally (Pr2 and Di2). From each strip of tissue, 4 full-thickness explants, each approximately 40 mg, were weighed and placed into culture for collagenase assay. The remaining tissue in each strip was homogenised and freeze-dried for hydroxyproline and protein assays.

Hydroxyproline and protein concentrations were calculated for each segment of colon by taking the mean values from duplicate assays of 5 aliquots of homogenate in each case. The results for groups of 5 animals were compared, with each animal providing its own control. Hydroxyproline data from preliminary experiments was available for a further 2 rabbits on the first post-operative day (total = 7) and 5 on the third day (total = 10) and protein results were available for a further 2 rabbits at three days (total = 7). In 5 animals at each time hydroxyproline was expressed as a percentage of total protein and these results were also compared.

The collagenase activity from each segment of colon was measured after each 24 hours in culture. Each
Figure IV.1.2
Diagram of dissection of anastomotic segment to give strips of tissue for hydroxyproline and collagenase determination.
CHAPTER IV.1 Investigation – biochemistry

Sample of medium was assayed in triplicate, at four dilutions, to give a value for active enzyme; a second set of dilutions was activated with APMA to obtain the value for total enzyme activity and latent activity was calculated by subtraction. The collagenase activity in any one segment of colon at any particular time in culture was derived from the mean activity in medium from three explants (medium from the fourth remained available for measurement if there was wide discrepancy in the results from the other three). Results were obtained from five rabbits at each post-operative time with each animal providing its own control values from the resected tissue. To avoid confusion in discussing these results, time in culture is always expressed in hours while time after operation is expressed in days.

Random samples of medium were incubated at 25°C and the reaction products examined by sodium dodecyl sulphate polyacrylamide gel electrophoresis, as detailed in Chapter III.3.iv, to confirm the specificity of the assay for mammalian collagenase.

In this way the changes in collagen and protein concentrations and the levels of collagenase activity, proximal and distal to the suture line, were assessed in normal colon and on the first and third days after
uncomplicated colonic anastomosis in the rabbit.

**Statistical Methods**

In view of the small numbers of samples to be analysed, it was considered appropriate to test the results by non-parametric methods. However, statistical tables are not available for comparison of this number of samples by non-parametric tests for paired data. The data were therefore transformed to natural logarithms to make them conform to a normal distribution. The results were then analysed by Student's 't'-test for paired and unpaired data, as appropriate (Diem and Lentner 1970).
(ii) RESULTS

COLLAGEN

Measurements of the hydroxyproline concentration of the colonic wall were made in 7 rabbits one day after anastomosis and in 10 on the third post-operative day. The results are detailed in the Appendix, in Tables A.4.1 to 3, which give the measured concentrations and the log transformed figures. Total protein concentration measurements were made in 5 rabbits after one day and 7 after 3 days: these results are shown with their log transformed equivalents in Tables A.4.4 and 5. Tables A.4.6 and 7 show hydroxyproline expressed as a percentage of the total protein concentration in the 5 animals at each post-operative time in which matching data was available. Table IV.1.3 provides a summary of this data, transformed to natural logarithms, for groups of 5 animals at each post-operative time.

One day post-op

Statistical comparison of the log transformed data showed no significant difference in hydroxyproline concentration between normal rabbit colon and any of the post-operative segments one day after anastomosis. There was, however, a tendency for the hydroxyproline concentration to decrease in Pr0, the proximal tissue.
CHAPTER IV.1 Investigation - biochemistry

Table IV.1.3

Hydroxyproline and Protein Concentrations
One and Three Days after Anastomosis
Mean Values (Ln) for Groups of 5 Rabbits

<table>
<thead>
<tr>
<th>Hydroxyproline Ln μg/mg</th>
<th>Protein Ln μg/mg</th>
<th>Hydroxyproline as percentage of protein (Ln)</th>
</tr>
</thead>
</table>

One Day Post-op

Control 2.29 ± 0.25 5.35 ± 0.11 1.55 ± 0.21
Pr2 2.42 ± 0.42 5.42 ± 0.16 1.61 ± 0.41
Pr0 1.98 ± 0.55 5.14 ± 0.55 1.45 ± 0.76
Di0 2.18 ± 0.48 5.35 ± 0.11 1.44 ± 0.56
Di2 2.07 ± 0.36 5.43 ± 0.11 1.26 ± 0.41

Three Days Post-op

Control 2.39 ± 0.13 5.39 ± 0.07 1.61 ± 0.18
Pr2 2.03 ± 0.26 *2.35 ± 0.33 ** 1.29 ± 0.39
Pr0 **1.81 ± 0.33 5.29 ± 0.12 1.13 ± 0.32
Di0 2.35 ± 0.42 5.30 ± 0.18 1.66 ± 0.53
Di2 2.49 ± 0.47 5.28 ± 0.07 1.82 ± 0.43

* P < 0.02 ** P < 0.005 vs controls
closest to the stitch line. Total protein concentration in the same segments of tissue showed no change on the first post-operative day and the ratio of hydroxyproline to protein did not alter significantly in any segment.

Three days post-op

On the third post-operative day the concentration of hydroxyproline in the colon proximal to the anastomosis fell significantly compared with normal colon. This was most marked in Pr0, the tissue closest to the suture line (P<0.005) but was still present in Pr2, two centimetres away (P<0.02). On the distal side, a decrease in hydroxyproline was seen in Di0 but this did not reach statistical significance. Total protein concentrations remained unchanged in all segments on the third post-operative day and, consequently, the ratio of hydroxyproline to total protein fell in segments Pr0 and Pr2, although the decreases failed to achieve statistical significance. The fall in hydroxyproline concentration was therefore not brought about by any change in total protein concentration which might have occurred in an inflammatory reaction.
COLLAGENASE

As shown in Chapter III.3.iv, the specificity of the biochemical assay for mammalian collagenase was demonstrated by sodium dodecyl sulphate polyacrylamide gel electrophoresis and this was confirmed for random samples of culture medium throughout the study period.

The measurements of collagenase activity in individual segments of colon, derived from the mean value in three explants, are given in detail for each period of culture and each post-operative time in Tables A.5.1 to 18 in the Appendix. The data are summarised in Tables IV.1.4 and 5, which give the mean values for 5 rabbits on the first and third post-operative days respectively. Tables IV.1.6 and 7 show the log transformed data with the statistical comparison in each set of 5 animals.

In keeping with the findings given in Chapter III.3.iii (when the required length of time in culture was determined), some collagenase activity was measured in nearly all samples of culture medium but the levels in medium from the first 24 hours in culture were considerably less than in later samples. This remained true whether the explants were taken from normal resected colon or from post-operative tissue. Similarly, for both pre- and post-operative tissue, the
Table IV.1.4

1 day post-op - Mean values from 5 rabbits
Total, Active and Latent Collagenase Activity
in Units x 10^-3/mg wet weight of tissue

<table>
<thead>
<tr>
<th>Time</th>
<th>Treatment</th>
<th>Total</th>
<th>Active</th>
<th>Latent</th>
<th>% Act ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>33.94</td>
<td>1.75</td>
<td>32.20</td>
<td>2.62 ± 4</td>
</tr>
<tr>
<td></td>
<td>24 h Pr2</td>
<td>32.73</td>
<td>3.00</td>
<td>29.73</td>
<td>6.65 ± 9</td>
</tr>
<tr>
<td></td>
<td>culture Pr0</td>
<td>23.61</td>
<td>0.27</td>
<td>23.34</td>
<td>1.62 ± 4</td>
</tr>
<tr>
<td></td>
<td>DiO</td>
<td>34.35</td>
<td>4.88</td>
<td>29.47</td>
<td>12.68 ± 12</td>
</tr>
<tr>
<td></td>
<td>Di2</td>
<td>54.69</td>
<td>27.83</td>
<td>26.86</td>
<td>42.83 ± 27</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>356.56</td>
<td>222.82</td>
<td>133.74</td>
<td>63.60 ± 10</td>
</tr>
<tr>
<td></td>
<td>48 h Pr2</td>
<td>421.92</td>
<td>261.01</td>
<td>160.91</td>
<td>62.39 ± 6</td>
</tr>
<tr>
<td></td>
<td>culture Pr0</td>
<td>391.34</td>
<td>189.14</td>
<td>202.20</td>
<td>53.66 ± 19</td>
</tr>
<tr>
<td></td>
<td>DiO</td>
<td>363.06</td>
<td>264.01</td>
<td>107.33</td>
<td>74.85 ± 21</td>
</tr>
<tr>
<td></td>
<td>Di2</td>
<td>398.22</td>
<td>269.05</td>
<td>129.16</td>
<td>69.63 ± 19</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>378.32</td>
<td>235.65</td>
<td>142.67</td>
<td>64.34 ± 11</td>
</tr>
<tr>
<td></td>
<td>72 h Pr2</td>
<td>528.31</td>
<td>282.95</td>
<td>245.36</td>
<td>57.14 ± 13</td>
</tr>
<tr>
<td></td>
<td>culture Pr0</td>
<td>351.80</td>
<td>172.18</td>
<td>179.61</td>
<td>56.68 ± 23</td>
</tr>
<tr>
<td></td>
<td>DiO</td>
<td>422.34</td>
<td>249.05</td>
<td>173.29</td>
<td>61.95 ± 22</td>
</tr>
<tr>
<td></td>
<td>Di2</td>
<td>468.14</td>
<td>249.58</td>
<td>218.56</td>
<td>57.48 ± 12</td>
</tr>
</tbody>
</table>
CHAPTER IV.1 Investigation - biochemistry

Table IV.1.5

3 days post-op - Mean values from 5 rabbits

Total, Active and Latent Collagenase Activity
in Units x 10^-3/mg wet weight of tissue

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Active</th>
<th>Latent</th>
<th>% Act ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>51.70</td>
<td>8.90</td>
<td>42.80</td>
<td>11.26 ± 12</td>
</tr>
<tr>
<td>24 h Pr2</td>
<td>96.77</td>
<td>31.44</td>
<td>65.33</td>
<td>22.42 ± 26</td>
</tr>
<tr>
<td>culture PrO</td>
<td>77.45</td>
<td>10.71</td>
<td>66.74</td>
<td>11.20 ± 8</td>
</tr>
<tr>
<td>DiO</td>
<td>101.90</td>
<td>41.00</td>
<td>60.91</td>
<td>30.61 ± 18</td>
</tr>
<tr>
<td>Di2</td>
<td>85.25</td>
<td>48.17</td>
<td>37.08</td>
<td>44.40 ± 37</td>
</tr>
<tr>
<td>48 h Pr2</td>
<td>691.17</td>
<td>489.07</td>
<td>202.10</td>
<td>65.59 ± 19</td>
</tr>
<tr>
<td>culture PrO</td>
<td>612.25</td>
<td>435.35</td>
<td>186.77</td>
<td>69.21 ± 22</td>
</tr>
<tr>
<td>DiO</td>
<td>672.05</td>
<td>458.10</td>
<td>213.94</td>
<td>64.78 ± 28</td>
</tr>
<tr>
<td>Di2</td>
<td>488.03</td>
<td>331.86</td>
<td>156.17</td>
<td>69.60 ± 19</td>
</tr>
<tr>
<td>72 h Pr2</td>
<td>573.06</td>
<td>417.48</td>
<td>155.58</td>
<td>72.92 ± 11</td>
</tr>
<tr>
<td>culture PrO</td>
<td>512.67</td>
<td>345.15</td>
<td>167.52</td>
<td>68.15 ± 17</td>
</tr>
<tr>
<td>DiO</td>
<td>621.20</td>
<td>420.60</td>
<td>200.60</td>
<td>68.88 ± 8</td>
</tr>
<tr>
<td>Di2</td>
<td>611.53</td>
<td>379.35</td>
<td>232.19</td>
<td>66.04 ± 26</td>
</tr>
</tbody>
</table>
CHAPTER IV.1 Investigation - biochemistry

Table IV.1.6

1 day post-op - Mean values from 5 rabbits

Log Transformed Collagenase Activity
in Ln Units x 10^-3/mg wet weight of tissue

<table>
<thead>
<tr>
<th></th>
<th>Total ± SD</th>
<th>Active ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>3.45 ± 0.46</td>
<td>0.31 ± 0.70</td>
</tr>
<tr>
<td>24 h Pr2</td>
<td>2.93 ± 1.67</td>
<td>0.78 ± 1.10</td>
</tr>
<tr>
<td>culture</td>
<td>2.92 ± 0.70</td>
<td>0.06 ± 0.13</td>
</tr>
<tr>
<td>Pr0</td>
<td>3.48 ± 0.39</td>
<td>1.12 ± 1.02</td>
</tr>
<tr>
<td>DiO</td>
<td>3.87 ± 0.53</td>
<td>+2.78 ± 1.15</td>
</tr>
<tr>
<td>Di2</td>
<td>5.88 ± 0.40</td>
<td>5.42 ± 0.32</td>
</tr>
</tbody>
</table>

| Control  | 5.87 ± 0.16| 5.40 ± 0.10 |
| 48 h Pr2 | 5.99 ± 0.39| 5.52 ± 0.37 |
| culture  | 5.92 ± 0.36| 5.24 ± 0.08 |
| Pr0      | 5.83 ± 0.44| 5.56 ± 0.22 |
| DiO      | 5.96 ± 0.26| 5.57 ± 0.25 |
| Di2      | 6.02 ± 0.24| 5.49 ± 0.27 |

| Control  | 5.88 ± 0.40| 5.42 ± 0.32 |
| 72 h Pr2 | 6.21 ± 0.36| 5.63 ± 0.22 |
| culture  | 5.73 ± 0.60| 5.09 ± 0.37 |
| Pr0      | 6.02 ± 0.24| 5.49 ± 0.27 |
| DiO      | 6.02 ± 0.47| 5.48 ± 0.32 |

*p < 0.005 vs controls

209
CHAPTER IV.1 Investigation - biochemistry

Table IV.1.7
3 days post-op - Mean values from 5 rabbits
Log Transformed Collagenase Activity
in Ln Units x 10^-3/mg wet weight of tissue

<table>
<thead>
<tr>
<th></th>
<th>Total ± SD</th>
<th>Active ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td>3.67 ± 0.85</td>
<td>1.28 ± 1.44</td>
</tr>
<tr>
<td><strong>24 h</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pr2</td>
<td>4.32 ± 0.88</td>
<td>2.16 ± 1.76</td>
</tr>
<tr>
<td>culture</td>
<td>4.15 ± 0.75</td>
<td>1.78 ± 1.20</td>
</tr>
<tr>
<td>Di0</td>
<td>4.22 ± 1.19</td>
<td>2.98 ± 1.80</td>
</tr>
<tr>
<td>Di2</td>
<td>3.96 ± 1.21</td>
<td>2.53 ± 2.12</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>5.86 ± 0.34</td>
<td>5.32 ± 0.48</td>
</tr>
<tr>
<td><strong>48 h</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pr2</td>
<td>6.46 ± 0.46</td>
<td>6.00 ± 0.69</td>
</tr>
<tr>
<td>culture</td>
<td>6.36 ± 0.36</td>
<td>5.97 ± 0.52</td>
</tr>
<tr>
<td>Di0</td>
<td>6.44 ± 0.40</td>
<td>5.90 ± 0.84</td>
</tr>
<tr>
<td>Di2</td>
<td>6.10 ± 0.50</td>
<td>5.70 ± 0.50</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>5.77 ± 0.27</td>
<td>5.27 ± 0.39</td>
</tr>
<tr>
<td><strong>72 h</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pr2</td>
<td>6.35 ± 0.09</td>
<td>6.02 ± 0.17</td>
</tr>
<tr>
<td>culture</td>
<td>6.21 ± 0.26</td>
<td>5.80 ± 0.34</td>
</tr>
<tr>
<td>Di0</td>
<td>6.41 ± 0.22</td>
<td>6.03 ± 0.15</td>
</tr>
<tr>
<td>Di2</td>
<td>6.39 ± 0.26</td>
<td>5.90 ± 0.32</td>
</tr>
</tbody>
</table>

*P < 0.05  **P < 0.02  ***P < 0.005  ****P < 0.002 vs controls
enzyme was predominantly in the latent form during the first 24 hours of culture but, after 48 and 72 hours culture, active collagenase formed between 54 and 75 per cent of the total activity. (Tables IV.1.4, IV.1.5)

**Normal colon** (Tables IV.1.4, IV.1.5)

Normal resected colon from the group of rabbits killed one day post-operatively secreted $33.94 \times 10^{-3}$ units of collagenase per milligram of wet tissue in the first 24 hours in culture and only 2.6 per cent of this was in the active form. Activity increased to $356.56 \times 10^{-3}$ units/mg after 48 hours culture and to $378.32 \times 10^{-3}$ after 72 hours, with 64 per cent of the enzyme being active at each time. Similar figures were measured in the control tissue from the group of animals killed 3 days post-operatively.

**One day post-op** (Tables IV.1.4, IV.1.6)

As for normal tissue, colon explants taken one day after anastomosis secreted less collagenase into the culture medium in the first 24 hours in culture than after longer periods and the enzyme was mostly in the latent form. An exception to this was seen in tissue from segment Di2, which secreted 43 per cent of the enzyme in the active form even during the first 24 hours
CHAPTER IV.1 Investigation - biochemistry

In culture (P<0.005).

Collagenase secretion increased in all segments after 48 and 72 hours of culture, when compared with the first 24 hours of culture, and at these times between 54 and 75 per cent of the enzyme was in the active form. For all segments of colon taken one day after operation the amount of collagenase secreted at these later times in culture tended to be higher than the corresponding values for unoperated colon but the increase did not reach statistical significance.

Three days post-op (Tables IV.1.5, IV.1.7)

At all culture times, tissue from colon on the third day after anastomosis secreted more collagenase than normal colon. This trend was not significant during the first 24 hours in culture but even at this stage the proportion of active enzyme tended to increase, ranging from 11 to 44 per cent post-operatively compared with 11 per cent in the control segments.

After 48 hours in culture the post-operative segments of colon secreted more collagenase than controls with an increase in both the total amount of enzyme and in the active form. This increase was only statistically significant, however, in the total
collagenase activity of the segments closest to the suture line, Pr0 and Di0 (P<0.05).

All segments of colon taken three days after anastomosis secreted significantly more collagenase than normal after 72 hours in culture and this was again due to a significant increase in the values for active enzyme. On the proximal side of the suture line the increase was significant in both the total and active values (P<0.05 for total activity in Pr0, P<0.02 for other proximal values). Distally, the significance was greater (P<0.005 for total activity in Di2 and active enzyme in Di0, P<0.002 for total activity in Di0), except in the active enzyme from colon 2 cm distal to the suture line.
(i) EXPERIMENTS

The immunohistochemical methods detailed in Chapter III.4 were applied to experiments on uncomplicated rabbit colon anastomoses to map the distribution of collagenase in the healing process over the first week. As with the biochemical experiments, histochemical investigation was carried out on normal resected colon and post-operative tissue one and three days after anastomosis, to study the times when the collagen concentration should be falling rapidly and then beginning to rise again. In addition, since the collagen concentration begins to fall within 3 hours of operation (Hendricks et al 1985), immunolocalisation was undertaken in anastomoses 12 hours after operation to look for early secretion of collagenase and after seven days to assess continued secretion in the remodelling phase when collagen levels have returned to normal.

Standard Operation

The standard colonic resection and anastomosis described in Chapter III.1 was performed in eleven rabbits. The resected segment from each was retained, washed and processed for immunohistochemistry and
routine histology. Two rabbits were killed twelve hours after operation and three each after one day, three days and seven days. Adhesions were separated carefully and a six centimetre segment of colon containing the anastomosis at its centre was taken from the animal. This was opened lengthwise along the anti-mesenteric border and any contents gently removed. The bowel was then divided transversely one centimetre distal to the suture line and the two centimetre segment below this was retained as the distal segment (Fig IV.2.1). A similar division was made one centimetre above the stitch line to provide a two centimetre segment containing the anastomosis and the two centimetre segment proximal to this.

After five washes in Hank's balanced salts solution containing antibiotics, as described in Chapter III.3.iii, the segments were transferred to the tissue culture hood and divided completely into strips for histochemistry. From each segment, one strip was then fixed directly in formal saline, embedded in paraffin wax, sectioned and stained with haematoxylin and eosin. The remaining strips were alternately processed immediately or cultured with monensin for 3 hours. Colon strips were then orientated on millipore filters, embedded in gelatin and frozen in liquid nitrogen as
CHAPTER IV.1 Investigation - histochemistry

Figure IV.2.1
Diagram of dissection of anastomotic segment to give strips of tissue for immunohistochemistry.
described in Chapter III.4. Filters were marked with indelible ink along the proximal edge to retain the orientation during further procedures.

All tissue blocks in gelatin were stored at -20°C and subsequently cut on at least one occasion. Whenever a block was cut, some sections were treated with anti-collagenase IgG, some with anti-TIMP IgG and some with normal sheep serum IgG to act as controls. This gave an even distribution of cultured and uncultured tissue around the circumference of the bowel and an even distribution of treatment with each antibody within each strip of tissue. Most blocks were cut two, three or more times to determine whether any significant change in staining pattern occurred on proceeding through the tissue. In this way, an overall picture of the distribution of collagenase and TIMP was obtained in normal colon, around an anastomosis, and in colon up to three centimetres proximal and distal to the stitch line, at four different post-operative times to provide a direct comparison with the results from the biochemical experiments.
CHAPTER IV.2 Investigation - histochemistry

'End-on' Anastomoses

A second set of experiments was carried out to compare the distribution of collagenase and TIMP in everted suture lines with the pattern in an 'end-on' technique of anastomosis. Twelve rabbits underwent resection and anastomosis using the edge-to-edge type of sutures described in Chapter III.1. Three rabbits were killed at each post-operative time (12 hours, one day, three days and seven days) and the tissue was handled in the same way as for the everted anastomoses. This gave a comparison of the histochemical localisation of collagenase and TIMP after everted and 'end-on' anastomoses.

Sham Operations

As a further set of controls, four rabbits underwent laparotomy with handling of the colon and identification of the usual resection site but no resection or anastomosis. Two animals were killed one day after operation and two after three days and colon from the 'anastomotic' site was processed for immunolocalisation of collagenase and TIMP to show whether laparotomy and handling of the bowel alone produced any change in the distribution of enzyme and inhibitor.
(ii) RESULTS

Immunohistochemical findings were consistent in animals investigated at the same post-operative time. In any one tissue block, areas of staining for collagenase and TIMP tended to appear and disappear gradually over 15 or 20 serial sections; the descriptions given below, therefore, represent the 'average' pattern of staining throughout the tissue. There were similar results proximal and distal to the suture line at all post-operative stages.

Resected colon

The histological appearance of normal rabbit colon is seen in Figure IV.2.2. No bright green fluorescence localising collagenase or tissue inhibitor of metalloproteinases was seen in any of the segments of normal colon resected at operation, whether cultured with monensin or not. Occasional yellow fluorescence was seen, the product of treatment with 4-chloronaphthol, identifying scattered inflammatory cells present in normal large bowel; this was more clearly seen in post-operative tissue when groups of inflammatory cells were found (Fig IV.2.3).
CHAPTER IV.2 Investigation - histochemistry

Figure IV.2.2

Normal appearance of rabbit colon.

Resected tissue, formalin-fixed and stained with H and E

Bar = 300μ
Anastomotic segment 3 days after operation, uncultured tissue, incubated with anti-TIMP. Bar = 5μ
Cell nuclei stain red with methyl green counter-stain while the yellow granules in inflammatory cells are produced by treatment with 4-chloronaphthol.
CHAPTER IV.2 Investigation - histochemistry

Standard Everted Anastomoses

Twelve hours post-operation

Twelve hours after operation the region of the anastomosis showed areas of haemorrhage and oedema (Fig IV.2.4.a). The gap between the two ends of bowel was filled with clot and fibrin and early inflammatory infiltration was seen. It should be noted that rabbit neutrophil polymorphonuclear leucocytes show relatively eosinophilic staining in their granules and can be difficult to distinguish from eosinophils (Weisbroth et al 1974). The region of the anastomosis showed positive staining for collagenase which was exclusively localised to the everted portions of bowel wall (Fig IV.2.4.b). The entire length of the everted parts contained extracellular enzyme on the interstitial matrix of the mucosal layer, confined mostly to the more superficial areas of this layer. Thus, green fluorescence outlined the superficial mucosal crypts in the everted regions as seen in Fig IV.2.5.a, which shows tissue incubated with anti-collagenase; this staining extended only 350 μ from the epithelial surface. Figure IV.2.5.b shows the same area on a control slide incubated with non-immune sheep serum. Figure IV.2.6 shows extracellular enzyme also found in the vicinity of suture material at this time.
CHAPTER IV.2 Investigation - histochemistry

Figure IV.2.4 Anastomosis 12 hours after operation.

a) Formalin-fixed tissue stained with H and E. Bar = 300μ

b) Diagram of region showing distribution of collagenase.

222
CHAPTER IV.2 Investigation - histochemistry

Figure IV.2.5 Collagenase localisation 12 hours post-op

a) Everted region of anastomotic segment, uncultured tissue, incubated with anti-collagenase. Bar = 50 μm
Red nuclei outline the mucosal crypts. On the left green fluorescence shows extracellular collagenase immediately beneath the epithelial surface (arrowed).

b) Similar region incubated with NSS showing control staining. Bar = 50 μm
Extracellular collagenase and inflammatory cells around suture material.

Everted region of anastomotic segment, 12 hours post-op, uncultured tissue, incubated with anti-collagenase and green fluorescence showing the secretory granules (Fig IV.2.7). Cells were found in an extensive area above and below the suture, with green fluorescence. Apart from the bar = 50μm, there are more yellow inflammatory cells visible around the suture material.
CHAPTER IV.2 Investigation – histochemistry

At higher powers, cells secreting collagenase could be identified, with bright red nuclei and speckles of green fluorescence within the cytoplasm showing the secretory granules (Fig IV.2.7). Cells were found in the same areas as extracellular collagenase and could be seen in tissue both with and without monensin treatment. Epithelial cells were never seen to secrete the enzyme but, within the interstitial matrix of the mucosal layer, cells with the appearance of fibroblasts showed positive staining. Secretion was not seen in cells which showed the yellow fluorescence of inflammatory cells.

Very little tissue inhibitor of metalloproteinases was visible twelve hours after anastomosis in any area of colon (Fig IV.2.8).

The distribution of collagenase in the tissue was similar both proximal and distal to the stitch line and was no more extensive above than below. Apart from the everted regions and the immediate vicinity of suture material, no staining was seen with either antibody in the segments containing the anastomosis or in the more proximal and distal segments.
CHAPTER IV.2 Investigation - histochemistry

Figure IV.2.7

Collagenase secreting cells 12 hours after anastomosis.

Everted region of anastomotic segment, incubated with monensin, treated with anti-collagenase. Bar = 10 μ
Green fluorescence identifies collagenase granules around the red nucleus of a secreting cell (arrow) and in the adjacent extracellular space.

No green fluorescence is seen with anti-TIMP antibody.

226
Figure IV.2.8 Absence of TIMP 12 hours after operation.

a) Everted region of anastomotic segment, uncultured tissue, incubated with anti-collagenase. Bar = 20μ
Higher power view of extracellular collagenase in mucosal layers.

b) Similar region, 4 sections further into block, incubated with anti-TIMP. Bar = 20μ
No green fluorescence is seen with anti-TIMP antibody.
CHAPTER IV.2 Investigation - histochemistry

One day post-operation

Twenty-four hours after operation the gap between the bowel ends was still plugged with fibrin but at this stage more inflammatory cells were collecting around the tips of the everted portions of bowel wall (Fig IV.2.9.a).

Extracellular collagenase was seen in the everted regions but was more discretely localised than at twelve hours since it was confined more closely to the cut ends (Fig IV.2.9.b). The enzyme was, however, seen to be spreading more deeply at these points so that it was found throughout the mucosal layer and was also identified in submucosa. Some collagenase was seen in the fibrin plug and some was again seen around strands of suture material. At one day, cells secreting collagenase could only be identified after culture with monensin to enlarge the secretory granules.

In contrast to the picture at 12 hours, TIMP staining was seen in tissue one day after operation and was found in the same regions as collagenase. The distribution of TIMP closely paralleled that of collagenase and TIMP secreting cells were similarly visible after monensin treatment (Fig IV.2.10).

As before, the pattern of staining seen proximal to
CHAPTER IV.2 Investigation - histochemistry

Figure IV.2.9 Anastomosis one day after operation.

a) Formalin-fixed tissue stained with H and E. Bar = 300µ

b) Diagram showing distribution of collagenase and TIMP.

collagenase and TIMP around suture material

secretion cells and extracellular collagenase and TIMP in mucosa
CHAPTER IV.2 Investigation - histochemistry

Figure IV.2.10 TIMP secreting cells one day after anastomosis.

Everted region of anastomotic segment, incubated with monensin, treated with anti-TIMP. Bar = 5 μm. Green fluorescence shows TIMP granules in the perinuclear regions of a group of TIMP secreting cells.
the stitch line was the same as that seen distally and no enzyme or inhibitor was found outside the vicinity of the anastomosis and suture material.

Three days post-operation

By the third post-operative day the fibrin plug filling the space between the two ends of bowel was larger and many inflammatory cells were seen infiltrating the tips of the everted parts, which were dead or dying (Fig IV.2.11.a). The serosal surface was completely sealed across the gap by a distinct layer of connective tissue over the plug. This was composed of granulation tissue with polymorphs and fibroblasts and evidence of new vessel formation.

Extracellular collagenase was once more found in the everted regions of the wall, adjacent to the dead parts and their inflammatory infiltrate but very little was seen within these infiltrated areas (Figs IV.2.11.b and IV.2.3). The enzyme activity now penetrated down to the muscle layers and collagenase can be seen in Figure IV.2.12.a in the interstitial spaces between dying muscle blocks. Figure IV.2.12.b shows the same area incubated with NSS and IV.2.12.c shows this area stained with haematoxylin and eosin. At this stage very few
CHAPTER IV.2 Investigation - histochemistry

Figure IV.2.11 Anastomosis three days after operation.

a) Formalin-fixed tissue stained with H and E. Bar = 500μ

b) Diagram showing distribution of collagenase and TIMP.
CHAPTER IV.2 Investigation – histochemistry

Figure IV.2.12

Collagenase in submucosa and muscle layers three days after anastomosis.

a) Everted region of anastomotic segment, uncultured tissue, incubated with anti-collagenase. Bar = 50μm
Green fluorescence shows extracellular collagenase in submucosa and spreading between dying muscle blocks.

b) Similar section treated with NSS as control. Bar = 50μm

c) Same section as a) restained with H and E. Bar = 25μm
collagenase secreting cells could be found, even after monensin treatment. Extracellular enzyme was still present around suture material.

Once more, TIMP distribution paralleled that of collagenase in the everted parts of the bowel and, similarly, few TIMP secreting cells were found in these areas. A new finding appeared at this time, however. Cells secreting TIMP were found within the layer of connective tissue which sealed the serosal surface of the anastomosis; this is seen at low power in Figure IV.2.13.a and higher power in IV.2.13.b. Once again, these cells had the appearance of fibroblasts. A chain of secreting cells stretched within this layer across the whole length of the anastomotic segments and could be identified in the segments of colon taken more proximally and distally. The cells continued to a distance of approximately 2 to 2.5 centimetres from the stitch line, to the point where this thickened protective layer narrowed into the normal serosal coat of the bowel wall; Figure IV.2.14 shows the H and E appearance of this coat in a proximal segment of colon. No extracellular TIMP was seen in this layer and there was no evidence of intra or extra cellular collagenase.

As at all other times, these findings were similar
Figure IV.2.13

TIMP cells in serosal layer three days post-op.

a) Serosal coat of anastomotic region, incubated with monensin, treated with anti-TIMP. Bar = 20 μ
Red nuclei show cells among strands of pale green collagen. A chain of nuclei are surrounded by bright green speckles identifying TIMP granules (arrows).

b) Similar region at higher power. Bar = 5μ
Higher power shows a TIMP secreting fibroblast with green granules adjacent to the spindle-shaped nucleus, alongside a pale green band of new collagen.

235
CHAPTER IV.2 Investigation - histochemistry

Figure IV.2.14

Serosal coat in proximal segment three days after anastomosis.

Formalin-fixed tissue stained with H and E. Bar = 300μ

Histology of the proximal segment shows the thickened serosal layer (arrow) in which TIMP secreting cells were found, extending approximately 2 cm from the suture line before narrowing into a normal serosal coat.
both above and below the anastomosis and it would not have been possible to distinguish the proximal and distal ends of the anastomotic segments without the aid of the identifying mark made previously on the millipore filter.

**Seven days post-operation**

One week after anastomosis the healing process had advanced sufficiently in some areas to restore epithelial continuity across the gap on the luminal surface of the bowel wall. In the deeper part of the gap, and in the regions where epithelial continuity was not yet restored, the plug was now replaced by organising granulation tissue; this contained fibroblasts, layers of collagen fibres and other connective tissue elements interspersed with scattered remnants of the everted regions of wall which were still being dismantled (Figs IV.2.15 and IV.2.16). Occasional buried mucosal crypts could be identified, usually dead or dying and accompanied by some inflammatory cells.

Aggregates of cells secreting collagenase and cells secreting TIMP were seen around these scattered remnants of mucosa and in small areas within the fibrous tissue; these were visible in both cultured and uncultured
Figure IV.2.15

Anastomosis seven days after operation.

a) Formalin-fixed tissue stained with H and E.
   Bar = 300μ

b) Diagram showing distribution of collagenase and TIMP.
CHAPTER IV.2 Investigation - histochernistry

Figure IV.2.16 Anastomosis seven days after operation.

a) Epithelial surface at site of anastomosis, uncultured tissue, frozen-section restained with H and E. Bar = 50μ
Epithelial continuity is restored in this region of the anastomosis at seven days.

b) Granulation tissue at site of anastomosis, formalin-fixed tissue, stained with H and E. Bar = 50μ
Higher power view of region in Figure IV.2.15.a showing suture material (s) and buried remnants of mucosa (m).
CHAPTER IV.2 Investigation – histochemistry

tissue (Fig IV.2.15.b). Figures IV.2.17 and IV.2.18 show a group of cells secreting collagenase adjacent to one such mucosal remnant with TIMP secreting cells in a similar region on another section. A few secreting cells were also found with small amounts of extracellular enzyme and inhibitor around residual suture material, which was seen to be more fragmented than at earlier times. No enzyme or inhibitor was found in the mucosal layers at this stage and none was seen in the proximal or distal segments of colon.
FIGURE IV.2.17 Collagenase and TIMP secreting cells seven days after anastomosis.

a) Area of granulation in anastomotic segment, uncultured tissue, incubated with anti-collagenase. Bar = 20 μm. Collagenase secreting cells with green granules cluster around a buried remnant of mucosa (m).

b) Same region, three sections further into tissue block, treated with anti-TIMP. Bar = 20 μm TIMP secreting cells seen alongside same mucosal remnant (m). Pale nuclear ghosts indicate cell death within the remnant.

c) Same region, four sections further into tissue block, treated with NSS Bar = 20 μm Control staining, same area.
CHAPTER IV.2 Investigation - histochemistry

Figure IV.2.18

Collagenase and TIMP secreting cells seven days after anastomosis.

a) Area of granulation tissue in anastomotic segment, uncultured tissue, treated with anti-collagenase. Bar = 5μ Higher power view of cluster of collagenase secreting cells from Figure IV.2.17.a.

b) Same region two sections further into tissue block, treated with anti-TIMP. Bar = 5μ Higher power view of TIMP secreting cells alongside mucosal remnant.
CHAPTER IV.2 Investigation - histochemistry

'End-on Anastomoses'

Twelve hours post-operation

The histological findings twelve hours after an edge to edge type of anastomosis are shown in Figure IV.2.19.a. The muscle layers of the bowel wall showed a small amount of inversion at the suture line while the mucosa, not included in the stitch, inverted across the gap with marked areas of haemorrhage and evidence of cell death in the glands. The inflammatory reaction, with initial infiltration by polymorphonuclear leucocytes, was similar to that after everted anastomosis and was almost entirely confined to the region between the points of passage of the sutures.

As with everted anastomoses, positive staining for collagenase was seen and was confined to the portion of bowel wall immediately adjacent to the cut edge (Figure IV.2.19.b). Bright green fluorescence appeared in the interstitial regions at the tip of the mucosa and, to a much lesser extent, in the muscle layer at the cut edge. Cells secreting collagenase could be seen in the mucosa and were identified in tissue both with and without treatment with monensin. A small amount of collagenase appeared around the sites penetrated by the sutures.

Very little tissue inhibitor of metalloproteinases was localised in 'end-on' anastomoses at twelve hours.

242a
Figure IV.2.19
End-on anastomosis 12 hours after operation.

(a) Formalin-fixed tissue stained with H and E. Bar = 300μ

(b) Diagram showing distribution of collagenase.
CHAPTER IV.2 Investigation - histochemistry

One day post-operation

One day after 'end-on' anastomosis the approximation of the bowel ends was closer than after everted anastomosis and therefore the fibrin plug was smaller, although the histological changes were otherwise similar with both techniques (Fig IV.2.20.a).

Extracellular collagenase was seen in the inverted mucosa of the edge to edge anastomoses and some cells secreting the enzyme were identified at higher power but these were more apparent in tissue which had been cultured with monensin. The distribution of collagenase was equivalent to that found in everted anastomoses at the same post-operative time (Fig IV.2.20.b).

TIMP fluorescence was seen one day after operation using the 'end-on' technique. TIMP appeared in the same areas as collagenase, although the fluorescence was less bright, and cells secreting TIMP were visible in tissue with and without monensin treatment.

The patterns of staining of enzyme and inhibitor were similar proximal and distal to the suture line and neither was identified in any area beyond the immediate vicinity of the anastomosis.
Figure IV.2.20

'End-on' anastomosis one day after operation.

a) Formalin-fixed tissue stained with H and E. Bar = 300μ

b) Diagram showing distribution of collagenase and TIMP.
Three days post-operation

Three days after anastomosis using the 'end-on' technique, the inflammatory reaction in the tissue was progressing in the same way as after everted anastomosis but the reaction appeared to be less florid in the deeper layers with the edge to edge technique since the amount of damaged tissue to be degraded was less (Figure IV.2.21.a). At this stage, the serosal surface was again completely sealed by a thick coat of newly formed connective tissue.

Extracellular collagenase was localised in the edges of the bowel wall at the junction between the healthy areas of tissue and the regions which were already dead and being removed by inflammatory cells (Fig VI.2.21.b). Very few cells secreting the enzyme could be identified but secretion was still seen around the suture material.

TIMP distribution paralleled that of collagenase in the same way that it did after everted anastomosis and once again TIMP secreting cells, without any evidence of collagenase, appeared in the complete serosal layer of new connective tissue. As at all other times, no distinction could be seen between the proximal and distal sides of the anastomosis.
Figure IV.2.21

'End-on' anastomosis three days after operation.

a) Formalin-fixed tissue stained with H and E. Bar = 300μ

b) Diagram showing distribution of collagenase and TIMP.
Seven days post-operation

After seven days the inflammatory reaction to 'end-on' anastomosis had given way to maturation of the granulation tissue into fibrous tissue (Fig IV.2.22.a). Less remodelling appeared to be required than after everted anastomosis since the layers of the bowel wall remained in better alignment and residual segments of the mucosa were not buried within the deeper layers. At the lumenal surface the inverted portions of mucosa had been degraded sufficiently to produce mucosal continuity in some places.

Little collagenase and TIMP activity could be localised at this stage. Small aggregates of secreting cells of both types were found within the fibrous tissue, mostly in relation to small groups of dead cells or to the suture material (Fig IV.2.22.b). Once again, these cells could be identified without monensin treatment of the tissue but they were less frequently seen than in everted anastomoses at the same stage and TIMP cells usually outnumbered those secreting collagenase.
Figure IV.2.22

'End-on' anastomosis seven days after operation.

a) Formalin-fixed tissue stained with H and E. Bar = 300μ

b) Diagram showing distribution of collagenase and TIMP.
Sham Operations

One and three days post-operation

Sham operation with handling of the bowel but no resection or anastomosis produced no histological changes in the bowel wall when the site which would normally have been resected was processed and examined. Similarly, no collagenase or TIMP could be localised in the tissue removed after sham operation, in the same way that none could be seen in resected normal colon.
DISCUSSION

Review of the literature has shown that the turnover of collagen in the colonic wall after anastomosis is an important aspect of the healing process and that the activity of the enzyme collagenase may be directly relevant to the quality of healing. Further investigation of the role of collagenase at this stage required the development of more specific methods of assessment of the activity of this enzyme in colonic tissue. This investigation set out to establish the appropriate methods for the determination of the role of the specific enzyme collagenase in the healing of colonic anastomoses in rabbits and to apply those methods to conditions of normal colonic healing.


The adaptation of a biochemical assay for collagenase for use in colonic tissue forms a major part of this work. It was first established that it was not possible to detect the activity of the enzyme in tissue taken directly from the animal, despite various extraction manoeuvres. Although direct extraction would be preferable to assess the changes in activity of collagenase 'in vivo', extensive experiments were unsuccessful; this may reflect the lack of stored enzyme in the tissue, the immediate activation and action of newly-synthesised latent enzyme in stimulated tissue or the binding of activated enzyme to inhibitors.

Once the need for tissue culture had been demonstrated, the conditions for the culture of explants of colonic tissue were determined, ensuring that bacterial contamination was eliminated. The specific assay described by Cawston and Barrett (1979) and the modifications of Terato et al (1976) were adapted for the measurement of collagenase activity in culture medium from colonic explants and the precise conditions
of culture, assay mixture and incubation details were established. The specificity of the assay system for mammalian collagenase was demonstrated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

Recognizing the limitations of an assay system which required tissue culture, an independent assessment of the distribution of collagenase in healing colon was made by immunohistochemical methods, using a specific antibody to rabbit collagenase. Further information on the activity of the enzyme in the healing tissue was obtained by immunohistochemical investigation of the naturally-occurring collagenase inhibitor, TIMP.

All three methodological approaches were applied to the colon of rabbits in which anastomotic suture lines were healing without complication, in order to investigate the role of collagenase in normal healing and provide a base-line for comparison with future studies into colonic anastomotic dehiscence.
MEASUREMENT OF HYDROXYPROLINE

The results obtained from assay of hydroxyproline in rabbit colon, before and after anastomosis, are in broad agreement with the findings of other workers. Normal levels of hydroxyproline concentration are similar to those found in rabbits by Hesp et al (1984a and b), Hendricks et al (1985) and Hawley (1970). A significant decrease in hydroxyproline concentration occurs in the colon wall on the third post-operative day in rats and rabbits (Cronin et al 1968a, Irvin and Hunt 1974a–c, Irvin 1976, Jönsson et al 1985a), although Hesp et al (1984a) and Hendricks et al (1985) have demonstrated that the fall is significant earlier than this in rabbits, with a trough level 48 hours after anastomosis. The present investigation shows that hydroxyproline concentration in rabbit colon decreases one day after anastomosis but the fall is not statistically significant at this time. Three days after operation a significant drop in concentration is found in the vicinity of the suture line. There is no major discrepancy when comparing these findings with the Dutch studies (Hesp et al 1984a, Hendricks et al 1985); measurements were not made at 48 hours in this investigation and the differences are only in the degree
of significance at any one time.

The changes in hydroxyproline demonstrated in rabbit colon are most marked on the proximal side of the anastomosis and extend for at least 2 cm in this direction. This disparity between proximal and distal tissue has been noted previously by several groups of workers (Cronin et al 1968a and b, Jiborn et al 1978a, 1980a and b, Hesp et al 1984a and b, Hendricks et al 1985) but no conclusive explanation for the discrepancy has been put forward. The present study demonstrates that the hydroxyproline concentration of unoperated colon does not vary significantly at different sites in the region to be used for anastomosis, so the distinction did not exist prior to operation. If the formation of an anastomosis produces an inevitable degree of intestinal obstruction at the level of the stitch line, then the consequent thinning of the wall on the proximal side might account for a reduced collagen concentration. In this investigation, however, any anastomosis showing macroscopic evidence of obstruction was discarded from the study and everted suture lines were employed deliberately to eliminate this bias. One possible explanation has been put forward by Jiborn et al (1980b), who point out that transection of the colon with circular anastomosis interrupts the parasympathetic
nervous supply to the proximal side of the stitch line, while leaving the sympathetic supply intact (Bessant and Robertson-Rintoul 1986). This could result in relative vasoconstriction in the proximal tissue (Hultén et al 1969), leaving it more susceptible to bacterial penetration and thus creating a greater stimulus for collagenase secretion. This hypothesis is supported by the finding that no asymmetry occurs after small bowel anastomosis (Jönsson et al 1985a, 1986, 1987) in a region which is still supplied by the vagus nerve. To test this proposition it would be necessary to compare the hydroxyproline changes around a circular colonic anastomosis with those in the vicinity of a longitudinal suture line, since the latter would leave both autonomic nerve supplies intact. However, the importance of research into collagen turnover in healing colon lies in its relevance to human large bowel surgery; colonic resection and circular anastomosis are common procedures in clinical practice while longitudinal suture lines are rarely feasible or desirable.

The importance of the hydroxyproline measurements in this study is two-fold. The agreement of the findings with those of other workers confirms that the animal model which has been employed does not differ significantly from those used in earlier contributions.
to the field. Confirmation that hydroxyproline concentration, and therefore collagen concentration, undergoes a significant decrease in the vicinity of a colonic suture line in the first few days after anastomosis, reaffirms the need to investigate the activity of the enzyme collagenase, which seems to hold the key position in the breakdown of collagen in mammalian tissue.
CHAPTER V.3 Discussion

BIOCHEMICAL MEASUREMENT OF COLLAGENASE

The work described in Chapter III.3.ii demonstrates that it was not possible to detect collagenase activity in homogenates of colonic tissue taken directly from the animal, nor in extracts of such homogenates. Kortmann and von Bary (1977) and von Bary et al (1978) applied the Wirl extraction (1975) to human colon, stirring the tissue in cold salt solution and then using ammonium sulphate precipitation. Although the high salt concentration and the use of sodium azide would act as anti-bacterial agents, the acceptance of tissue up to 24 hours post mortem must be questioned. Even with refrigeration tissue necrosis begins within this period, particularly in areas with a high cell turnover such as colonic mucosa. Weeks et al (1979) criticised the Wirl process, since it extracted only 50 per cent of the collagenase in involuting uterus compared with their own method. Kortmann and von Bary did not use any further means, such as SDS-PAGE, to confirm the specificity of their assay system and have not published any subsequent data from it, raising doubts about the reliability of the method.

Brennan and colleagues (1984) homogenised post-operative rat colon in Tris buffer containing
calcium. The resulting mixture was assayed for collagenase activity by the method of Cawston and Barrett (1979). Their paper provides insufficient detail for the methodology to be criticised but again no tests for specificity were made and no subsequent reports from the method have appeared. Their findings could not be reproduced in the present study. These groups of workers entered the study of collagenase activity from the field of intestinal healing; the variability of their results, the failure to demonstrate enzyme specificity and the lack of subsequent use of the methods all indicate the difficulties encountered in trying to extract collagenase from fresh colon and raise considerable misgivings about the reliability of their findings. Collagenase extraction from fresh aortic wall is reported in the rat by Cohen et al (1984) but similar criticisms apply to this paper.

Menashi et al (1987), Powell et al (1988) and Powell and Greenhalgh (1989) extracted collagenase from the media of human aortic wall using molecular sieve chromatography to remove any TIMP. Even with this manoeuvre, no enzyme was detected in most of the samples and collagenase activity was measured only in extracts from ruptured aortic aneurysms. Eisenberg et al (1984) and Kishi et al (1984) extracted the enzyme from skin
but workers in the collagenase field routinely use culture systems to study the enzyme. Although steps to remove TIMP from the extracts were not considered in the present study, in retrospect it might have been wiser to discount entirely the questionable reports of extraction from colon and accept the findings of Oyamada and colleagues (1983) that tissue culture is necessary for the measurement of collagenase in rabbit colon. Nonetheless, attempts at extraction are still being made. van der Stappen et al (1989), working in a centre which has produced much information about collagen turnover in colonic anastomoses, have recently reported the application of the Wirl extraction system to rat intestine before and after anastomosis. Although they used the Cawston and Barrett assay (1979), they claim only to have measured collagenolytic activity in the tissue. Inter-animal variation in the results was so great that they have compared the ratios of activity in post-operative and control tissues rather than the actual values. Once again there is no electrophoretic confirmation of the specificity of the system and their results cannot be taken to be measurements of collagenase itself.

Collagenase is a specific enzyme which is only synthesised on demand, must be activated from its latent
form and is then rapidly inhibited by tissue inhibitor of metalloproteinases. All these factors contribute to the inability to detect the enzyme in uncultured tissues; this is confirmed by the histochemical experiments in the present study, which failed to demonstrate any collagenase in uncultured normal colon.

Secretion of collagenase by normal colon in culture

Explants of normal rabbit colon were shown to secrete a specific enzyme which fulfils the criteria for mammalian collagenase and produces the characteristic pattern of breakdown products on SDS-PAGE. This was first demonstrated in rabbit colon by Oyamada et al (1983), who maintained explants in Tyrode's solution. They detected collagenase in the culture fluid within 12 hours of culture, reaching a peak level between 48 and 92 hours. The results of the present study cannot be compared directly with those of Oyamada et al because of considerable differences between the two systems. Firstly, the volume of medium used by this group was much less per unit weight of tissue than in the present study while their medium was a balanced salts solution rather than a nutrient culture medium. The addition of serum to the medium introduces factors which are necessary for the optimum growth and maintenance of the
tissue; these have not been identified fully but have
been shown to be important in the culture of tissue for
collagenase measurements (Fell et al 1986, 1989). The
serum, however, must be specifically processed to remove
$\alpha_2$-Macroglobulin and this was carried out by
collagenase activity in medium which had been
centrifuged and diluted and expressed activity per ml of
this enzyme solution: in the present investigation,
collagenase activity was related to the weight of the
original tissue explant. Assay conditions also
differed: the measurement of collagenase activity at
35°C in this study cannot be directly compared with the
activity at 37°C recorded by Oyamada et al because of
the marked increase in activity of the enzyme with a
rise in temperature in this range (Harris et al 1984).

Despite these major differences in methodology,
some important similarities have been shown between the
present results and those of Oyamada et al (1983).
Normal rabbit colon secretes a specific collagenase
which can be detected in culture medium within 24 hours
and which produces the characteristic 3/4 and 1/4
fragments of collagen. Activity reaches a peak between
48 and 72 hours of culture. This pattern is similar to
the secretion of collagenase in culture by pig synovium
(Fell et al 1986), bovine dental pulp (Kishi et al 1984), human gingiva (Heath et al 1982) and rheumatoid synovial cells (Werb et al 1977). All these tissues show a similar lag phase in collagenase secretion in the first 24 hours of culture which is thought to be due to 'de novo' synthesis of the enzyme when the stimulation of placing the tissue into culture is applied (Harris and Krane 1974a, Eisen et al 1970). The presence of $\alpha_2$-Macroglobulin in the tissue may also be an inhibitory factor initially (Harris and Vater 1980, Peacock 1980). In addition, Sellers et al (1979) found that explants of rabbit bone produced TIMP in the first days in culture, which then gradually disappeared; similar findings were observed in cultures of skin and uterus (Murphy et al 1977). This would mask the presence of any collagenase in the medium initially.

The detection of collagenase from normal colon in culture highlights the first major discrepancy between the biochemical and histochemical approaches used in this study. No collagenase was immunolocalised in normal colon, even after six hours in culture and with the enhancement of monensin treatment. This is also true for non-gravid uterus and normal articular cartilage, in which collagenase does not immunolocalise (Hembry et al 1986) but from which enzyme secretion can
be stimulated in culture (Hunter et al 1984, Pasternak et al 1986, Murphy et al 1977). Inactive skin scars show no histochemical evidence of collagenase activity (Hembry and Erlich 1986) but extracts of uncultured skin have measurable activity (Eisenberg et al 1984). It is possible that this is due to the precise specificity of the antibodies used for immunolocalisation, which were prepared in the same way in all these studies, but each biochemical investigation adequately demonstrated the specificity of the enzyme measured, using SDS-PAGE. Similarly, it is unlikely that the histochemical method is less sensitive to the presence of small amounts of enzyme, since it is possible histochemically to demonstrate several separate granules of collagenase within individual secreting cells. However, it must be remembered that the immunohistochemical investigation cannot be considered a quantitative assessment of the enzyme activity: any comparison of the amounts of collagenase in different pieces of tissue is purely subjective. There is clearly a major difference in approach between the two methods which leads to results which are irreconcilable.

Returning to the biochemical findings in rabbit colon, collagenase is initially secreted almost entirely in its latent form, 93 per cent being latent in the
first 24 hours in this study. Later, active enzyme is more evident, with the proportion reaching about two thirds of the total after 48 hours culture, in both this study and in Oyamada and colleagues' investigations (1983). This is in contrast to the secretion of collagenase by other tissues. Synovial cells (Fell et al 1986), dental pulp (Kishi et al 1984) and normal gingiva (Heath et al 1982) secrete almost entirely latent collagenase even after several days in culture. The findings suggest that the colonic tissue itself produces a factor which can activate the latent enzyme. This was demonstrated in skin by Eisenberg et al (1984) who showed the presence of active enzyme in the tissue. Such an activator might have been supplied by the serum in the colon culture medium (Fell et al 1986) but the agreement with the findings of Oyamada et al (1983), who did not add serum to their cultures, suggests a different mechanism. One plausible explanation for the activation is that rabbit colon secretes plasminogen activator, as has been shown in human and rat colon samples (Gelister et al 1986, 1987a and b). This would then produce plasmin by its action on plasminogen, which could be derived either from the tissue or from the serum. Plasmin has been shown to activate collagenase and is thought to be the 'in vivo' activator in some
circumstances (Werb et al 1977, Eeckhout and Vaes 1977, He et al 1989). Trauma must be applied to the tissue to place explants into culture and this may constitute a stimulus for the secretion of both plasminogen activator and collagenase. Alternatively, the high turnover of cells in colonic mucosa may account for the difference from other tissues. A higher protein turnover indicates greater trypsin activity and trypsin, used as a collagenase activator ‘in vitro’, may also contribute to collagenase activation ‘in vivo’ (Werb and Aggeler 1978).

Post-operative secretion of collagenase

The biochemical evidence from this investigation suggests that colonic tissue secretes more collagenase than normal after an anastomosis. The total amount of enzyme measured from tissue removed one day after operation shows an increase which does not reach statistical significance. By the third post-operative day the increase is significant in all tissue measured after 72 hours culture and in the tissue closest to the stitch line after 48 hours culture. Within the first 24 hours of culture there is some increase in the proportion of active enzyme but the range of results is very wide. The later increase is mostly in the active
form. This is not surprising since most of the enzyme is active after 48 hours in culture but it does indicate that there is no shortage of activating agent in the system. This finding could be anticipated because any increase in enzyme is in response to a need for greater activity, and therefore it is the active form of the enzyme which is required.

In the interpretation of results in culture it is difficult to distinguish the effects of culture itself from those in the tissue before it is placed in culture and it is possible that this investigation has demonstrated this particular point more clearly than it has thrown light on the biochemical activity of collagenase in healing anastomoses. Thus it might be difficult to prove that the differences found post-operatively in this study are truly due to the effects of the operation rather than to some other factor. However, the handling of the tissue up to the point of culture and the conditions in which the explants were cultured were identical for pre- and post-operative tissue and the only intentional difference between the explants was whether they were taken from the colon before or after an anastomosis. Similar studies have shown differences in the secretion of collagenase by explants from normal and abnormal
human gingiva (Heath et al 1982) and Barnes and Douglas (1985) have demonstrated a correlation between collagenase secretion and certain pathological conditions of the vulva. This may be an indication of the degree of inflammatory reaction in the tissue concerned: inflamed gingiva secreted more enzyme than normal (Heath et al 1982) and an inflammatory reaction certainly occurs at a colonic suture line. Rheumatoid synovium, again involved in an inflammatory process, produces large quantities of collagenase (Werb et al 1977, Cawston et al 1984), although here the inflammation is inappropriate and is the mediator of the pathological process.

Ostensibly, therefore, the results of the biochemical investigations in this study tend to support the original work by Hawley (1970) and indicate that collagenase plays a part in the healing process in colon. Collagen concentration decreases in the first few days after an anastomosis and this is accompanied by, or even preceded by an increase in the secretion of the major collagenolytic enzyme, collagenase. In the present study both the increase in collagenase and the decrease in hydroxyproline are not significant until the third post-operative day and the coincidence in timing
CHAPTER V.3 Discussion

might well suggest a causal relationship; it could be argued that an increase in collagenase activity should precede any change in the collagen concentration but the timing of samples may have been inappropriate to demonstrate this in this instance.

However, the correlation does not hold true when comparing the site of collagen decrease with the increase in collagenase. The fall in hydroxyproline, in this study and in others, is demonstrated only proximal to the suture line. Collagenase secretion increases symmetrically and is recorded at least two centimetres distal to the anastomosis, where the hydroxyproline concentration in tissue from the same strips of colon does not decrease; indeed, the measured changes in collagenase are more significant distally than proximally. This discrepancy is hard to explain. The difference between the proximal and distal changes in collagen has been established by several different workers (Cronin et al 1968a, Jiborn et al 1978a, Hesp et al 1984b, Hendricks et al 1985). No other specific measurements of collagenase have been made from post-operative colon and the findings may indicate that the apparent cause and effect relationship is not valid after all. Alternatively, some other factor may be responsible for the hydroxyproline results which remains
operative for all the studies concerned. Since the overall level of collagen in the tissue is the result of the balance between synthesis and lysis, it is possible that a more marked increase in synthesis occurs on the distal side of an anastomosis, resulting in an overall steady level of hydroxyproline despite an increase in lysis. This was not shown in the studies of Cronin et al (1968b) and Jiborn et al (1980a and b), who measured collagen synthesis by the uptake of labelled proline, since they found a more marked increase in synthesis on the proximal side, where uptake rose between 8 and 15 fold compared to 3.5 and 5 fold distally. Nor is there any discrepancy in the normal level of hydroxyproline across this region, as shown by the unoperated group of animals in the present study. Further possible explanations lie in a difference in normal collagenase activity at these sites, across a distance of two or three centimetres, a difference in TIMP activity after operation or in the contribution of other proteolytic enzymes to collagenolysis.
IMMUNOLOCALISATION OF COLLAGENASE AND TIMP

The application of immunohistochemical techniques to rabbit colonic anastomoses provides an assessment of the distribution of collagenase and tissue inhibitor of metalloproteinases in the region of the operation over the first seven post-operative days. These results cannot be directly compared to any previous studies in this field since no comparable investigation has been reported. The findings must therefore be interpreted in relation to known histological features in order to understand the processes which are taking place in this healing reaction. The sequence of histological changes in this study is in general agreement with the descriptions of healing anastomoses given by several other workers in the past (Hesp et al 1985, Stein and Barry 1983, Foster and Leaper 1984, Getzen and Holloway 1966, Trueblood et al 1969, Herrman et al 1964). Although minor differences are seen, and have previously been reported in relation to everted rather than inverted suture lines (Ravitch et al 1981), the broad sequence of events is unchanged and the healing reactions investigated in this study followed the normal pattern for colonic healing.

Comparing the changes in everted and 'end-on'
anastomoses, the inflammatory reaction in the mucosa of the 'end-on' type tended to be more florid than in eversion but the response was more closely localised to the site of anastomosis and was almost completely confined to the region between the points of penetration of the suture material. Since it is known that the degree of inflammatory reaction at an anastomosis varies at different points around the circumference (Herrmann et al 1964), this difference between the two techniques is not necessarily significant.

The availability of a specific antibody to rabbit collagenase allows investigation of the distribution of the enzyme in tissue after an anastomosis has been formed. In addition, it is possible to define the distribution of its inhibitor, TIMP, to show the control under which collagenase is acting in these circumstances. The findings using this method are not entirely uniform. When collagenase is localised in tissue blocks, positive staining can be found in the same area of several sections in succession and the descriptions given in Chapter IV.2.i represent the 'average' distribution of the enzyme through the tissue. However, some sections in each block show less extensive staining than this, so that serial sections show enzyme gradually appearing and then disappearing in any one
area. The interpretation of this must be speculative but it suggests that the stimulus to increase collagenase secretion is not uniform and consequently the visible response is not uniform either. In such a localised tissue reaction, with an enzyme normally as tightly controlled as collagenase, it is not surprising that at any one time of sampling the secretion is somewhat patchy. Adjacent negative areas might well be positive if the tissue were collected slightly earlier or slightly later in relation to the time of operation. It is also reasonable to expect a gradual transition from one picture to another between sample times. The same explanation accounts for minor variations in the pattern of staining between one rabbit and the next, although the agreement between animals was very close, hence the limited number of animals studied.

Neither collagenase nor TIMP can be localised in normal rabbit colon which has been resected at operation, nor in colon after sham operation. This is in agreement with findings in non-gravid rabbit uterus and normal articular cartilage (Hembry et al 1986) and in inactive scar tissue in skin (Hembry and Ehrlich 1986). The collagen status of normal bowel is not static, since, like all biological systems, the components of colon are under continual gradual
turnover; synthesis and lysis of collagen presumably occur at such low levels that far greater numbers of tissue sections would need to be examined in order to find the occasional cell which is secreting collagenase or TIMP. This is an interesting finding, since it was increased levels of collagenolytic activity in normal unoperated colon which first implicated the enzyme (Hawley 1970). In addition, Hawley found that the levels of collagenolytic activity in colon rose after sham laparotomy. In the present study, no collagenase (or TIMP) was localised in colon one or three days after sham operation; a further discrepancy in findings which suggests that collagenase may not be the enzyme Hawley was seeking.

Within twelve hours of colonic resection and anastomosis there has been a stimulus to increase collagenase synthesis, which can then be immunolocalised both in secreting cells and extracellularly. Synthesis occurs in the parts of colon closest to the sites of damage, which are the cut edges and the regions pierced by the passage of sutures. The stimulus for synthesis may be provided by the damaged tissue itself, which releases plasminogen activator. This would stimulate the formation of plasmin, a recognised activator of collagenase (Eeckhout and Vaes 1977, He et al 1989),
which also induces synthesis (Werb and Aggeler 1978). In addition, the damage to blood vessels and the presence of clot implies a concentration of polymorphonuclear leucocytes in the region of damage. Polymorphonuclear leucocytes themselves synthesise collagenase in most species but they may be more important in this situation as a source of cytokines, such as Interleukin-1, a substance which has been shown to stimulate collagenase synthesis in all systems so far investigated (Murphy and Reynolds 1985, Pasternak et al 1986).

Whatever the stimulus, collagenase synthesis and secretion occur in colon within twelve hours of the standard operation and can be seen in the mucosal layers of the everted portions of bowel wall. By the time the healing and remodelling processes are finished, these everted parts will have been completely removed and the tissue layers will be once more in alignment. Mucosal layers heal, in ideal circumstances, without scarring, since epithelial cells grow across the defect to meet and join those from the other side. Muscle does not regenerate but the gap in the submucosa and muscle layers will fill with new connective tissue which will, in time, contract down to a narrow scar line (Hesp et al 1985, Stein and Barry 1983, Foster and Leaper 1984,
CHAPTER V.4 Discussion


Initially, the gap fills with clot and debris produced by the trauma of transection. Collagenase appears first in the damaged but relatively intact tissue alongside the plug, starting in the mucosal layers. By twenty-four hours, clot is resolving into fibrin and the enzyme is seen in small amounts within this plug. Synthesis is less extensive, since secreting cells can now be seen only after using the enhancement technique of short-term culture with monensin. Extracellular enzyme now spreads less far away from the cut edge but, at the ends of the everted tissue, it extends more deeply into the tissue layers. Inflammatory cell infiltration is more noticeable but still does not extend into the areas producing collagenase. Thus, after an initial mucosal reaction to damage, collagenase distribution becomes even more specific and the enzyme acts only on the tissue immediately adjacent to the cut edge, gradually spreading down through mucosa into submucosa and muscle.

Within twenty-four hours, collagenase activity is matched by TIMP. Since active collagenase is rapidly inhibited by TIMP, localisation of the inhibitor alongside the enzyme indicates a strict limitation of
enzyme activity in those areas. This parallel activity of TIMP continues through all the other times studied. The function of TIMP is to regulate the extracellular activity of metalloproteinases (Cawston et al 1981, Murphy and Reynolds 1985, Cawston and Mercer 1986, He et al 1989) and these findings indicate that degradation of collagen in these areas is tightly controlled.

By the third post-operative day, the process of removing the everted regions is clearly under way, since the tips of these parts are dying and completely infiltrated by inflammatory cells. Collagenase activity has moved further along the everted parts and precedes the wave of inflammatory cells as they progress deeper into the tissue. This suggests that the enzyme initiates collagen breakdown in these areas, leaving tissue which is more susceptible to phagocytosis and to the proteolytic enzymes secreted by inflammatory cells. Although Hesp and colleagues (1985) propose the granulocytes themselves as the source of collagenase after colonic anastomosis, in this study the secreting cells had the appearance of fibroblasts and were seen in areas yet to be infiltrated by inflammatory cells. In addition, the secretion of collagenase by rabbit polymorphs has not yet been demonstrated (G Murphy and R M Hembry personal communication).
CHAPTER V.4 Discussion

Three days after operation, the plug itself has increased in size and connective tissue formation is seen in a thickened layer on its outer surface, which helps to seal the lumen of the bowel from the peritoneal cavity. Maintenance of this layer must therefore be continued until the underlying fibrin has been replaced by more substantial scar tissue. This is confirmed by finding TIMP secretion within the layer, unaccompanied by collagenase which is clearly not desirable in this region at this stage. TIMP inhibits gelatinase and stromelysin as well as collagenase (Sellers et al 1979, Cawston et al 1981, Welgus et al 1985a, He et al 1989), so TIMP activity here will help to prevent premature breakdown of this protective outer layer and therefore contribute to the prevention of anastomotic dehiscence.

At the seven day stage, replacement of the fibrin plug by collagenous scar is well under way. This area is filled with active fibroblasts and, within the granulation tissue, remnants of undegraded mucosa and other tissues remain. These are invaded by inflammatory cells when they are clearly dead but, while they are still viable, they are the site of localised collagenase and TIMP activity, produced by small groups of secreting cells. This is similar to the findings in hypertrophic skin scars where regions of active remodelling contained
collagenase and TIMP in close proximity to negative, inactive areas (Hembry and Ehrlich 1986). In colon, at 7 days, the fibrous scar has yet to mature and contract, while remnants of unwanted elements are still being removed. Remodelling of the region occurs piecemeal, in small areas at a time and may continue in the same fashion for some time to come. As the need for remodelling decreases, collagenase and TIMP activity are likely to decrease also, until they return to such low levels that they are undetectable, as in normal colon.

The distribution of collagenase and TIMP after 'end-on' anastomosis is very similar to that after the standard operation with an everted suture line. Collagenase appears within 12 hours, particularly in the inverted mucosa, and can be identified extracellularly and in secretory granules within fibroblasts. Even at 12 hours TIMP is found in the same areas as collagenase and this evidence for control of the enzyme activity continues to the end of the study. Once again, the activity of both enzyme and inhibitor remain confined to the immediate vicinity of the cut edge of the bowel. Collagenase appears in the inverted mucosa which rapidly dies and must be degraded and removed to produce a smooth join. Less activity is apparent in the muscle layers with this technique since less remodelling of
these layers is required to complete the realignment. As before, TIMP secreting cells are found in the serosal coat at three days. There is no collagenase activity in this layer after either type of operation and the presence of TIMP presumably helps to maintain the seal between the lumen of the bowel and the peritoneal cavity.

There is no significant difference in the pattern of collagenase and TIMP secretion when comparing the everted and 'end-on' techniques of colonic anastomosis. The sequence of events which has been demonstrated by the findings of these immunohistochemical experiments is that of an uncomplicated healing reaction and the locations and extent of collagenase production are those that might be predicted in a normal healing response. The response remains extremely localised at all times after both techniques, since collagenase was never found more than a few millimetres from the cut edge of the bowel wall. A similar localised sequence of changes is seen, on an even smaller scale, around the sites of damage created by suture material, which also provokes a foreign body reaction. Enzyme activity is sufficient to remodel the region of the anastomosis and remove the damaged and dying parts of the bowel but is under the control of TIMP within a day of operation and remains
confined to the immediate vicinity of the suture line at all times. Hendricks et al (1985) and others (Cronin et al 1968a, Jiborn et al 1978a, Hesp et al 1984a and b, Hawley 1970) measured a significant fall in collagen after anastomosis which extended for several centimetres along the colon wall in a proximal direction but no collagenase activity was immunolocalised in the bowel wall more than a few millimetres from the cut edge, either proximally or distally. Apart from these changes at the very margin, the results of immunohistochemistry indicate that the decrease in collagen after anastomosis is not due to increased collagenase activity.
CRITICISMS AND IMPLICATIONS OF FINDINGS

It is clear from the foregoing discussion that the results of the biochemical and histochemical experiments in this study are not compatible. The biochemical data support Hawley's original hypothesis that collagenase may contribute to colonic anastomotic breakdown, since the enzyme secreted by explants in culture increases significantly in the post-operative period and there is an overall loss of collagen in the colon wall. The histochemical evidence, however, points to an extremely localised and entirely appropriate reaction to tissue damage, with collagenase initiating the degradation of those tissue elements which are not going to contribute to the healed suture line. It is therefore necessary to compare and criticise these two approaches in order to interpret fully the findings of this investigation.

Many criticisms can be levelled at the biochemical assay which was employed in this study. The requirement for tissue culture inevitably creates opportunities for unwanted effects to influence the results. A marked lag phase occurs before collagenase can be detected in the culture medium; this is in keeping with other tissue culture studies but it suggests that the stimulus of culture is required before detectable enzyme is produced.
This correlates with the lack of histochemically detectable collagenase in uncultured normal colon; however, positive staining was seen in post-operative tissue which had not been cultured even though the lag phase remained a feature of secretion in both pre- and post-operative tissue. It is difficult to believe that a significant increase in collagenase activity, sufficient to account for the fall in collagen and to contribute to the incidence of anastomotic dehiscence, would not abolish or at least considerably diminish this lag phase when the tissue is placed into culture.

Considering this discrepancy from another angle, the total extent of positive staining in post-operative tissue was never more than a few millimetres from the suture line; in dissection of the tissue to provide explants for culture, such a positive area could only represent a fraction of any individual explant and the sensitivity of the biochemical assay may be insufficient to demonstrate the small increase because the effects of culture override it. This could also explain the wide variation in the proportion of active enzyme secreted in the first 24 hours in culture by explants taken 3 days after anastomosis.

A further consideration is the trauma applied to the tissue while preparing explants for culture. Although the explants were supported on stainless steel grids to reduce...
any unwanted tendency to remodel in the culture conditions, it is impossible to prepare an explant of manageable size without creating a cut edge around its perimeter. The effect of such trauma may completely overwhelm the more localised effects of the presence of a suture line at one edge of the explant. It has been shown previously that collagenolytic activity (measured by a synthetic substrate) can be increased, not only by the creation of a suture line or the passage of various types of sutures, but also by the simple passage of a needle through the tissue (Lunstedt et al 1984). Fell et al (1986) have shown that minced pig synovium, when placed in culture, produces collagenase which breaks down the collagen within the explant itself.

The principal objective of the present study was to investigate the activity of collagenase in colonic healing; the complexity of the biochemical methods involved precluded the measurement of inhibitory activity by TIMP. It is possible that TIMP is acting in the tissue when it is first removed from the animal and that the gradual appearance of measurable collagenase really represents a gradual disappearance of TIMP. In some studies in which TIMP has been measured, it has been secreted by cells in monolayer culture but not from explants (Welgus et al 1979, Heath et al 1982).
Histochemically it was possible to show the presence of TIMP 24 hours after operation; the antibody to collagenase remains effective for enzyme which is already bound to inhibitor but the biochemical assay would detect only unbound collagenase. Menashi et al (1987) removed TIMP in order to detect collagenase activity in extracts of aortic wall. Fell et al (1986) have suggested that biochemical measurements of collagenase may represent only the overspill of enzyme which has not been bound to TIMP and therefore may not truly reflect the amount of activity, since enzyme which has acted and then been inactivated will not be registered. Similarly, it may be true that TIMP cannot be detected in a system unless it is present in excess over collagenase (Heath et al 1982).

One further point is of particular importance in evaluating the results of the biochemical assay. The viability of individual tissue explants in culture was not examined in this study. Since collagenase production is dependent on viable cells, this is clearly directly relevant to the production of enzyme but systems in which the uptake of dye is used to demonstrate the percentage of cells remaining viable in a monolayer culture are not applicable to explants of full wall thickness. The structure of the tissue was certainly lost in culture, since the explants became soft and pulpy and difficult to
handle by 72 hours; visible tissue loss after only six hours in culture has been demonstrated by the histological findings shown in Chapter III.4. This supports the findings of Fell et al (1986), who showed extensive destruction of connective tissue within explants of pig synovium in culture; the collagenase activity measured in culture medium may represent only the residual enzyme when the collagen within the explant has already been degraded. It is reasonable to assume that the ability of a tissue explant to secrete collagenase is directly relevant to its viability. In this study viability was not assessed; for the moment it must be assumed that the percentage of tissue lost is relatively uniform for each explant and that uniformity of handling of the explants produces, on average, a similar loss of cells from both pre- and post-operative tissues. Only if this is so will the differences in secretion truly reflect the effects of operation on the tissue rather than the way that it was handled subsequently. However, the absence of such uniformity may account for the wide spread of results in any one group of explants. This was minimised by taking the mean activity from three separate explants as the value for any one piece of tissue but it indicates that further studies with this method should include some attempt to assess the viability of the explants.
Thus, there is no compatibility between the histochemical findings of increased collagenase in localised areas of tissue from 12 hours after operation, which is beginning to decrease by the third post-operative day, and the biochemical evidence for increased collagenase activity which begins one day after operation and is greater after three days. Even more at odds is the measurement of increased activity in tissue taken 2 cm away from the stitch line when histologically the changes are confined to a region only a few millimetres wide. In this respect the biochemical evidence from the hydroxyproline assay cannot be correlated either with that from the collagenase assay or with the histochemical data.

In the face of these major discrepancies it is necessary to give more weight to some results than others in evaluating the findings of this investigation. Based on the above analysis, and the knowledge that the immunohistochemistry reflects the 'in vivo' situation, the data from the immunohistochemical studies appear to be a closer representation of the true activity of the enzyme collagenase in the healing colonic anastomosis. The data from the biochemical studies cannot lightly be discarded but must be viewed with more reservations. For the future, further biochemical assessments of the collagenase activity in colon should include demonstration of the
degree of viability of the tissue, given that culture cannot be avoided. It must not be forgotten that this assay does represent a quantitative assessment of activity: the immunohistochemical data are qualitative and any assessment of relative amounts of activity in the sections can only be subjective. Furthermore, the antibody used for localisation reacts with both latent and active collagenase and with collagenase bound to collagen or TIMP (Hembry et al 1986). For pharmacological investigations in this field, which will be necessary if the use of enzyme inhibitors is to be pursued, quantitative assay is vital and distinction between the different forms of the enzyme is equally important. In addition, relevant information would be provided by the inclusion of an assay for TIMP activity; enzyme-linked immunosorbent assays (ELISA) for both TIMP and collagenase have now become available (J J Reynolds, personal communication, Yoshioka et al 1987, Kodama et al 1989). The ability to measure the activity of both enzyme and inhibitor promises a much clearer understanding of the situation, since a delicate shift in balance between the two may be all that is required to produce a significant change in collagen (Reynolds 1986, Kerwar et al 1986).
As a result of this comparison of methods, the role of collagenase in uncomplicated colonic anastomotic healing must be deduced from the results of the immunohistochemical experiments. Collagenase is present in a tightly controlled system, playing a part which might be predicted for a normal healing response. In the formation of an anastomosis the cut edges of the bowel wall are turned either inwards or outwards to ensure good approximation of the edges and to help keep the suture line water- and air-tight until true healing occurs. These edges are furthest from the remaining blood supply to the wall and are compromised by the pressure of the suture material. As time passes they are broken down and replaced by fibrous scar tissue which in turn gradually remodels until the junction of the two ends of bowel is smooth. Collagenase becomes active in these localised regions only, initiating collagen breakdown of the parts which will then be degraded by other proteolytic enzymes. This response is independent of the precise configuration of the suture line. It may be the action of collagenase which allows subsequent infiltration by polymorphonuclear leucocytes, the source of the other proteinases.

This system is rapidly brought under control by the appearance of tissue inhibitor of metalloproteinases; within 24 hours of operation collagenase is never seen
unless accompanied by TIMP. The process of degradation remains strictly limited to the immediate region of trauma but TIMP has an added role in maintaining the protective coat which appears on the outer surface of the anastomosis by the third post-operative day. Once scar tissue is present, both enzyme and inhibitor act in small areas at a time, mopping up residual elements of the original wall and remodelling the fibrous tissue into a mature scar.

The source of collagenase for this process is of interest. It has been suggested that the inflammatory cells which collect at the site of trauma are the secretors of collagenase (Hesp et al 1985, Mastboom et al 1989). Granulocytes certainly appear at an anastomosis and can be identified by staining with haematoxylin and eosin and by the chloronaphthol technique used in this study. Human granulocytes do indeed secrete collagenase and are the only cells known to store the enzyme in anticipation of need (Weiss and Peppin 1986). However, immunolocalisation demonstrates that collagenase is found only in areas which have not yet been infiltrated by granulocytes. The enzyme originates in interstitial cells within the mucosal layer which have the appearance of fibroblasts; fibroblasts are certainly a common source of collagenase in other situations (Werb and Aggeler 1978, Welgus et al 1979, Welgus et al 1985b, Pettigrew et al
1980). Similar cells secrete TIMP, although, without double-staining techniques, it is not possible to show whether the same cells are releasing both enzyme and inhibitor.

This is perhaps not conclusive evidence that the collagenase at colonic anastomoses originates in fibroblasts. However, considerable work has been carried out to try to demonstrate collagenase in rabbit polymorphonuclear leucocytes, without success (G Murphy and R M Hembry, personal communication). Not only do neutrophil collagenases show immunological differences from the tissue enzymes (Murphy and Sellers 1980, Weiss and Peppin 1986, Mainardi et al 1987), so that the anti-collagenase antibody used in this study might not react with a granulocyte enzyme, but rabbit granulocytes appear to be devoid of collagenase altogether. In the animals used in this particular investigation, therefore, the collagenase must be secreted by cells other than granulocytes. This may suggest that the model system used for this investigation is inappropriate, since granulocytes in other species, notably man, do store and secrete the enzyme (Weiss and Peppin 1986). Some advantage can be gained, however, by studying a system with one source of enzyme before proceeding to the more complicated situation. In addition, much of the previous
work in this field, including the original evidence which implicated collagenase in anastomotic dehiscence, was carried out in this very rabbit model.

The role of the inflammatory cells must not be dismissed simply because they do not secrete collagenase in this instance. A small number of granulocytes are always present in the colon wall and large numbers collect at the site of an anastomosis (Hesp et al 1985, Keshavarzian et al 1986). They release cathepsins and other cysteine and serine proteinases which contribute to the further degradation of collagen, once collagenase has produced its initiating split. The same enzymes can act directly on any collagen which has been damaged by the trauma of resection and the passage of sutures. Högström and Haglund (1986) have shown that neutrophils act via oxygen free radicals and proteinases to reduce the suture-holding capacity of the post-operative intestinal wall. Granulocytes are also, and perhaps more importantly, the source of cytokines, such as Interleukin-1. The cytokines are mediators of many responses, including stimulation of both collagenase and TIMP synthesis and secretion. The attraction of granulocytes to the site of operation may result in the release of cytokines which then stimulate local fibroblasts to synthesize collagenase. As suggested
before (Chapters V.3 and V.4), it may be the release of plasminogen activator by damaged tissue which interacts with plasminogen in serum to produce plasmin, thus activating the newly-synthesised collagenase. Since TIMP release is also promoted by the cytokines, the degradatory process remains controlled, while reducing the unwanted collagen to a state in which it can be fully degraded by other granulocyte enzymes.

If this interpretation of events is accurate then collagenase plays a precise and closely regulated role in uncomplicated colonic healing and there is nothing to suggest any uncontrolled or excessive activity in normal circumstances. The results indicate that no benefit can be expected from the prophylactic administration of a collagenase inhibitor to all subjects undergoing colonic anastomosis and no such benefit was demonstrated in a clinical trial of aprotinin (Young and Wheeler 1984).

This does not explain the marked changes in hydroxyproline which have been found in the anastomotic region in many investigations and confirmed by this study. As discussed earlier (Chapter V.2), these changes may be artefactual, since no satisfactory explanation has yet been found for the discrepancy between the proximal and distal sides of the anastomosis. Alternatively, the changes in hydroxyproline could be accounted for by some process
other than the action of collagenase.

The evidence from the biochemical assay of collagenase used in this study does not contribute to this argument, since strenuous efforts were made to ensure that the activity measured was specific to collagenase. The original assay used by Hawley (1970) did not carry such safeguards and the excess collagenolysis recorded in this work may well have been due to the presence of other proteolytic agents. Other enzymes, usually secreted by inflammatory cells, are certainly known to attack partially denatured collagen. Cathepsin B and other cysteine proteinases are known to be more effective in acidic conditions (Peacock 1980, Sellers and Murphy 1981, Murphy and Reynolds 1985) and pockets of ischaemia and acidity may easily exist in the region of a newly-formed anastomosis. Phagocytic removal of undegraded collagen fibres may also take place (Laurent 1989).

The decrease in hydroxyproline concentration need not therefore be entirely discredited in the analysis of healing colon. Not only has collagen concentration been shown to decrease but collagen synthesis is known to be markedly increased. These findings indicate a need to look elsewhere for the factors involved in anastomotic dehiscence. Many other proteolytic enzymes may contribute to the process and should be investigated to determine
their role in colonic healing. The encouraging effects of non-specific inhibitors, such as aprotinin and soy bean trypsin inhibitor in animals (Delaney and Lalor 1976, vonBary et al 1976, Young and Wheeler 1982, 1983), may be due to their effects on these other enzymes rather than on collagenase (Eisenberg et al 1984).

Further work is required in the investigation of colonic anastomotic breakdown since this study provides no evidence for excessive collagenase activity in normal circumstances; application of the immunohistochemical methods to anastomoses in the presence of obstruction, ischaemia or peritonitis would confirm whether this holds true when there is a high risk of dehiscence. Studies in other species could determine the role of granulocyte collagenase. It may prove more profitable, however, to investigate the activity of other enzymes: gelatinase, stromelysin, the cathepsins and other proteases all contribute to the breakdown of connective tissues in some circumstances and it is important to define the reactions which are taking place before trying to assess the effect of inhibitory agents.

In a wider context, this study has established the use of immunohistochemistry to examine the distribution of proteases in colonic healing. Antibodies are now available for the study of collagenase, gelatinase,
stromelysin and TIMP (R M Hembry, personal communication). Investigation of their role in other colonic conditions, such as inflammatory bowel disease and tumour invasion, may not only elucidate the pathological processes involved but might also lead to the development of more specific therapeutic measures.
CHAPTER VI Conclusions

CONCLUSIONS

This study set out to re-appraise the role of collagenase in colonic anastomotic healing. Comparison of the findings shows that immunohistochemistry is the most reliable of the specific methods available. Collagenase activity is found in rabbit colon after anastomosis, confined to a narrow margin at the cut ends of the bowel wall. It appears in the mucosa within 12 hours of operation and gradually progresses more deeply through the damaged edges, initiating collagen breakdown in the tissue prior to its infiltration by granulocytes. By 24 hours collagenase secretion is already beginning to wane and TIMP appears, to keep the enzyme activity under tight control. Three days after operation, TIMP is also helping to maintain anastomotic integrity by preventing protease degradation of the serosal sealing layer. After 7 days both enzyme and inhibitor are active only in small areas where dying tissue remains to be destroyed and where remodelling is taking place. The enzyme is performing a predictable and carefully controlled process of breakdown of unwanted tissue and there is no evidence for excessive collagenase activity in normal colonic anastomotic healing.
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Table A.1.1

Comparison of Hydroxyproline Concentrations in wet and dry tissue, before and 3 days after colonic anastomosis.

<table>
<thead>
<tr>
<th>Rabbit D</th>
<th>Mean ± SD</th>
<th>Coefficient of variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Res</td>
<td>1.07 ± 0.42</td>
<td>39.25</td>
</tr>
<tr>
<td>Pr5</td>
<td>0.54 ± 0.24</td>
<td>44.44</td>
</tr>
<tr>
<td>Pr2</td>
<td>0.82 ± 0.35</td>
<td>42.68</td>
</tr>
<tr>
<td>Pr0</td>
<td>1.06 ± 0.72</td>
<td>67.92</td>
</tr>
<tr>
<td>Di0</td>
<td>1.13 ± 0.43</td>
<td>38.05</td>
</tr>
<tr>
<td>Di2</td>
<td>1.89 ± 1.20</td>
<td>63.49</td>
</tr>
<tr>
<td>Di5</td>
<td>0.94 ± 0.19</td>
<td>20.21</td>
</tr>
</tbody>
</table>

Mean Co var for wet tissue = 45.15

<table>
<thead>
<tr>
<th>Measured in dry tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Res</td>
</tr>
<tr>
<td>Pr5</td>
</tr>
<tr>
<td>Pr2</td>
</tr>
<tr>
<td>Pr0</td>
</tr>
<tr>
<td>Di0</td>
</tr>
<tr>
<td>Di2</td>
</tr>
<tr>
<td>Di5</td>
</tr>
</tbody>
</table>

Mean Co var for dry tissue = 26.05
Table A.1.2

Comparison of Hydroxyproline Concentrations in wet and dry tissue, before and 3 days after colonic anastomosis.

<table>
<thead>
<tr>
<th>Rabbit</th>
<th>Mean ± SD</th>
<th>Coefficient of variation</th>
</tr>
</thead>
<tbody>
<tr>
<td>E</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Res</td>
<td>1.50 ± 0.45</td>
<td>30.00</td>
</tr>
<tr>
<td>Pr5</td>
<td>0.72 ± 0.29</td>
<td>40.28</td>
</tr>
<tr>
<td>Pr2</td>
<td>2.03 ± 1.07</td>
<td>52.71</td>
</tr>
<tr>
<td>Pr0</td>
<td>1.44 ± 0.76</td>
<td>52.78</td>
</tr>
<tr>
<td>Di0</td>
<td>2.02 ± 1.69</td>
<td>83.66</td>
</tr>
<tr>
<td>Di2</td>
<td>1.56 ± 0.32</td>
<td>20.51</td>
</tr>
<tr>
<td>Di5</td>
<td>1.13 ± 0.59</td>
<td>52.21</td>
</tr>
</tbody>
</table>

Mean Co var for wet tissue = 47.45

| Res    | 12.81 ± 1.49   | 11.63   |
| Pr5    | 4.96 ± 1.77    | 35.69   |
| Pr2    | 13.11 ± 4.32   | 32.95   |
| Pr0    | 7.66 ± 1.88    | 24.54   |
| Di0    | 10.13 ± 0.71   | 7.01    |
| Di2    | 11.65 ± 2.99   | 25.67   |
| Di5    | 17.65 ± 2.96   | 16.77   |

Mean Co var for dry tissue = 22.04
APPENDIX

Table A.1.3
Comparison of Hydroxyproline Concentrations in wet and dry tissue, before and 3 days after colonic anastomosis.

<table>
<thead>
<tr>
<th>Rabbit G</th>
<th>Mean ± SD</th>
<th>Coefficient of variation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Measured in wet tissue</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Res</td>
<td>1.99 ± 0.88</td>
<td>44.22</td>
</tr>
<tr>
<td>Pr5</td>
<td>0.73 ± 0.12</td>
<td>16.44</td>
</tr>
<tr>
<td>Pr2</td>
<td>0.87 ± 0.13</td>
<td>14.94</td>
</tr>
<tr>
<td>Pr0</td>
<td>1.47 ± 0.26</td>
<td>17.69</td>
</tr>
<tr>
<td>Di0</td>
<td>1.68 ± 0.67</td>
<td>39.88</td>
</tr>
<tr>
<td>Di2</td>
<td>1.34 ± 0.32</td>
<td>23.88</td>
</tr>
<tr>
<td>Di5</td>
<td>1.72 ± 1.54</td>
<td>89.53</td>
</tr>
<tr>
<td>Mean Co var for wet tissue =</td>
<td>35.23</td>
<td></td>
</tr>
</tbody>
</table>

| Measured in dry tissue              |           |                          |
| Res      | 11.51 ± 1.12 | 9.73                    |
| Pr5      | 9.54 ± 2.59  | 27.15                   |
| Pr2      | 7.22 ± 1.82  | 25.21                   |
| Pr0      | 8.58 ± 2.08  | 24.24                   |
| Di0      | 8.73 ± 1.39  | 15.92                   |
| Di2      | 7.86 ± 3.71  | 47.20                   |
| Di5      | 11.31 ± 3.87 | 34.22                   |
| Mean Co var for dry tissue =        | 26.24      |
APPENDIX

Table A.1.4

Comparison of Hydroxyproline Concentrations in wet and dry tissue, before and 3 days after colonic anastomosis.

<table>
<thead>
<tr>
<th>Rabbit J</th>
<th>Mean ± SD</th>
<th>Coefficient of variation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Measured in wet tissue</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Res</td>
<td>1.16 ± 0.20</td>
<td>17.24</td>
</tr>
<tr>
<td>Pr5</td>
<td>1.20 ± 0.30</td>
<td>25.00</td>
</tr>
<tr>
<td>Pr2</td>
<td>1.52 ± 0.13</td>
<td>8.55</td>
</tr>
<tr>
<td>Pr0</td>
<td>1.16 ± 0.41</td>
<td>35.34</td>
</tr>
<tr>
<td>Di0</td>
<td>1.15 ± 0.16</td>
<td>13.91</td>
</tr>
<tr>
<td>Di2</td>
<td>1.45 ± 0.40</td>
<td>27.59</td>
</tr>
<tr>
<td>Di5</td>
<td>1.23 ± 0.63</td>
<td>51.22</td>
</tr>
<tr>
<td>Mean Co var for wet tissue</td>
<td>25.55</td>
<td></td>
</tr>
</tbody>
</table>

| Measured in dry tissue | | |
| Res      | 11.79 ± 5.57 | 47.24                   |
| Pr5      | 10.11 ± 1.75 | 17.31                   |
| Pr2      | 7.35 ± 1.99  | 27.07                   |
| Pr0      | 9.84 ± 1.88  | 19.11                   |
| Di0      | 11.29 ± 3.41 | 30.20                   |
| Di2      | 11.71 ± 4.06 | 34.67                   |
| Di5      | 12.08 ± 3.18 | 26.32                   |
| Mean Co var for dry tissue | 28.85 |
Table A.1.5

Comparison of Hydroxyproline Concentrations in wet and dry tissue, before and 3 days after colonic anastomosis.

<table>
<thead>
<tr>
<th>Rabbit</th>
<th>Mean ± SD</th>
<th>Coefficient of variation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Measured in wet tissue</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Res</td>
<td>1.76 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>Pr5</td>
<td>1.04 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>Pr2</td>
<td>0.70 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>Pr0</td>
<td>1.27 ± 0.44</td>
</tr>
<tr>
<td></td>
<td>Di0</td>
<td>1.62 ± 0.53</td>
</tr>
<tr>
<td></td>
<td>Di2</td>
<td>1.98 ± 0.34</td>
</tr>
<tr>
<td></td>
<td>Di5</td>
<td>1.72 ± 0.64</td>
</tr>
<tr>
<td>Mean Co var for wet tissue =</td>
<td>25.77</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Measured in dry tissue</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Res</td>
<td>10.13 ± 1.48</td>
</tr>
<tr>
<td></td>
<td>Pr5</td>
<td>7.91 ± 1.45</td>
</tr>
<tr>
<td></td>
<td>Pr2</td>
<td>6.83 ± 1.12</td>
</tr>
<tr>
<td></td>
<td>Pr0</td>
<td>8.18 ± 0.95</td>
</tr>
<tr>
<td></td>
<td>Di0</td>
<td>11.84 ± 2.85</td>
</tr>
<tr>
<td></td>
<td>Di2</td>
<td>10.41 ± 4.07</td>
</tr>
<tr>
<td></td>
<td>Di5</td>
<td>9.75 ± 3.78</td>
</tr>
<tr>
<td>Mean Co var for dry tissue =</td>
<td>23.27</td>
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</tbody>
</table>
Table A.2

**HANK'S BALANCED SALTS SOLUTION**

<table>
<thead>
<tr>
<th>Component</th>
<th>g/l</th>
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<tbody>
<tr>
<td>sodium chloride</td>
<td>8.00</td>
</tr>
<tr>
<td>potassium chloride</td>
<td>0.40</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>0.185</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.10</td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>0.10</td>
</tr>
<tr>
<td>Na₂HPO₄ anhydrous</td>
<td>0.048</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.06</td>
</tr>
<tr>
<td>glucose</td>
<td>1.00</td>
</tr>
<tr>
<td>sodium bicarbonate</td>
<td>0.35</td>
</tr>
<tr>
<td>HEPES</td>
<td>4.766</td>
</tr>
</tbody>
</table>

plus antibiotics: 1x 10x

<table>
<thead>
<tr>
<th>Component</th>
<th>60 mg</th>
<th>600 mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>penicillin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>streptomycin</td>
<td>100 mg</td>
<td>1 g</td>
</tr>
<tr>
<td>amphotericin B</td>
<td>2.5 mg</td>
<td>25 mg</td>
</tr>
</tbody>
</table>

332
### Table A.3

**DULBECCO'S MODIFICATION OF EAGLE'S MEDIUM**

<table>
<thead>
<tr>
<th>Substance</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>L arginine HCl</td>
<td>84.0 mg/l</td>
</tr>
<tr>
<td>D-Ca pantothenate</td>
<td>4.0 mg/l</td>
</tr>
<tr>
<td>L cystine</td>
<td>56.78 mg/l</td>
</tr>
<tr>
<td>choline chloride</td>
<td>4.0 mg/l</td>
</tr>
<tr>
<td>disodium salt</td>
<td>folic acid</td>
</tr>
<tr>
<td>glycine</td>
<td>30.0 mg/l</td>
</tr>
<tr>
<td>i-inositol</td>
<td>7.0 mg/l</td>
</tr>
<tr>
<td>L histidine HCl</td>
<td>42.0 mg/l</td>
</tr>
<tr>
<td>nicotinamide</td>
<td>4.0 mg/l</td>
</tr>
<tr>
<td>L isoleucine</td>
<td>104.8 mg/l</td>
</tr>
<tr>
<td>pyridoxal HCl</td>
<td>4.0 mg/l</td>
</tr>
<tr>
<td>L leucine</td>
<td>104.8 mg/l</td>
</tr>
<tr>
<td>riboflavin</td>
<td>0.4 mg/l</td>
</tr>
<tr>
<td>L lysine</td>
<td>146.2 mg/l</td>
</tr>
<tr>
<td>thiamin HCl</td>
<td>4.0 mg/l</td>
</tr>
<tr>
<td>L methionine</td>
<td>30.0 mg/l</td>
</tr>
<tr>
<td>CaCl₂.2H₂O</td>
<td>264.9 mg/l</td>
</tr>
<tr>
<td>L phenylalanine</td>
<td>66.0 mg/l</td>
</tr>
<tr>
<td>Fe(NO₃)₃.9H₂O</td>
<td>0.1 mg/l</td>
</tr>
<tr>
<td>L serine</td>
<td>42.0 mg/l</td>
</tr>
<tr>
<td>KCl</td>
<td>400.0 mg/l</td>
</tr>
<tr>
<td>L threonine</td>
<td>95.2 mg/l</td>
</tr>
<tr>
<td>MgSO₄.7H₂O</td>
<td>200.0 mg/l</td>
</tr>
<tr>
<td>L tryptophan</td>
<td>16.0 mg/l</td>
</tr>
<tr>
<td>NaCl</td>
<td>6400.0 mg/l</td>
</tr>
<tr>
<td>L tyrosine</td>
<td>89.5 mg/l</td>
</tr>
<tr>
<td>NaH₂PO₄.2H₂O</td>
<td>141.3 mg/l</td>
</tr>
<tr>
<td>disodium salt</td>
<td>D glucose</td>
</tr>
<tr>
<td>L valine</td>
<td>93.6 mg/l</td>
</tr>
<tr>
<td>sodium pyruvate</td>
<td>110.0 mg/l</td>
</tr>
<tr>
<td>phenol red</td>
<td>15.0 mg/l</td>
</tr>
<tr>
<td>HEPES</td>
<td>20 mM</td>
</tr>
<tr>
<td>penicillin</td>
<td>60.0 mg/l</td>
</tr>
<tr>
<td>L glutamine</td>
<td>292.0 mg/l</td>
</tr>
<tr>
<td>streptomycin</td>
<td>100.0 mg/l</td>
</tr>
<tr>
<td>sodium bicarbonate</td>
<td>500.0 mg/l</td>
</tr>
<tr>
<td>amphotericin B</td>
<td>2.5 mg/l</td>
</tr>
<tr>
<td>acid-treated foetal calf serum</td>
<td>1.0 per cent (v/v)</td>
</tr>
</tbody>
</table>
Table A.4.1

1 day post-op
Hydroxyproline Concentration in μg/mg dry tissue

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Pr2</th>
<th>Pr0</th>
<th>Di0</th>
<th>Di2</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3</td>
<td>7.44</td>
<td>8.48</td>
<td>5.33</td>
<td>5.59</td>
<td>8.98</td>
</tr>
<tr>
<td>C4</td>
<td>12.78</td>
<td>8.95</td>
<td>4.45</td>
<td>9.08</td>
<td>9.67</td>
</tr>
<tr>
<td>C5</td>
<td>11.14</td>
<td>8.27</td>
<td>5.40</td>
<td>5.83</td>
<td>5.87</td>
</tr>
<tr>
<td>C7</td>
<td>7.75</td>
<td>13.47</td>
<td>9.30</td>
<td>10.53</td>
<td>5.18</td>
</tr>
<tr>
<td>A2</td>
<td>11.55</td>
<td>21.69</td>
<td>16.97</td>
<td>17.80</td>
<td>12.23</td>
</tr>
<tr>
<td>B1</td>
<td>9.88</td>
<td>22.89</td>
<td>16.40</td>
<td>22.74</td>
<td>16.54</td>
</tr>
<tr>
<td>B2</td>
<td>17.16</td>
<td>18.04</td>
<td>17.90</td>
<td>7.42</td>
<td>9.01</td>
</tr>
<tr>
<td>Mean</td>
<td>11.10</td>
<td>14.54</td>
<td>10.82</td>
<td>11.28</td>
<td>9.64</td>
</tr>
</tbody>
</table>

Log transformed figures

<p>| | | | | | |</p>
<table>
<thead>
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<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>C3</td>
<td>2.01</td>
<td>2.14</td>
<td>1.67</td>
<td>1.72</td>
<td>2.19</td>
</tr>
<tr>
<td>C4</td>
<td>2.55</td>
<td>2.19</td>
<td>1.49</td>
<td>2.21</td>
<td>2.27</td>
</tr>
<tr>
<td>C5</td>
<td>2.41</td>
<td>2.11</td>
<td>1.69</td>
<td>1.76</td>
<td>1.77</td>
</tr>
<tr>
<td>C7</td>
<td>2.05</td>
<td>2.60</td>
<td>2.23</td>
<td>2.35</td>
<td>1.64</td>
</tr>
<tr>
<td>A2</td>
<td>2.45</td>
<td>3.08</td>
<td>2.83</td>
<td>2.88</td>
<td>2.50</td>
</tr>
<tr>
<td>B1</td>
<td>2.29</td>
<td>3.13</td>
<td>2.80</td>
<td>3.12</td>
<td>2.81</td>
</tr>
<tr>
<td>B2</td>
<td>2.84</td>
<td>2.89</td>
<td>2.88</td>
<td>2.00</td>
<td>2.20</td>
</tr>
<tr>
<td>Mean</td>
<td>2.37</td>
<td>2.59</td>
<td>2.23</td>
<td>2.29</td>
<td>2.20</td>
</tr>
</tbody>
</table>
Table A.4.2

3 days post-op

Hydroxyproline Concentration in μg/mg dry tissue

<table>
<thead>
<tr>
<th>Control</th>
<th>Pr2</th>
<th>Pr0</th>
<th>Di0</th>
<th>Di2</th>
</tr>
</thead>
<tbody>
<tr>
<td>C8</td>
<td>9.12</td>
<td>5.20</td>
<td>4.72</td>
<td>5.2</td>
</tr>
<tr>
<td>C13</td>
<td>12.35</td>
<td>8.08</td>
<td>5.74</td>
<td>10.43</td>
</tr>
<tr>
<td>C15</td>
<td>12.30</td>
<td>10.21</td>
<td>4.28</td>
<td>15.41</td>
</tr>
<tr>
<td>C10</td>
<td>11.23</td>
<td>8.96</td>
<td>9.00</td>
<td>12.71</td>
</tr>
<tr>
<td>K</td>
<td>10.13</td>
<td>6.83</td>
<td>8.18</td>
<td>11.84</td>
</tr>
<tr>
<td>D</td>
<td>17.22</td>
<td>9.84</td>
<td>10.22</td>
<td>13.12</td>
</tr>
<tr>
<td>E</td>
<td>12.81</td>
<td>13.11</td>
<td>7.66</td>
<td>10.13</td>
</tr>
<tr>
<td>G</td>
<td>11.51</td>
<td>7.22</td>
<td>8.58</td>
<td>8.73</td>
</tr>
<tr>
<td>J</td>
<td>11.79</td>
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3 days post-op - Log transformed figures

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1 day post-op

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3 days post-op

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338
Table A.4.6

Ratio of Hydroxyproline to Protein
1 day post-op

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Protein Concentration in \( \mu g/mg \) dry tissue

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APPENDIX

Table A.5.1

1 day post-op, 24 h culture

Active Collagenase Activity

in Units x 10^-3/mg wet weight of tissue

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## Table A.5.2

1 day post-op, 24 h culture

Latent Collagenase Activity

in Units x 10^{-3}/mg wet weight of tissue

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APPENDIX

Table A.5.3

1 day post-op, 24 h culture

Total Collagenase Activity

in Units x 10^-3/mg wet weight of tissue

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### APPENDIX

Table A.5.4

1 day post-op, 48 h culture

**Active Collagenase Activity**

in Units x 10^{-3}/mg wet weight of tissue

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Table A.5.5

1 day post-op, 48 h culture

Latent Collagenase Activity

in Units x 10^-3/mg wet weight of tissue

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APPENDIX

Table A.5.6

1 day post-op, 48 h culture

Total Collagenase Activity

in Units x 10^-3/mg wet weight of tissue

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Table A.5.7

1 day post-op, 72 h culture

Active Collagenase Activity

in Units x 10^-3/mg wet weight of tissue

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### APPENDIX

Table A.5.8

1 day post-op, 72 h culture

**Latent Collagenase Activity**

in Units x 10^-3/mg wet weight of tissue

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### Table A.5.9

1 day post-op, 72 h culture

Total Collagenase Activity

in Units x 10^-3/mg wet weight of tissue

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Table A.5.10

3 day post-op, 24 h culture

Active Collagenase Activity

in Units x 10^-3/mg wet weight of tissue

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### APPENDIX

Table A.5.11

3 day post-op, 24 h culture

Latent Collagenase Activity

in Units x 10^-3/mg wet weight of tissue

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Table A.5.12

3 day post-op, 24 h culture

Total Collagenase Activity
in Units x 10^-3/mg wet weight of tissue

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Table A.5.13

3 day post-op, 48 h culture

Active Collagenase Activity

in Units x 10^-3/mg wet weight of tissue

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APPENDIX

Table A.5.14

3 day post-op, 48 h culture

Latent Collagenase Activity

in Units x 10^-3/mg wet weight of tissue

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Table A.5.15

3 day post-op, 48 h culture

Total Collagenase Activity
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355
Table A.5.16

3 day post-op, 72 h culture

Active Collagenase Activity

in Units x 10^-3/mg wet weight of tissue

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Table A.5.17

3 day post-op, 72 h culture

Latent Collagenase Activity

in Units x 10^-3/mg wet weight of tissue

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### Table A.5.18

3 day post-op, 72 h culture

**Total Collagenase Activity**

in Units x 10^-3/mg wet weight of tissue

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Paper accepted 21 November 1987

Multiple authorship

The Editors remain concerned about the number of papers submitted to the Journal which appear to carry an inappropriate number of authors. We whole-heartedly endorse the recent statement by the International Committee of Medical Journal Editors, namely that authorship 'should be based only on substantial contributions to (a) conception and design, or analysis and interpretation of data; (b) drafting the article or revising it critically for important intellectual content; and (c) final approval of the version to be published'. It must be stressed that these three criteria must all be fulfilled. Those submitting material to The British Journal of Surgery should be warned that the Editors intend to try to enforce these guidelines wherever possible.
Role of collagenase in colonic anastomoses: a reappraisal

Increased collagenolysis, with reduction in collagen concentration, has been incriminated in the breakdown of colonic anastomoses but previous studies have measured only collagen levels and non-specific collagenolytic activity. Collagenase, the initiating enzyme in collagen degradation, is synthesized on demand and controlled by tissue inhibitor of metalloproteinases (TIMP). Antibodies to collagenase and TIMP were applied to colonic anastomoses in rabbits to investigate the role of the enzyme during healing. Within 12 h of operation, secreting cells and extracellular collagenase were identified at the everted edges of the bowel wall. After 24 h, collagenase activity was accompanied by TIMP secretion in the same localized regions, and by the third postoperative day very few cells were still synthesizing enzyme in these areas, although extracellular activity remained visible. TIMP-secreting cells, however, were seen in a layer of connective tissue sealing the serosal surface of the anastomosis. At 7 days, both enzyme and inhibitor were found only in small aggregates of secreting cells in the deeper layers. The localization and extent of collagenase and TIMP activity accorded well with a normal healing response as, at all times, the enzyme was confined to the immediate vicinity of the suture line.

Keywords: Collagenase, tissue inhibitor of metalloproteinases, anastomosis, colon

Dehiscence of a large bowel anastomosis is a major complication of colonic surgery, prolonging hospital stay and increasing mortality from 7 per cent to 22 per cent1. Collagen is the major structural protein of the colon wall and excess collagenase activity was first implicated in the dehiscence of colonic anastomoses by indirect evidence from measurements of bursting pressure and of collagen. Collagen concentration in the colon wall decreases in the early postoperative period2-4, reaching a trough after 48 h in rabbits. This corresponds to the time when the anastomosis is at its weakest. The assumption that the fall in collagen was due to an increase in collagenase was supported by Hawley6, who showed that explants of normal colon produced greater lysis of collagen gels than did other parts of the gastrointestinal tract and confirmed a 145 per cent increase in collagenolytic activity 3 days after a colonic anastomosis.

Such lysis, however, may be due to a variety of proteolytic enzymes and is not specific to collagenase, the metalloproteinase which normally initiates extracellular collagen breakdown7. Increased understanding of collagenolysis has shown that mammalian collagenase has a very precise action, cleaving the triple helix at a single locus to produce three-quarter and one-quarter fragments which are then susceptible to degradation by other proteinases and cathepsins. Collagenase is secreted on demand, in a latent form, and must be activated extracellularly. Tissue inhibitor of metalloproteinases (TIMP), found in most tissues and cells in culture, forms a tight complex with the enzyme during healing. Within 12 h of operation, secreting cells and extracellular collagenase were identified at the everted edges of the bowel wall. After 24 h, collagenase activity was accompanied by TIMP secretion in the same localized regions, and by the third postoperative day very few cells were still synthesizing enzyme in these areas, although extracellular activity remained visible. TIMP-secreting cells, however, were seen in a layer of connective tissue sealing the serosal surface of the anastomosis. At 7 days, both enzyme and inhibitor were found only in small aggregates of secreting cells in the deeper layers. The localization and extent of collagenase and TIMP activity accorded well with a normal healing response as, at all times, the enzyme was confined to the immediate vicinity of the suture line.

Materials and methods

Preparation of tissue

Segments of colon 2 cm in length were resected from a standard site in the distal colon of 11 female New Zealand White rabbits (weight 1·5–2·5 kg), under pentobarbitone anaesthesia. Single-layer, everted anastomoses were then carried out with interrupted 6/0 silk sutures. Getzen and Holloway8 have demonstrated better healing with eversion than inversion in dog colon anastomoses and subsequent animal experiments have provided evidence in favour of both options, although clinical trials indicate that inversion produces less complications in man9. However, inversion in our hands created a variable degree of obstruction in some rabbits; since obstruction considerably alters the collagen dynamics during colonic healing3, a reproducible everted anastomosis was employed so that uncomplicated healing could be investigated.

Two animals were killed 12 h after operation and three each after 24 h, 3 days and 7 days. At the time of death a 2-cm segment containing the anastomosis at its centre, and 2-cm segments proximal and distal to this, were removed. Resected and postoperative segments were washed in five changes of Hank's balanced salts solution containing antibiotics and cut into 3-mm strips. One strip of tissue from each segment of colon was fixed immediately in formalin for staining with haematoxylin and eosin. The other strips from each segment were processed entirely for histochemistry as follows: alternate strips were orientated on Millipore filters, embedded in 7 per cent gelatin and snap-frozen for 90s in liquid nitrogen; remaining strips were cultured for 3 h in Dulbecco's modification of Eagle's medium, containing 5 µm monensin and antibiotics, before embedding in gelatin and freezing as before. Monensin prevents the translocation of secretory proteins, including collagenase9, and the consequent intracellular accumulation assists the identification of synthesizing cells. In this way the entire circumference of the bowel was examined for a distance of 3 cm from the suture line in each direction.

Immunolocalization

Frozen sections, 4-7 µm thick, were cut from each tissue block, fixed for 30 min in 4 per cent paraformaldehyde in phosphate buffered saline (PBS) and washed in three changes of PBS (5 min each wash). The tissue was made permeable with 0·1 per cent Triton X-100 for 5 min to allow the antibodies to penetrate into cells. The tissue was then washed and treated for 10 min with 4-chloronapthol (2·8 mM in methanol/PBS with 0·01 per cent H2O2), to prevent the non-specific binding of fluorescein by inflammatory cells11. After further washing, sections were incubated in a moist atmosphere for 30 min with 5 µg of anti-collagenase IgG12, anti-TIMP IgG13, or non-immune sheep serum IgG14 to act as controls for non-specific staining. The anti-collagenase and anti-TIMP IgG were prepared by the immunization of sheep with purified preparations of either rabbit collagenase or rabbit TIMP. Specificity of these antisera and their use in other immunolocalization studies have
been described previously. Excess antibody was washed off and 375 μg of FITC-labelled pig anti-sheep antibody applied for 30 min. Sections were treated with the nuclear counter-stain methyl green, 1 mg/ml for 2 min, and mounted in glycerol/PBS mounting fluid containing additives to reduce fading (Citifluor Ltd., London, UK). Slides were examined by epifluorescence with either a Leitz Dialux 20 photomicroscope or a Zeiss Photomicroscope III, using standard wide and narrow band filters. Fluorescent photomicrographs were taken on Kodak Ektachrome 400 film, uprated to 1600 ASA, and haematoxylin and eosin photomicrographs on Plus X Pan black and white film.

Results

All rabbits were healthy at the time of death and no anastomotic dehiscence was seen. The histochemical findings were consistent around the circumference of each anastomosis and were reproducible in different animals investigated at the same time after operation. The staining patterns proximal and distal to the suture line were similar at all postoperative stages.

Resected colon

No green immunofluorescence localizing collagenase or TIMP was seen in any of the segments of normal colon resected at operation and culture with monensin did not show any secreting cells. Inflammatory cells were seen to have yellow granules after treatment with 4-chloronapthol.

Twelve hours after operation

Twelve hours after operation the gap between the two ends of bowel was filled with clot and fibrin and early inflammatory cell infiltration was seen. The region of the anastomosis showed positive immunofluorescence for collagenase which was exclusively localized in the everted portions of bowel wall (Figure 1). The entire length of the everted parts contained extracellular enzyme on the interstitial matrix between the superficial mucosal crypts (Figure 2a). Many actively secreting cells, with fine speckles of fluorescence in the perinuclear region, could be identified without the need for short-term culture with monensin (Figure 2b). Some extracellular enzyme was also present in the vicinity of suture material.

Very little TIMP was visible 12 h after anastomosis, in any area of colon. No staining was seen with either antibody outside the everted regions or in the immediate vicinity of the suture line.

Twenty-four hours after operation

Twenty-four hours after operation the gap between the bowel ends was still plugged with fibrin and a greater inflammatory infiltrate was present. Extracellular collagenase was seen in the everted regions but was more discretely localized than at 12 h and it was confined more closely to the cut ends. At these sites enzyme spread more deeply through the tissue layers into the submucosa and into the fibrin plug (Figure 3). Cells secreting collagenase were fewer and could only be identified after culture with monensin.

However, TIMP staining was seen at 24 h and was found in the same regions as collagenase. The distribution of TIMP closely paralleled that of collagenase, and TIMP-secreting cells (Figure 2c) were similarly visible after monensin treatment.

Three days after operation

By the third postoperative day the fibrin plug filling the space between the two ends of bowel was larger and inflammatory cells were concentrated around the tips of the everted bowel ends. The serosal surface was completely sealed across the gap by a distinct layer of connective tissue over the plug (Figure 4). Extracellular collagenase was once more found in the everted regions of the wall, adjacent to the inflammatory infiltrate, and very few collagenase-secreting cells could be found, even after monensin treatment. Extracellular enzyme was still present around suture material.

TIMP distribution paralleled that of collagenase but, in addition, cells secreting TIMP were found within the layer of connective tissue which sealed the serosal surface of the anastomosis (Figure 2d). A chain of secreting cells stretched within this thickened protective layer to the point where it narrowed into the normal serosal coat of the bowel wall. No extracellular TIMP or intra- or extracellular collagenase was seen in this layer.

Seven days after operation

One week after anastomosis the healing process had advanced sufficiently in some areas to restore mucosal continuity. In the deeper layers, the plug was organized into maturing fibrous tissue but occasional buried mucosal crypts and suture remnants could still be identified (Figure 5). Around these scattered remnants, and in small areas within the fibrous tissue, were aggregates of cells containing collagenase (Figure 2e) and cells containing TIMP (Figure 2f) which were visible in both monensin-treated and uncultured tissue. Little extracellular enzyme or inhibitor was seen in any area.

Discussion

This histochemical study used specific antibodies to collagenase and TIMP to investigate their distribution in colon during the first week after anastomosis. As with previous studies, neither collagenase nor TIMP could be localized in normal colon which had been resected at operation but within 12 h of anastomosis collagenase was visible both in secreting cells and extracellularly. Synthesis occurred in those areas closest to the sites of damage, the cut edges and the regions penetrated by the
Collagenase in colonic anastomoses: N. L. Chowcat et al.

Figure 2  Immunofluorescence of collagenase and TIMP in rabbit colon anastomoses. a Mucosa from everted region of anastomosis 12 h after operation treated with anti-collagenase. Red nuclei outline the mucosal crypts. In the upper part of the picture green fluorescence shows collagenase on the interstitium, extending only 350 μm from the epithelial surface (e). Arrow indicates interstitial matrix not staining for collagenase, confirming very localized distribution of this enzyme. Bar = 50 μm. b Everted region of anastomosis 12 h after operation; incubated with monensin and treated with anti-collagenase. Green fluorescence identifies intracellular collagenase granules around the red nucleus of a secreting cell (arrow) and in the adjacent extracellular space. Bar = 10 μm. c Everted region of anastomosis 1 day after operation; incubated with monensin and treated with anti-TIMP. Green fluorescence shows TIMP granules in the perinuclear regions of three secreting cells. Bar = 5 μm. d Connective tissue layer sealing serosal surface of anastomosis 3 days after operation; incubated with monensin and treated with anti-TIMP. Many red nuclei are surrounded by bright green granules (arrows) forming a chain of TIMP-secreting cells within this serosal layer. Bar = 20 μm. e Anastomotic region 7 days after operation with buried mucosal remnant (m), treated with anti-collagenase. Collagenase-secreting cells cluster around the outside of the mucosal remnant. Bar = 20 μm. f Anastomotic region 7 days after operation, with same mucosal remnant as e a few sections further into block of tissue; treated with anti-TIMP. Groups of TIMP-secreting cells are also seen around the buried mucosa (m). Pale red nuclear ghosts indicate cell death within the remnant. Bar = 20 μm.
Collagenase in colonic anastomoses: N. L. Chowcat et al.

By 24 h the collagenase distribution was less extensive and was accompanied by TIMP. The degradative process now appeared to be under tight control since the inhibitor rapidly inactivates the enzyme and is thought to be produced by cells to regulate the extracellular activity of collagenase and other metalloproteinases. This parallel localization of TIMP and collagenase was seen at all subsequent times. The process of removing the everted regions continued through the third postoperative day, with collagenase and TIMP appearing to precede the inflammatory cells as they progressed deeper into the tissue. Connective tissue formation was seen in a thickened layer on the serosal surface, sealing the peritoneal cavity from the lumen of the bowel until the underlying fibrin had been replaced by more substantial scar tissue. TIMP secretion, without any evidence of collagenase, was found within this layer, and probably contributes to the maintenance of anastomotic integrity by preventing premature degradation of this protective coat.

At 7 days after operation the fibrin plug had been replaced by a collagenous scar which had still to mature and contract. Remnants of undegraded mucosa and other tissues were seen adjacent to small groups of cells secreting collagenase and TIMP. This is similar to the findings in hypertrophic skin scars, where regions of active remodelling contained collagenase and TIMP in close proximity to negative, inactive regions. A similar sequence of changes was seen, on a smaller scale, around the sites of damage created by suture material. This very localized remodelling is likely to continue for some time, until normal tissue organization has been fully restored.

Although granulocytes have been proposed as the source of collagenase responsible for the loss of collagen from colonic anastomoses, there is no definitive evidence in the literature for a specific collagenase in rabbit polymorphonuclear leucocytes and neither a specific collagenase nor a TIMP-like collagenase inhibitor was detectable in rabbit polymorphonuclear leucocytes in this laboratory (U. Bretz and G. Murphy, unpublished observations). The secreting cells in this study had...
the appearance of fibroblasts and enzyme was localized in areas which were only later infiltrated by inflammatory cells. The stimulus for synthesis may come from the secretion of cytokines, such as interleukin 1, by inflammatory or connective tissue cells, while the damaged tissue itself releases plasmin, a collagenase activator. The sequence of events which has been outlined is that of an uncomplicated healing reaction and the locations and extent of collagenase production are those that might be predicted in a normal healing response. Hendricks and colleagues measured the collagenase concentration of the colon wall following uncomplicated anastomosis in the rabbit and found a 38 per cent decrease by 48 h; this significant fall extended for several centimetres along the colon in a proximal direction. However, in this study we found no collagenase localized in the bowel wall more than a few millimetres from the cut edge, either proximally or distally: both collagenase and TIMP were confined to the immediate vicinity of the suture line at all times. These findings suggest that the apparent decrease in collagen concentration is not due to extensive collagenase activity.

The role of collagenase appears to be to initiate the breakdown of non-redundant tissue, an activity which is confirmed by the restoration of continuity. This tissue can then be further degraded and removed by inflammatory cells. This investigation, however, has been confined to uncomplicated anastomoses and the enzyme distribution in circumstances of dehiscence has yet to be determined. These methods can now be applied to situations in which anastomotic breakdown is more likely, such as ischaemia or perforation, to show whether the production of collagenase is then more extensive or the balance between enzyme and inhibitor is lost. Although the administration of proteinase inhibitors appeared to improve anastomotic healing in animals, the results of a clinical trial were less convincing. This study indicates that little benefit is to be expected from a specific inhibition of collagenase in uncomplicated anastomoses, since the enzyme is already closely controlled by TIMP. However, if in further studies the balance between collagenase and TIMP is shown to be disturbed during anastomotic dehiscence, then the administration of synthetic TIMP-like inhibitors might be a valuable therapeutic measure.

Acknowledgements

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References