# Aspects Of Colonic Sulphur Metabolism In Health And Disease

James W.E. Moore M.B.B.S., F.R.A.C.S.

Department of Surgery
The Queen Elizabeth Hospital

A Thesis for the Degree of Doctor of Medicine

Submitted September 1996

For

Angus and Victoria

# Contents

**Summary**

The aetiology of ulcerative colitis remains unclear although most authors would now accept that events occurring in the colonic lumenal environment, the colonic epithelium and the mucosal immune compartment are of importance. A number of possible contributing lumenal factors have been proposed in the past such as infectious agents, dietary antigens and proinflammatory substances of bacterial origin. More recently, the role of colonic lumenal sulphur metabolism in the aetiology of ulcerative colitis has received some attention. Reduced sulphur compounds impair oxidation of n-butyrate in colonocytes in a manner analogous to that seen in ulcerative colitis and the levels stool sulphide are increased in one animal model of colitis in which the delivery of sulphate to the colon and the presence of an intact anaerobic microflora are required. Organisms capable of sulphide production are found in the human colon and are present in increased numbers in ulcerative colitis. Sulphatase activity, which may increase the availability of sulphate in the colonic lumen, is increased in the stool of patients with ulcerative colitis. Finally, levels of sulphide in stool may be elevated in patients with ulcerative colitis, although this has been disputed. Such evidence has led to the **general hypothesis that lumenal sulphides are aetiologically important in ulcerative colitis** and is reviewed in Chapter 2.

As studies indicating a deleterious effect of sulphide on colonic epithelial metabolic performance have been performed *in vitro*, an *in vivo* model to test this hypothesis was established (Chapter Three). Adult Sprague Dawley rats had ante-grade Roux-en-Y colostomies fashioned to allow "in-flow" access to the bowel. Animals were treated with 2 ml sodium hydrosulphide (10, 20, 30 mM) or saline control twice daily via the stoma for four or ninety days and then sacrificed. Isolated colonic epithelial cell suspensions were prepared and incubated at 37°C in the presence of [1-$^{14}$C] labelled n-butyrate (5mM) or [6-$^{14}$C] glucose (5 mM). Metabolic performance of cell suspensions was measured radiometrically ($^{14}CO_2$ production)

Sulphide treatment produced a highly significant reduction in $^{14}CO_2$ production from n-butyrate in all groups compared to control in both four and ninety day experiments. Ketone body formation from n-butyrate was significantly reduced in treated animals compared to controls. Lactate production was not significantly reduced between control and treated animals. There was no difference between groups with respect to histologic appearance in either four or ninety day experiments. These results suggest that lumenal sulphide, while capable of producing a reduction in n-butyrate oxidation of a degree similar to that observed in quiescent ulcerative colitis, is not of itself sufficient to produce mucosal inflammation.

The cause of diminished n-butyrate oxidation by the colonic epithelial cell in patients with ulcerative colitis is not clear. Short chain acyl-dehydrogenase (SCAD) is an important regulatory enzyme in beta oxidation and SCAD prepared from non colonic sources can be inhibited by sulphides. Studies of acyl-CoA ester profiles in human colonic epithelial cells in health or ulcerative colitis have not been performed. Should an abnormal pattern of acyl-CoA ester concentrations, consistent with SCAD inhibition, be shared by human ulcerative colitis and an animal model utilising sulphide exposure, then this might be regarded as further supportive evidence for the role of sulphides in the pathogenesis of colitis.

The studies described in Chapter 4 examine the acyl-CoA ester profiles in isolated rat colonic epithelial cells treated *in vitro* with sodium hydrosulphide (NaHS). Isolated rat colonic epithelial cell suspensions were incubated for ten minutes in the presence of [1-$^{14}$C] n-butyrate (5 mM), with and without NaHS (1.5 mM). Incubations were carried out both in the presence and the absence of exogenous CoA and ATP. Metabolic performance was assessed by $^{14}CO_2$ production and by acyl CoA ester production measured by HPLC with UV detection.

In incubations carried out in the presence of exogenous CoA and ATP, treatment with NaHS significantly diminished $^{14}CO_2$ production and was associated with

increased levels of butyryl-CoA and a reduction in levels of crotonyl-CoA. The mean concentration of acetyl CoA in the reaction mix at ten minutes was not significantly different between control and sulphide treated incubations. These results support the suggestion that sulphides inhibit n-butyrate oxidation in colonic epithelial cells by inhibiting short chain acyl dehydrogenation of activated fatty acids.

The demonstration of elevated concentrations of sulphide in the stool of patients with ulcerative colitis would provide support for the hypothesis of sulphide induced damage in this disease. Furthermore, if such a hypothesis were to be true, it might be expected that stool sulphide would be elevated in patients with quiescent colitis, as the metabolic abnormality is observed in this group also. Evidence concerning lumenal sulphide in ulcerative colitis has been conflicting and it seemed worthwhile to re-examine stool sulphides and sulphate in control and colitic subjects (Chapter 5). Fifteen control and nineteen patients with ulcerative colitis were enrolled for study. Eleven colitic subjects had disease limited to the left colon and thirteen had inactive disease. Six patients were not taking salicylate therapy. Stool sulphide was measured using a direct spectrophotometric method and by reversed phase high performance liquid chromatography (RPHPLC) with absorbance detection. Mean free and total stool sulphide levels did not differ significantly between control and colitic subjects. Neither disease extent nor disease activity was observed to influence stool sulphide The level of free stool sulphide in colitic patients who were taking salicylate medication was not significantly different from either control patients or colitic patients not on salicylates. Similarly, free and bound stool sulphate, measured by RPHPLC with conductivity detection, was not influenced by presence of disease, disease extent, disease activity or salicylate medication. Breath methane was measured in all subjects using gas chromatography. Methane was detectable in 32 % of subjects studied. The mean free stool sulphide was higher in non-methanogenic individuals but this was not significantly different.

The ability of the colonic mucosa to detoxify sulphide may be an equally important determinant leading to sulphide induced damage of the colonic mucosa. Detoxification of sulphide by the colonic mucosa may be by oxidation, cationic and protein binding, sulphane sulphur transferase reactions or methylation. Of these, methylation by the enzyme thiol methyltransferase (TMT) was considered worthy of study. It may be hypothesised that the degree of sulphide effect on the metabolic performance of the colonic epithelium depends on the activity of mucosal TMT. The experiments described in Chapter 6 assessed the role mucosal detoxification of sulphide by thiol methyltransferase (TMT) mediated methylation may play in protecting the healthy human colonic mucosa from the adverse effects of lumenal sulphide. Colonic epithelial cell suspensions from healthy human proximal (n = 9) and distal colon (n = 10) were incubated in the presence of [1-$^{14}$C] labelled n-butyrate (5 mM) alone , butyrate plus NaHS (1.5 mM), or butyrate plus NaHS plus S adenosyl-[1-$^{14}$C methyl]-methionine (SAMe) (5 mM). The end points of the study were $^{14}CO_2$ production and mucosal TMT activity. Incubation with NaHS produced a significant decrease of $^{14}CO_2$ production compared to control incubations which was similar for cell suspensions from the proximal and distal colon. SAMe reversed this effect completely in cell suspensions from the proximal colon but not the distal colon, suggesting a greater susceptibility of the distal colon to sulphide damage. Median whole mucosal TMT values did not differ between proximal and distal colonic mucosa, but a bimodal distribution of TMT values was observed in the distal colon. However, neither the degree of inhibition of $^{14}CO_2$ production due to sulphide nor the degree to which SAMe reversed this inhibition correlated with whole mucosal TMT activity. Regional differences in TMT activity mediating S-methylation may help to explain the predilection for ulcerative colitis to occur in the distal colon but this cannot be explained solely on the basis of TMT activity.

The studies described provide only qualified support for the general hypothesis as previously stated. While lumenal sulphide produces a metabolic abnormality in rat colonic mucosal tissue consistent with the selective inhibition of n-butyrate oxidation seen in human ulcerative colitis, stool levels of sulphide are not elevated

in patients with ulcerative colitis, even when allowing for possible effects of drug therapy. Future studies should perhaps focus on the role of sulphide detoxification by the colonic mucosa in both health and disease.

## DECLARATION

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no materila previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

11.2.97

**Acknowledgments**

Entering the world of fulltime research just over two years ago, it was apparent to me that this was a world of great opportunity and yet one of great uncertainties. Opportunity to provide a contribution, however modest, to the understanding of the clinical conundrum that is idiopathic ulcerative colitis; uncertainties as to exactly what to do and how to do it. As a clinician, comfortable in the setting of the bedside and operating theatre, having to "crawl before I could walk" again was a great frustration. That I was not entirely alone in this respect was some help; Michael Eaton's position was a similar one and the time spent in support of one another at various times over the last two years has been a great benefit.

The number of people who have helped create the circumstances that made it possible to undertake this work is considerable and to them all I offer my sincere thanks. Professor Glynn Jamieson was the first to seed the idea of working under Professor Bill Roediger's supervision. Professor Guy Maddern, amongst the many other worthy changes he has wrought at The Queen Elizabeth Hospital, provided facilities and an environment which proved highly conducive to completing this work appropriately and on time. Financial support for myself and family by The Royal Australasian College of Surgeons, through the Phyllis and Thornell Shore Memorial Scholarship (for both 1994 and 1995) is gratefully acknowledged as is The R.A.C.S. Research Foundation and The Ileostomy and Colostomy Association of South Australia for financial support to fund the costs associated with performing these studies. The help of the secretarial staff in the Department of Surgery, particularly Sandra Ireland and Lyn Martin, in all those "little tasks" that have cropped up over the last two years has been indispensable. Ken Porter and his staff in the Animal House at The Queen Elizabeth Hospital provided superb service in terms of animal care and were a great help regarding one's dealing with Animal Ethics Committees. The support of all the surgeons and gastroenterologists at The

Queen Elizabeth Hospital who allowed me to study their patients is gratefully acknowledged.

Inevitably, however, there are a few individuals whose particular contributions warrant special mention. The help and support of Sue Millard and Wendy Babidge has been absolutely indispensable. Both these talented scientists have exhibited extremes of patience and reserve in dealing with the novice they found inhabiting their laboratory. Sue's instruction in the techniques of colonic epithelial cell isolation and incubation provided the technical backbone to many of the studies that follow and Wendy's contribution through her mastery of HPLC made much of the analytic work possible. Both were excellent "sounding boards" for ideas and direction and I gratefully acknowledge their contributions.

Bill Roediger has an international reputation in the field of colonic epithelial metabolism and for this reason alone I would have been lucky to have had him as a supervisor. Bill's encouragement and enthusiasm, however, for the work I have undertaken and the ability to keep me on the right track when ideas started to outrun the capabilities to achieve, in the time frame available to me, have proved to be the aspects of his contribution for which I am particularly grateful.

Finally I must thank my family and in particular, my wife Cassandra. The never ending round of changes, both geographic and clinical, that has characterised our life together continued with our return from Sydney for me to take up this research post. Cassandra's help and support in this undertaking was never lacking, even in the most adverse circumstances.

Adelaide

March 1996

# Chapter 1

## General Introduction

*Books are where questions are explained to you, life is where they aren't.*

*"Flaubert's Parrot", Julian Barnes*

Inflammatory bowel disease has been actively studied for over 100 years, and yet it is probably the reverse of the quotation above that is correct today, for with every new finding described, new questions arise, new controversies are revealed. Controversy has never been far away in the history of inflammatory bowel disease and it has not always been confined to differences of clinical opinion. The unpleasant circumstances surrounding what is usually regarded as the first reported case of ulcerative colitis (by Sir Samuel Wilks, 1859) have been described by Hawkins (1983) in some detail. The attending Wilks described a fatal diarrhoeal illness, now believed to be ulcerative colitis, in a young woman which he believed had been induced by unworthy attempts by the victim's lover (also a physician) to induce an abortion. The unfortunate doctor concerned was charged, found guilty of murder and sentenced to death, only to be reprieved at Her Majesty's mercy. Such controversial opinion as was expressed by Wilks is not hard to understand when it is remembered that such routine clinical aids as the ability to inspect the rectal mucosa (the rigid sigmoidoscope) were more than 45 years in the future.

Much intensive research, particularly in the last thirty years, has enriched our understanding of the epidemiology, natural history and pathology of ulcerative colitis and Crohn's disease. There have been significant advances in the understanding of the principles of medical management and of the place of surgery for unresponsive or complicated disease. Yet a clear understanding of the aetiology and pathogenesis of both these conditions remains elusive and has limited the development of newer forms of medical therapy. Indeed, opinion has differed as to whether ulcerative colitis and Crohn's disease affecting the colon are two different ends of the spectrum of the same disease process or whether they represent two (or

perhaps more) distinct maladies. A major problem in identifying important aetiologic and/or pathogenetic factors has been the distinction between primary aetiologically important events and "epiphenomena", or those observed abnormalities arising as a consequence of, but not contributing to the cause of inflammation of the colonic mucosa and /or deeper layers. Important, but by no means exclusive, examples of this include the demonstration of altered T cell numbers in peripheral blood lymphocytes in patients with Crohn's disease (Strickland 1974), abnormal neutrophil motility in patients with ulcerative colitis (Hermanowicz 1985) and increased mucosal (Pacheco 1987) and lumenal prostaglandins and their precursors (Lauritsen 1988).

With respect to the aetiology of ulcerative colitis, three major areas of research interest have evolved over recent years: that examining events and factors in the mucosal immune compartment, that in the epithelium and that in the colonic lumen. It seems probable that a single abnormality that explains the aetiopathogenesis of ulcerative colitis does not exist. Current hypotheses suggest that a combination of genetic, lumenal, epithelial and immune factors or events all contribute to the development of the pattern of colonic inflammation that is recognised clinically as idiopathic ulcerative colitis. Of these factors, it might be argued that the least explored is the role of the lumenal contents in disease initiation and progression. The colonic lumen and the activities of its microbial flora have gained an important place in our understanding of colonic physiology and should receive equal attention in the study of colonic disease. The role of lumenally derived antigens or soluble factors in the pathogenesis and / or aetiology of colitis has often been raised but little explored.

The implication of any given factor in the aetiology or pathogenesis of either of the major phenotypes of inflammatory bowel disease would ideally require the demonstration of its presence in the bowel, an abnormal level of the factor or an abnormal response by the mucosa to it, a longitudinal time course of the factors activity consistent with disease activity, and disease specificity. Several lines of

investigation, to be reviewed herein, suggest that reduced sulphur compounds may be important in the aetiology of ulcerative colitis. It is the general hypothesis of the studies that follow that lumenal mercaptides, particularly hydrogen sulphide, derived from microbial sulphate reduction (and/or the fermentation of some amino acids) contribute to the aetiology of ulcerative colitis by the inhibition of short chain fatty acid oxidation. Mercaptides may induce such an abnormality by abnormally elevated lumenal levels in patients with colitis or by differences in the detoxification of sulphide between patients with colitis and healthy individuals. The aims of the studies to be described were

1. To assess the effect of topically administered sulphide on the rat colonic mucosa both in terms of metabolic and histologic outcomes and so to potentially develop a novel animal model of colitis.
2. To assess the effect of in vivo sulphide exposure on the intermediary metabolism of the rat colonic epithelium by measurement of CoA ester profiles.
3. To measure stool sulphide and sulphate in patients with ulcerative colitis and healthy control subjects.
4. To assess the role of the enzyme thiol methyltransferase (TMT)in the colonic epithelial detoxification of lumenally derived sulphide.

The review that follows will aim first to outline the evidence for the primacy of short chain fatty acid metabolism in the health of the colonic mucosa and for the abnormality of fatty acid oxidation observed in ulcerative colitis. Animal models of colitis will be reviewed as several lines of investigation support a role for sulphur containing compounds in the generation of experimental colonic inflammation. Consideration will then be made of the biology of sulphur, particularly colonic sulphur metabolism, the sources of potentially harmful mercaptides in the colonic lumen and the role that such compounds may play in the pathogenesis of ulcerative colitis. As the studies reported herein are, in part, concerned with measurement of sulphide and sulphate in a complex organic matrix (ie faeces) some consideration of the reported methodologies for such measurement will follow.

# Chapter 2

# Introductory Reviews

## 2.1 Intestinal and Colonic Epithelial Metabolism

### 2.1.1 Enterocytes

Interest in mucosal metabolism began with studies of small bowel, directed initially at aspects of absorptive capacity of amino acids. Studies of the absorption of casein in the rat revealed portal venous levels of amino acids that would be expected given the amino acid composition of this protein with the exception of glutamic acid, levels of which were significantly reduced, with elevated levels of alanine suggesting transamination of glutamic acid during absorption (Wiseman 1953). Subsequent studies utilising various animal models (Windmuller 1975, Frizzell 1974, Hanson 1977, Pritchard 1977) have demonstrated that both vascularly and gut derived glutamine enter a common intracellular metabolic pool, with approximately 60% of a [1-$^{14}$C] label appearing as $CO_2$, 20% as lactate and the rest as various amino acids including alanine, citrulline and proline (Windmuller, 1975). That glucose is an energy substrate for small bowel mucosa has long been known by virtue of the demonstration of reduced ATP levels and increased glucose uptake and lactogenesis in vascularly perfused rat small intestinal mucosa with reduced arterial oxygen concentrations (Pasteur effect) (Lamers 1972).The relatively unimportant role of glucose however, has been suggested by several different studies of oxidative metabolism in strips of rat ileal mucosa (Dickens 1941), rabbit ileal mucosal strips (Frizzell 1974) and canine jejunal mucosal strips (Lester 1975), showing a respiratory quotient (RQ) for these mucosal preparations of the order 0.7–0.8 oxidising endogenous substrate (suggesting an endogenous substrate of lipid-like nature) and increasing to 0.9 on addition of glucose, with a concomitant but variable increase in total oxygen consumption and $CO_2$ production. Oxidation of glucose accounts for relatively little of this utilisation with only 12–15 % of glucose carbon appearing as $CO_2$ and 60–80 % as lactate in the fed state and only 8% as $CO_2$ during fasting. During the fed state in perfused rat small intestine, lumenally derived glucose accounts for a significantly higher proportion of

substrate uptake than in the fasted state, when vascularly derived glutamine is the major respiratory fuel (Hanson 1977). The RQ data above has suggested a lipid like substrate is important as an energy source and several studies have shown that ketone bodies are quantitatively important in this respect (Windmueller 1978, 1980). Ketone bodies derived from the vascular perfusate in an isolated, perfused segment of rat jejunum completely inhibit glucose oxidation but not glucose uptake in fasted rats, all glucose being converted to lactate (Hanson 1978). This preservation of glucose uptake and reduction of oxidation is thought to be a useful method of glucose conservation via the process of hepatic gluconeogenesis during fasting (Hanson 1978). Studies of human small bowel mucosal metabolism have been limited by the inacessibility of the human small bowel for study. The development of techniques to incubate and study mucosal metabolism in biopsy specimens (Williams 1992, Finnie 1993, Chapman 1994) has overcome this problem to some extent. Chapman et al (1995) have reported higher rates of $^{14}CO_2$ production from [$^{14}$C] n-butyrate than either [$^{14}$C] glucose or [$^{14}$C] glutamine in human terminal ileal mucosa but did not examine the utilisation or production of ketone bodies.

### 2.1.2 Colonocytes

#### 2.1.2.1 Role of Short Chain Fatty Acids in Colonic Epithelial Metabolism

Interest in the metabolic substrates for colonic epithelial cells (colonocytes) followed a similar path. Observations that the portal venous levels of butyric acid were significantly lower than rumenal fluid whilst acetate and propionate levels were similar in ruminants (Annison 1957) suggested that butyrate was being metabolised by the mucosa. This was confirmed in work examining ketogenesis in rabbit mucosa (Henning 1972) wherein a relatively constant ability to metabolise [1-$^{14}$C] n-butyrate was found along the length of the colon but with a proximal predilection for ketogenesis. The premier status of n-butyrate as a respiratory fuel was confirmed by Roediger (1980b) using an isolated rat colonic epithelial cell method; in the presence of 10 mM n-butyrate, 95% of glucose underwent glycolysis and glucose oxidation was significantly reduced by the presence of

n-butyrate. 86% of oxygen consumption could be accounted for by oxidation of n-butyrate although the relative contribution of ketone bodies could not be directly measured due to their possible and unmeasured place in lipogenesis. These findings have in large measure been confirmed in several other studies of rat mucosal metabolism (Ardawi 1985, Fleming 1991) although the latter were unable to demonstrate a fractional preference for butyrate on $O_2$ consumption largely by reason of large intersample variability. Nevertheless both studies were able to demonstrate a preference for colonic mucosal butyrate oxidation as measured by $^{14}CO_2$ production.

The development of methods to isolate human colonocytes led to the assessment of these nutrients in healthy human tissue. These methods initially utilised the isolation of colonocytes from substantial areas of resected specimens (Roediger 1979a,b). A similar pattern of utilisation to that seen in rat colonic mucosa was found; a high level of glucose utilisation and aerobic glycolysis, low glucose oxidation (particularly in the presence of n-butyrate) and with significant increases in $O_2$ consumption produced by n-butyrate (10 mM) and acetoacetate (5 mM) (Roediger 1980a). Examination of substrate utilisation by region revealed that while n-butyrate oxidation (measured either as $^{14}CO_2$ production or as % contribution to $O_2$ consumption) was similar in right and left colons, ketogenesis was less marked in the left colon, as suggested by Henning (1972), and that glucose oxidation was more pronounced in the right colon (lactogenesis being relatively similar) (Roediger 1995) This led to the suggestion that SCFA (in particular n-butyrate) are more important as an energy source in the distal colon (Roediger 1980a). Subsequent studies have developed methods to utilise small biopsy forcep derived samples (Williams 1992, Chapman 1993, Finnie 1993) to allow assessment of mucosal metabolic performance in both health and disease and repeatedly in the same individual. Whilst these methods inevitably include some metabolic activity which can be ascribed to non epithelial tissue (lamina propria lymphocytes, smooth muscle, fibroblasts) the results obtained concur with earlier findings that n-butyrate is the preferred substrate for colonic epithelium.

Apart from the importance as a metabolic fuel, n-butyrate exhibits other important effects on the colonic mucosa. Butyrate stimulates differentiation of a number of different cell lines *in vitro* as evidenced by loss of transformed phenotype in colorectal tumour cell lines (Tsao 1983, Whitehead 1986), induction of haemoglobin production in erythroleukaemic cells (Leder 1975) and the ectopic production of βHCG in HeLa and ChaGo cell lines (Chou 1977). Its effect on regulation of gene expression may be in part expressed through its effect on histone acetylation (Sealy 1978, Kruh 1982). Other important effects include changes to cytoskeletal structure including increased fibrillar fibronectin expression in Kirsten virus transformed NRK cells (Hayman 1980) and growth arrest early in G1 (Kruh 1980). The important role of butyrate in the colonic mucosa is further supported by the recent description of butyrate dependent apoptosis in colonic mucosa (Hague 1995).

### 2.1.2.2 Colonocyte Metabolism in Disease: Ulcerative Colitis and Pouchitis

The study of colonic mucosal metabolism in disease (in particular ulcerative colitis) has produced some divergence of opinion regarding alterations in n-butyrate metabolism.. Initial studies using isolated colonocytes (Roediger 1980c) found a significant reduction in n-butyrate (10 mM) oxidation as measured by both $O_2$ consumption and $^{14}CO_2$ production in cells isolated from the colon of patients undergoing resection for ulcerative colitis compared to controls. The degree of this reduction correlated with the severity of disease, although the percentage viability of cells in the active colitis group was significantly lower than in controls or quiescent colitis groups. Ketone body production mirrored this reduction in butyrate oxidation, as might be expected. There was an associated increase in glucose utilisation in both active and quiescent ulcerative colitis which could not be accounted for by lactogenesis. Metabolism of glutamine to ammonia in acute colitis was almost double that of control values with an increase flux of glutamate across glutamate dehydrogenase to α-oxoglutarate (and hence the Krebs cycle) suggesting an increase in glutamine oxidation in acute but not quiescent colitis. These findings led to the hypothesis that ulcerative colitis may represent an "energy deficient

disease" (Roediger 1980c), a view supported by the finding of reduced ATP levels in quiescent colitis (Kameyama 1984). Since these findings were reported there have been five other studies examining the role of butyrate metabolism in the pathogenesis of ulcerative colitis. Chapman et al (1994) confirmed the above findings with respect to the reduction in butyrate oxidation in ulcerative colitis. Using the methodology described above to assess metabolic performance in biopsy samples, butyrate oxidation to $CO_2$ was reduced by approximately 50% in quiescent and mildly active colitic specimens, a finding of a similar order of magnitude to that found by Roediger (1980c). A small reduction in glucose and glutamine oxidation was found but these were not statistically significant. Finnie (1993) reported no reduction in butyrate oxidation (measured as $^{14}CO_2$ production) in patients with quiescent ulcerative colitis but a significant increase in the metabolism of [U-$C^{14}$]glutamine to $^{14}CO_2$ in the descending colon of patients with colitis. The intersample variation in $CO_2$ production in this study was large (20–23%) and half the control group smoked compared to none of the patients under study. Recently a study by Clausen and Mortensen (1995) has reported no significant difference in either glucose or n-butyrate oxidation in colonic epithelial cell suspensions derived from 8 control and 14 ulcerative colitic subjects. $^{14}CO_2$ production values reported for control incubations were, however, significantly lower then those previously reported (Roediger 1980, Chapman 1994). No direct assessment of isolated cell viability was made, rather a linear $^{14}CO_2$ production rate over 60 minutes was taken as evidence of efficient metabolic performance. Two other studies supporting the reduction of butyrate metabolism in ulcerative colitis have been published in abstract form. Ireland (1990) again found a significant reduction in $^{14}CO_2$ production ex butyrate in colitic mucosa compared to normals, and confirmed the finding of Roediger (1980c) and Chapman (1994) that macroscopically uninvolved mucosa exhibited a similar although less severe reduction of butyrate oxidation suggesting that this defect predates the development of overt colitis. This study also demonstrated no effect of 5-ASA on butyrate oxidation in either normal or colitic mucosae. Finally, Williams (1992) found a profound reduction in butyrate oxidation in two cases with active rectal inflammation. It should be noted that in

none of these studies (apart from Roediger (1980c)) were ketone body formation determined nor was the contribution of substrate oxidation to oxygen utilisation examined. Nevertheless, the evidence would seem compelling that a reduction in mucosal butyrate metabolism is a feature of ulcerative colitis.

Several factors lend support to the proposition that this reduction in butyrate oxidation may be pathogenically important in ulcerative colitis. First, the finding that the distal colonic mucosa is relatively more dependent on butyrate oxidation by virtue of a reduction in ketogenesis and lower glutaminolysis (Roediger 1980a) may explain the observation that U.C. begins as a distally based disease. Second, a reduction of butyrate availability such as is seen in defunctioned colons can produce a colitis not dissimilar to ulcerative colitis on both macroscopic and microscopic grounds (diversion colitis) (Glotzer 1981). Such colitis can be successfully treated by the use of butyrate rich short chain fatty acid enemata (Harig 1989). The lumenal levels of n-butyrate have been measured in ulcerative colitis with conflicting results. Total SCFA levels were reported as being elevated in ulcerative colitis (Roediger 1982) in keeping with the observed reduction in SCFA oxidation reported by the same author in colitis (Roediger 1980c), but Vernia (1988a) reported lower levels of total SCFA content in colitic patients, perhaps due to the number of active colitics studied (Vernia 1988b). These studies are of limited value, however, as they only reflect the resulting balance between microbial fermentation and SCFA production and mucosal uptake. SCFA enemata as a treatment for active ulcerative colitis have been examined in several trials. Breuer (1991) reported that mixed SCFA enemata improved 9 of 10 patients with moderately active distal disease treated for six weeks. Scheppach (1992) reported a randomised single blind trial of n-butyrate (100 mM) enemata in ten patients with distal disease. Symptoms and macroscopic indices of disease activity were all improved with butyrate enema treatment. SCFA enemata may be as effective as conventional (ie 5-ASA or steroid) topical therapy (Senagore 1992) Thirdly, reduction of mucosal fatty acid oxidation, produced by inducing pantothenic acid deficiency (reducing Co-A availability) or through the use of specific inhibitors of β oxidation such as

2-bromo-octanoate, has been associated with the development of mucosal inflammation (Wintrobe 1943, Roediger 1986b). Finally, changes seen in the small bowel of patients with ulcerative colitis both before and after surgical management suggest that the metabolic welfare of the mucosa may be aetiologically important. Chapman (1995) has reported a reduction in butyrate metabolism in the macroscopically and microscopically normal terminal ileum of patients with ulcerative colitis compared to controls. The condition of pouchitis, symptomatically indistinguishable from active ulcerative colitis is recognised with increasing frequency following ileo-anal pouch procedures (Nicholls 1989). Whilst some cases have been ascribed to recurrent/persistent ulcerative colitis in "retained"rectal mucosa (Koltun 1991), ischaemia (Rauh 1991) stasis with or without anastomotic stricture (Fonkalsrud 1990) or unrecognised Crohn's disease, the evidence for a unifying aetiology of this distressing complication of pouch surgery remains unclear. It is clear however that the incidence of pouchitis is much higher when the procedure was performed for ulcerative colitis than for familial adenomatous polyposis (FAP) (Lohmuller 1990). The observation that the ileal pouch mucosa comes to resemble colonic mucosa in histologic appearance (Shepherd 1989) and, in some cases, mucin type (Shepherd 1987, Veress 1995) has led to the suggestion that pouchitis represents the re-emergence of an ulcerative colitis like condition in the pouch mucosa (Shepherd 1989) and as such may be a useful model for ulcerative colitis. This view is supported by the finding of increased numbers of RFD9+ macrophages in the mucosa of ileoanal pouches with pouchitis (DeSilva 1991), a finding thought to reflect the specific effector mechanisms operative in inflammatory bowel disease (Allison 1988). In this setting the finding of reduced terminal ileal n-butyrate oxidation in patients with ulcerative colitis (Chapman 1995)and a further reduction in glutamine oxidation in ileoanal pouch mucosa (Chapman 1993b) may be of particular importance.

Ideally, if the observed defect in fatty acid oxidation is to assume a central role in the pathogenesis of U.C., disease specificity should be demonstrated. No study has specifically addressed this issue, ie by examining butyrate metabolism in other

colitides. Unpublished work from this laboratory, however, suggests that this disease specificity is present with the oxidation of butyrate in two Crohn's colitics being no different to controls (pers. comm, W.E.W. Roediger).

### 2.1.2.3 Experimental Models Of Beta Oxidation Inhibition In The Colon

Attempts have been made to reproduce the metabolic abnormality ascribed to the mucosa of ulcerative colitis *in vitro* and *in vivo*. Roediger (1986b) treated rats with intrarectal administration of sodium 2-bromo-octanoate and found active colonic inflammation associated with impaired n-butyrate oxidation. Lumenal nitrite levels have been reported as being elevated in active ulcerative colitis (Roediger 1986c) and are presumed to be derived from inflammatory cells (Iyengar 1987). Roediger (1986a) reported no deleterious effect of intraluminal nitrite on colonic mucosal metabolism (rather found an increase in butyrate oxidation in colons treated with supraphysiological concentrations of nitrite), nor on mucosal histology. Several sulphur containing compounds have been known for some time to alter fatty acid metabolism in other tissues. Mercaptoacetate is known to inhibit β oxidation of fatty acids in the liver (Bauche 1983) and mercaptopropionate has a similar effect in the heart (Sabbagh 1985). Roediger (1990) reported a reduction in butyrate oxidation and ketogenesis in colonocytes induced by mercapto fatty acids (mercaptoacetate and mercaptopropionate) and sodium sulphite without apparent effect on glucose oxidation or lactogenesis. Later work from this group examined further the role of sulphur compounds (mercaptides) in the generation of this metabolic derangement. In experiments using isolated rat colonocytes (Roediger 1993b), sodium hydrosulphide (NaHS) (0.1–0.5 mM) was found to significantly reduce n-butyrate oxidation and ketogenesis without significant impairment of glucose oxidation. At higher concentrations (1–2 mM) glucose oxidation was significantly reduced. Further work using isolated human cells (Roediger 1993b) confirmed this action with a more marked effect being noted in the distal colon. These observations have served to recruit mercaptides (particularly NaHS) as candidates for further study as agents capable of producing an *in vivo* model of ulcerative colitis.

## 2.2 Acyl CoA Esters and Colitis

The majority of the work reported (and here reviewed) describing the metabolic state of colonocytes in health and disease has used either oxygen consumption (Roediger 1980) or $^{14}CO_2$ production (Roediger 1979a, Finnie 1993, Chapman 1994) as endpoints of metabolic activity. A further useful method of assessing the metabolic welfare of the colonocyte is the measurement of the intermediates of short chain fatty acid oxidation, the short chain acyl-CoA esters. The measurement of such species would be a useful means to examine the level at which colonocyte beta oxidation is impaired, both in the model of sulphide treated colonocyte suspensions previously discussed (Roediger 1993a) and in human ulcerative colitis.

### 2.2.1 Relevance to Disease

Co A and acyl CoA metabolism is of interest in the pathogenesis of ulcerative colitis when the work of Ellestad-Sayed et al (1976) is recalled. Pantothenic acid levels were found to be normal in patients with ulcerative colitis, but CoA levels in colonic mucosal specimens taken from patients undergoing resection for ulcerative colitis were found to be significantly depressed compared to controls. The CoA assay used in this study may have overestimated free CoA as tissue heating was used to disrupt cells in at least some of the specimens. This study included equal numbers of patients with ulcerative colitis and "granulomatous colitis" and whilst there did not seem to any difference between these groups on the raw data presented, no criteria for their diagnosis were given and the question of disease specificity of their findings remains open. While the authors concluded that the reduced CoA levels seen in their colitis patients was most likely due to a block in the synthesis of CoA somewhere between the first phosphorylation of pantothenic acid and the decarboxylation of 4-phospho-pantothenyl-cysteine, an alternative explanation may be sequestration of CoA in bound form ie as an acyl CoA ester. Arguing against such a sequestration, however, is the recent very small study of three patients by Loftus (1995) in which topical pantothenic acid (as a nightly enema for 4 weeks) failed to improve clinical or histologic status in mild to moderately active ulcerative colitis.

### 2.2.2 Effect of Sulphide on Aspects of Short Chain Fatty Acid Metabolism

In the context of the above, the level of any putative sulphide action on fatty acid oxidation requires consideration. That sulphide acts on the electron transport chain is well established (see 2.3.2) and thought to account for the rapid, fatal effect of industrial exposures to significant atmospheric levels (> 700 ppm) of $H_2S$ (Beauchamp 1984). The observation that in sulphide treated (1.0 mM) isolated colonic epithelial cell suspensions, ketogenesis derived from crotonate, (the enoyl derivative of n-butyrate) was significantly greater than that observed from n-butyrate suggests that the FAD dependent butyryl-CoA dehydrogenase step may be important (Roediger 1993a). This dehydrogenation is thought to represent the major regulatory step in beta oxidation of short chain fatty acids, with strong substrate affinity (Km 1–2 μM) and product inhibition, by both crotonyl CoA and acetoacetyl CoA (Bremer 1984) and may therefore be of interest in conditions with associated diminished fatty acid oxidation such as ulcerative colitis. In situ hybridisation studies suggest that the content of colitic mucosal NADH dehydrogenase mRNA is elevated which may argue against a NADH dependent abnormality in colitis (Mayall 1992). Coenzyme A may bind to sulphides to form acyl CoA persulphides which have been suggested to act as an inhibitor of acyl CoA dehydrogenase (Shaw 1987, Williamson 1982). Finally, Stein (1995) have reported that an abnormality of butyrate uptake may be important in thiol mediated mucosal metabolic abnormalities following studies showing inhibition of bicarbonate dependent uptake into isolated apical membrane vesicles derived from rat colon by mercaptopropionate.

### 2.2.3 Measurement of Acyl-CoA esters

CoA esters are found ubiquitously in nature, forming one of the many compounds used for energy transfer. The thioester linkage in such compounds has a large free energy ($\Delta G_o = -7.5$ kcal/mol) and is utilised in a large number of biological reactions. Important amongst these is the activation of fatty acids and α-keto acids in the oxidation of fats and amino acids and in triglyceride synthesis. Accordingly, much interest has centred on the development of suitable assays for the detection of

these compounds. Enzymatic methods have been described (Michal 1974) using either 3-hydroxy-acyl dehydrogenase (measured by NADH extinction) and PTA (phospho-transacetylase) (measured by change in absorption at 233 nm by acetyl CoA) but are unable to quantify acyl CoA intermediates of fatty acid oxidation as they determine either only free CoA (PTA assay) or free CoA, CoA disulphide (CoA-S-S-CoA), dephospho CoA and other CoA precursors (HOADH assay). Long chain acyl CoA esters have been measured by absorption onto $Al_2O_3$, washing with organic solvents to separate neutral lipid followed by analysis by gas liquid chromatography with flame ionisation detection (Prasad 1987) and by paper chromatography (Smith 1978). These last three methodologies would seem to have been superceded by the application of HPLC techniques although enzymatic methods are still regarded as being useful in certain situations (Waku 1992).

Reverse phase HPLC methods have been used by a number of groups, and have generally used either UV (Corkey 1981, DeBuysere 1983, King 1985, Hosokawa 1986, Hovik 1991, Demoz 1995) or radiometric (Singh Kler 1991, Eaton 1994) detection. A review of these published reports highlights several important features of these methods. First, medium and long chain fatty acyl CoA esters and neutral fats (such as membrane lipid) are acid insoluble whereas short chain acyl CoA esters (C2-6) are acid soluble, making their isolation from cell homogenates possible by acid extraction. Second, many reports neutralise acid extracts to ensure column longevity. CoA esters are extremely pH labile; hydrolysis of the thioester linkage at physiological pH occurs readily meaning that neutralisation of acid extracts will incur a reduction in acyl CoA with a corresponding increase in "free" CoA (reduced CoA, CoA-SH) and glutathione CoA (not susceptible to alkaline hydrolysis), a finding demonstrated by DeBuysere and Olson (1983). A further disadvantage of neutralisation of acid extracts is the additional salt load introduced and requiring separation. This problem, however, can be overcome either through the use of a "pre column" (Hosakawa 1986) or the use of an organic acid such as trichloroacetic acid, rather than using perchloric acid, which is then extracted through an organic solvent such as ether (Corkey 1988a). A further argument in

favour of the omission of neutralisation is the stability of CoA ester values over time when this step is omitted (Demoz 1995). Finally, HPLC conditions can be created to separate virtually all acyl CoA esters, long medium and short chained through the selection of an appropriate mobile phase buffer and organic solvent concentration (Debuysere 1983), eluant gradient (Demoz 1995) and pH, flow rate and column characteristics (Corkey 1988a).

The presence of acyl CoA intermediates of fatty acid oxidation has been examined predominantly in hepatic tissues including isolated rat hepatocytes and mitochondria (Corkey 1981, Eaton 1994, Demoz 1995) and human liver biopsies snap frozen in liquid $N_2$, (Corkey 1988b) but also in other organs known to utilise fatty acids including the rat heart (Corkey 1981) and rat and human kidney and skeletal muscle (Veerkamp 1986). The C4 and C2 intermediates of fatty acid oxidation have all been demonstrable in hepatocytes of various mammalian species with the single exception of 3-hydroxy-butyryl-CoA. The acyl CoA profiles of colonic epithelial cells, in which fatty acid oxidation is known to be of great importance in the metabolic welfare of the cell, have not been examined, either in health or disease.

## 2.3 Animal Models of Inflammatory Bowel Disease

The study of many disease processes has benefited from the development of suitable animal models and many models of experimental intestinal inflammation have been developed. Nevertheless, no single animal model has proved entirely satisfactory, and in particular, no animal model has been examined with respect to the metabolic abnormality described in ulcerative colitis. The following aims to review some of the historical context of animal models of inflammatory bowel disease, and then to focus on lines of evidence that suggest for an interactive role between the anaerobic colonic flora and colonic sulphur metabolism in the genesis of at least several models of experimental colitis.

### 2.3.1 Naturally Occurring Models

Numerous naturally occurring inflammatory bowel diseases have been reported in animals but many are inappropriate models for human IBD due to proven infectious aetiology (Citrobacter freundii colitis of mice, Brennan 1965) infrequent occurrence (such as many of those listed below) or inappropriate size of host (equine colitis). These models are of interest, however, as they establish that a range of infectious and other agents can induce an inflammatory response that is similar to that seen in IBD.

**Siamang gibbon colitis**

Sporadic reports (Lapin 1962) exist of a naturally occurring relapsing diarrhoeal illness occurring in gibbons. Its acute form can be readily reproduced in experimental animals by G.I. inoculation of *Shigella* species but the naturally occurring form is more complex with a chronic carrier state which often shows histological appearances indistinguishable from chronic inactive U.C. with a shortened mucosal crypt length , distorted glandular architecture and psuedopolyps (Stout 1969) Approximately 1/3 of cases are found to harbour the organism in stool but a causal relationship is unclear as 8% of monkeys harbour *Shigella* without evidence of disease and successful eradication of the organism does not lead to resolution of diarrhoea or histologic changes. Furthermore, in Stouts series of four cases all occurred at times of "emotional stress", namely during introduction of a new animal to a cage or following the death of a longstanding partner. Shigellosis could be ruled out in only one case with any certainty but the parallels to the role of psychological stress to relapses of human U.C. are apparent. Professor Basil Morson of St Marks Hospital, London was unable to distinguish the histologic appearances of one of these specimens from human U.C., underlining the belief that the these appearances are shared by a number of separate disease processes (Strober 1985).

**Colitis of Cotton Top Tamarins**

The colitis reported in Siamang gibbons (Stout 1969) and other non human primates (Scott 1975) whilst having some features in common with human ulcerative colitis tends to run a more fulminant acute course without a tendency to chronicity. More recently, a wasting syndrome associated with a chronic colitis has been described in cotton top tamarins (*Sanguinus oedipus*) (Madara 1985). Like U.C. this condition is associated with an increased risk of colonic and rectal adenocarcinoma (Chalifoux 1981) and is accompanied by a reduction in type IV mucin subfraction (Podolsky 1985) as seen in human U.C. (Podolsky 1984). Such a reduction was not observed in other non human primate species which are not prone to the development of a spontaneous colitis (Podolosky 1985). The degree of inflammation seen even in acute flares of this condition is dissimilar to the human condition with at most minor ulceration; severe disease is regarded as being characterised by the presence of crypt abscesses alone (Madara 1985). It remains unclear whether cotton top tamarin colitis is a disease seen in the wild as rectal biopsies at the time of capture do not appear to have been studied. This may be important as an as yet unidentified pathogen may be acquired following human contact.

**Rabbit mucoid enteritis**

A subacute disease occurring in natural populations of rabbits characterised by the passage of large volumes of mucus in the stool has been described (Flatt 1974). Its cause has never been clearly elucidated but it can be reproduced experimentally by ligation of both colon (distal to the ileo-caecal junction) and the caecum (proximal to the ileocaecal junction) (Toofanian 1983) and attenuated by intracaecal injection of cholestyramine or oxytetracycline, suggesting a bacterial metabolite as the cause. This condition is not associated with significant mucosal inflammation and can only be regarded as of minimal value as a model for human inflammatory bowel disease.

**Swine enteritis and dysentery**

An endemic disease of herd swine, swine enteritis more closely represents Crohn's disease than U.C. with a transmural inflammatory reaction affecting predominantly the ileocaecal region with superficial mucosal ulceration but no fissuring (Biester 1928). Herd histories strongly support an infectious aetiology and this condition has been ascribed to primary infection with *Salmonella supestifer* with secondary invasion by *Actinomycoses necrophorus* (Biester & Schwarte 1931). Swine dysentery macroscopically resembles human U.C. more so than swine enteritis but is thought to be due to infection with *Treponema hyodyserenteriae* acting synergistically with host colonic flora (Gillespie and Timoney 1981).

**Chronic ulcerative caecitis of rats**

A spontaneous disease occurring in laboratory rats, mainly of Wistar and hooded buff strains, featuring caecal oedema, inflammation and ulceration but without intestinal obstruction or fistulation has been described.(Stewart 1941). This was an autopsy study and no microbiological studies were performed. The disease was confined to several animal houses strongly suggesting an unidentified pathogen. A striking pathologic feature was marked mural oedema. Similar appearances have been produced through the intralymphatic injection of particulate silica, producing a chronic lymphangitis (Reichert 1936).

**Colitis in Dogs**

Sporadic reports of spontaneous enterocolitis occurring in many different species of dog exist. (Van Kruiningen 1967,1972, Strande 1954). The spectrum of disease extends from a transmural ileocolitis resembling Crohn's disease through a colitis cystica profunda like syndrome to a mucosal colitis with some appearances similar to U.C. These have mainly been case reports and only two conditions seem to have received any detailed examination. A relapsing colitis has been described in boxer dogs (Kennedy 1966). It usually has its onset in puppyhood and runs a relapsing and remitting course into adulthood. Macroscopic appearances are not dissimilar to U.C. with erythema and contact bleeding. Like ulcerative colitis, it tends to be more

marked distally and to spread proximally as the disease progresses. It responds to salazapyrine (Kennedy 1966). Histological appearances are, however significantly different (Van Kruiningen 1967). The inflammatory process extends through the lamina propria into the submucosa and is characterised by the accumulation of large pale macrophages containing abundant P.A.S. positive material. Ulceration tends to occur over such areas and crypt abscesses, pseudopolyps or glandular architectural distortions are not a feature. Whilst these appearances have been likened to those of Johnes disease (Van Kruiningen 1967), the macrophages do not contain acid fast bacilli. Perhaps a closer human condition would be Whipples disease. The origin of the intracellular P.A.S. positive material remains unclear but may represent remains of a coccoid infectious agent or Chlamydial inclusion bodies (Van Kruiningen 1972). Two cases of a segmental enterocolitis affecting Cocker Spaniels were reported by Strande (1954) both with disease strikingly similar to Crohn's disease of the terminal ileum on macroscopic description. One case had a "skip" lesion involving the rectum. Histologically both cases had inflammation predominantly affecting the submucosa with a striking eosinophillia and lymphatic neutrophillic infiltration. Despite a careful search for potential pathogens none were identified, although the appearances are strongly suggestive of a parasitic agent.

### 2.3.2 Induced Models of IBD

Many different approaches have been described to produce intestinal inflammation in animals in the hope of developing a useful model of human IBD. The diversity of these attempts and their outcome are analysed, focussing on models of colonic inflammation.

#### 2.3.2.1 Abdominal Sympathectomy in Dogs

Berger and Lium (1960) performed abdominal postganglionic sympathectomy in mongrel dogs, producing a shortlived (approximately two weeks) illness characterised by diarrhoea and transient bloody stools, attributed to the abolition of the "restraining influence on the secretory and kinetic activities of the bowel". Histology revealed a prominence rather than depletion of mucus cell activity and

the mucosal cellular infiltrate was predominantly haemorrhagic, explained by the authors as being due to venous occlusion by vigorous muscular contraction. The features of this model are not in keeping with ulcerative colitis and it may be regarded as being of historical interest only.

#### 2.3.2.2 Immunologic Models

These may be conveniently considered as being of two types: antibody mediated and cell mediated models. The first attempts to induce intestinal inflammation by immunologic means were reported by Kirsner et al in the 1950s and early 1960s and utilised the induction of a localised Arthus and Schwartzmann reaction in the colon of rabbits (Kirsner 1961). In general these produced haemorrhagic areas in a perivascular distribution that progressed to ulceration but swiftly resolved. This same group (Kraft 1963) subsequently reported the "Auer" reaction whereby a colitis was induced by the local application or systemic administration of an antigen (egg albumin) to which the animal had previously been sensitised, in the presence of altered mucosal permeability induced by topical application of dilute formalin. Immune complexes could be demonstrated in the bowel wall. This model suggests that local deposition of immune complexes can initiate an inflammatory cascade producing mucosal inflammation. The finding that antigen-antibody reactions with subsequent fixation of complement may play a role in human U.C. (Hodgson 1977) led to attempts to develop an animal model using immune complexes. Hodgson et al (1978) induced an acute colitis by intravenous administration of soluble immune complexes to animals whose rectal mucosa had previously been injured with dilute formalin. This failed to progress to a chronic process however, a characteristic achieved by Mee et al (1979) using a similar protocol but with prior sensitisation with the enterobacterial antigen of Kunin. These authors concluded that exposure to lumenal antigens of bacterial origin may be an important prerequisite for the development of a chronic inflammatory response. A model mediated by autoantibodies has been described by Nairn and co-workers (Nairn 1979). Foetal colon implants placed beneath the renal capsule and in subcutaneous tissue in syngeneic adult rats resulted in the development of inflammatory changes

characterised by the mucosal accumulation of anticolon antibodies (which were specific for both adult and foetal rat colonic mucosa but not for other gastrointestinal tissues). This model is interesting in that it is the only described model, immunologically, chemically or otherwise produced, where a role for lumenal antigens cannot be postulated.

Dinitrochlorobenzene (DNCB) has long been known to stimulate T cell mediated immune reactions. Prior sensitisation to DNCB by cutaneous application, followed by re-exposure in the colonic lumen produces a colitis in rabbits (Rabin 1978) and guinea pigs (Glick 1981) with some features similar to ulcerative colitis. Again, it was found that continued intermittent lumenal administration of DNCB was required to prolong the inflammatory response. Rabin (1978) demonstrated cell mediated skin hypersensitivity reactions in these animals to adult rabbit colon extracts and postulated that these sensitised lymphocytes may play a role in perpetuating the response. Prior induction of such cell mediated hypersensitivity did not influence either the severity or duration of inflammation, however (Rabin 1978).

The suggestion that mucosal permeability may play role in the aetiology of Crohn's disease (Ward 1977, Shorter 1972) has led to attempts to utilise altered mucosal permeability to develop animal models of I.B.D. Morris et al (1989) described a model using a mucosal "barrier breaker" (50% ethanol) to induce a permeability abnormality in rat colons followed by intralumenal administration of a hapten (TNBS) which in concert with "tissue proteins" is known to elicit a cell mediated immune response (Little 1966). This model resulted in the development of transmural, granulomatous inflammation of the colon treated with TNBS with ulceration persisting at least 8 weeks. The appearances of this model would seem, perhaps not surprisingly to be more akin to Crohn's disease.

### 2.3.2.3 Inflammatory bowel disease in transgenic mice

Recent observations in several transgenic species of mice have afforded an interesting model of intestinal inflammation. Mombaerts et al (1993) described transgenic mice lacking the genes for $\alpha$ or $\beta$ T cell receptor. Such mice developed inflammation of the lamina propria of the colon with crypt abscesses but without ulceration whilst RAG 1 mice (deficient in the recombinase gene and therefore deficient in both T and B cell function) housed in the same facility did not, suggesting a requirement for B cell activity for inflammation to occur. Mice with an interrupted interleukin 2 (IL-2) gene have been reported to develop IBD (Sadlak 1993) associated with highly activated T and B cell subsets and anti-colon antibody. When raised in a germ free environment, these animals did not develop the illness again suggesting the importance of the lumenal microflora in the pathogenesis of colonic inflammation. Similar findings have been reported for interleukin 10 deficient mice (Kuhn 1993). Harmer et al (1990) in an attempt to produce a rat model of HLA B27 associated spondylo-arthritides found that 100% of one transgenic germ cell line successfully expressing HLA B27 and human $\beta_2$ microglobulin and 6/7 of another developed a pan intestinal inflammatory condition, the colon being most severely affected. These animals also developed a wide spectrum of manifestations associated with HLA B27 in humans (peripheral arthritis, uveitis, nail changes, orchitis). These findings are of interest given the development of ulcerative colitis in a percentage of HLA B27 positive individuals.

### 2.3.2.4 Colitis Induced by Chemotactic Agents

Inflammatory bowel diseases are characterised by the accumulation of inflammatory cells within the bowel wall. The suggestion that neutrophil motility, in particular chemotaxis, may be abnormal in Crohn's disease (Segal 1976) has led to attempts to develop an animal model of intestinal inflammation using chemo-attractant molecules. It should be noted however that several studies have now been published which do not support the contention that an abnormality of phagocytic cell activity is responsible for the pathogenesis of Crohn's disease or ulcerative colitis. (Rhodes 1983, O'Morian 1981, DeAmelio 1981). These studies have all

used peripheral blood monocytes and neutrophils and studies of mucosal phagocytic cell chemotaxis have yet to be reported.

Formylated peptides such as formyl-methionyl-leucyl phenylanaline (FMLP) and formyl-norleucyl-leucyl-phenylanaline (FNLP) are synthetic analogues of naturally occurring bacterial products which attract and activate neutrophils to release inflammatory mediators *in vitro* and *in vivo* (Painter 1984). Chester (1985) described the use of FMLP and FNLP in isolated rat colonic segments and whole mouse colons producing an acute colitis in a dose and time dependent manner. Whether the concentrations used (0.1–10 mM) were physiologically representative of those observed in either controls or colitic subjects is unclear. Inflammation was transmural and , at higher concentrations, produced mural necrosis. Formylated peptides such as those described above are inactivated, at least in part, by a myeloperoxidase catalysed oxidation of methionine contained within the molecule (Clark 1980). This may account for the observation that FMLP is less chemotactic than FNLP which contains no methionine, both *in vitro* (Clark 1980) and *in vivo* (Chester 1985). Clark and Szot (1982) have demonstrated that neutrophils activated by either phorbol myristate acetate or opsonised zymosan secrete a factor(s) which inactivates neutrophil chemotaxis (as measured by a modified Boyden chamber technique). This inactivation was inhibited by free methionine (acting as a competitive antagonist) and by various reducing agents such as ascorbic acid and thio-ethers. The reducing agent hydrogen sulphide was not specifically examined however.

Several bacterial cell wall derived compounds are known to exhibit strong chemotactic properties and have been explored as agents capable of inducing experimental colonic inflammation. Peptidoglycan-polysacharride (PG-PS ) polymers are major cell wall constituents for almost all bacteria. Intramural injection of such compounds in susceptible species (Lewis, Sprague Dawley rats, (Elson 1995)) results in chronic granulomatous inflammation of the entire bowel wall (Sartor 1985, Yamada 1993) which may be associated with extraintestinal

manifestations. The histologic features are not, however consistent with a useful model of ulcerative colitis.

#### 2.3.2.5 Acetic acid

The use of dilute acetic acid to induce mucosal injury was first described by Takagi (1970) when used to induce chronic gastric ulceration and later modified by Okabe (1971) in the production of duodenal ulceration. This work was extended to the induction of colonic mucosal injury by MacPherson and Pfeiffer (1978) who used varying concentrations of acetic acid applied serosally (10–50%) and intralumenally (10–35%). Bloody diarrhoea developed in all rats within 3 days accompanied by macroscopic and histologic evidence of a colitis affecting the treated segment. Healing of colons treated with intralumenal 10% acetic acid proceeded to normal appearances over 21 days. Serosal application led to the development of marked peritoneal adhesions as well as the colitis particularly at higher concentrations. A similar study was carried out by Fabia et al (1992). They used an isolated segment of rat colon (exteriorised as twin end stomas) to examine concentration and duration of exposure. Exposure to 4% acetic acid for 20 seconds reproducibly created appearances similar to those seen by MacPherson et al (1978) but this model may be criticised due to the possible confounding influence of faecal diversion. The mechanism of acetic acid injury remains unclear. Acetic acid has been shown to induce mucosal endothelial injury with microvascular sludging and stasis (Koo 1990) and the acetic acid model may be regarded as being analagous to ischaemic colitis. The effects of acetic acid cannot be simply due to the effect of hydrogen ions as the instillation of hydrochloric acid of appropriate pH does not induce similar changes. (Zeitlin 1984).

#### 2.3.2.6 Carrageenan

Carrageenan is a highly sulphated polygalactoside (MW 100-800,000) derived from marine algae of the class Rhodophyceae (red seaweeds) including *Euchema spinosum, Gigartina acicularis* and *Chondrus cryspus* (Ishioka 1986). Carrageenan is widely used as an emulsifying agent in food preparation. Through a process of

acid hydrolysis, three distinct co-polymers can be derived ,designated iota, kappa and lambda. These differ with respect to their degree of sulphation ($\kappa < \iota < \lambda$). Marcus and Watt (1969) first described the administration of degraded carrageenan (1% in drinking water, 5% in diet) to guinea pigs, rats, rabbits and mice, producing a colitis characterised by the development of ulceration and inflammatory changes very like that of U.C. These changes tend, however to be most marked in the caecum and proximal colon of treated animals. Engster and Abraham (1976) compared the ability of each co-polymer to produce inflammation in the guinea pig using 1% carrageenan in drinking water. Kappa and lambda fractions were found not to produce inflammation or ulceration irrespective of MW used and inflammation only occurred using iota carrageenan of MW 21–107,000. The mechanism of induction of inflammation in this model has been extensively studied by Abraham (1974). The ability to produce caecal ulceration was closely related to the ability of lamina propria macrophages to endocytose carrageenan. Ulceration appeared to occur when carrageenan was taken up by intra phagocytic lysosomes with subsequent release of lysosomal enzymes. This is supported by Allison (1966) and Cantanzaro (1971), who found that cytotoxicity induced by carrageenan was consequent upon macrophage lysosomal uptake of carrageenan.

Several observations suggest that other factors are involved in the pathogenesis of this model. Onderdonk (1978), using a guinea pig model, administered 5% carrageenan in drinking water preceded by three days of antibiotic treatment. Animals treated with oral gentamicin (not absorbed orally) and sulphamethoxazole-trimethoprim (systemically absorbed) did not differ from control animals with respect to macroscopic or microscopic caecal and proximal colon appearances but animals pretreated with oral metronidazole failed to develop ulceration. Such an effect was not apparent however, if the metronidazole was given three days after carrageenan was begun. Similar results have been reported using oral clindamycin in hamsters (Onderdonk 1977). These findings may need to be interpreted with care, as it has been reported that metronidazole given to mice receiving dextran sodium sulphate (see below) reduced their water and hence their DSS intake,

suggesting that the reduction in inflammation seen in metronidazole animals may be due to a reduced DSS exposure (Murthy 1995). That the colonic flora is important in the pathogenesis of the carrageenan model is supported by the finding that germ free animals do not develop ulceration when treated with carrageenan (Onderdonk 1981). The same study found that associating germ free animals with simplified inocula of microflora (consisting of approximately 10 species only) led to the development of carrageenan induced ulceration only when those innocula were derived from colitic animals. *Bacteroides vulgatus* was observed to be a common inoculum capable of inducing colitis even in the absence of carrageenan. Subsequent work from the same group (Onderdonk 1983) using non germ free animals suggested that whilst pre-immunisation with killed *B. vulgatus* did not influence the severity of carrageenan induced ulceration, feeding viable *B. vulgatus* to animals previously immunised to this organism significantly accentuated the inflammatory response compared to animals similarly fed carrageenan and the viable organism but not previously immunised. The significance of these findings is lessened however in that nonimmunised animals developed antibody titres to *B. vulgatus* following oral challenge and by the small numbers of animals in the *B. vulgatus* immune group compared to the nonimmune group. That factors other than the simple administration of carrageenan influence this model is further evidenced by the finding that the inflammatory response is accentuated in scorbutic animals (Langman 1985).

Finally, a recent study has shown that faecal sulphide levels are almost doubled in rats treated with 5% carageenan in drinking water (Florin 1995). Despite the reported effects of sulphide on colonic epithelial metabolism (Roediger 1993a,b), studies of mucosal n-butyrate oxidation have not been performed in this animal model.

### 2.3.2.7 Other Sulphated Polysaccharide Models

Several other sulphated polysaccharides including Dextran sodium sulphate (DSS)(Okayasu 1990) amylopectin sulphate (Watt 1972) have been used to induce

a colitis similar in morphology to that of the carrageenan model, except that disease is more distally based. Importantly, administration of the non sulphated analogues of these compounds does not produce inflammation or ulceration in treated colons (Ishioka 1986). Chronic DSS induced colitis was able to be engendered with repeated cycles of DSS administration and was associated with an increase in faecal *Bacteroides* sp. counts (Okayasu 1990). Metronidazole treatment confers a protective effect in this model similar to that observed in the carageenan model (Ohkusa 1987). With regards to the mechanism of induction of colitis, macrophage uptake of DSS has been observed (Okayasu 1990) but less markedly than in carrageenan colitis. This may be species related, less of a feature of DSS colitis or an indication that macrophage uptake is an unrelated epiphenomenon. Interestingly, it is only very recently that studies of DSS colitis have been performed in germ free animals. Germ free NMRI/KI mice were found to develop DSS colitis in the same manner to conventional mice, although data confirming that these animals were in fact germ free was not included (Axelsson 1995). Indeed, sulphasalazine treatment of the animals in this study reduced the incidence of mucosal inflammation. As diazo bond reduction by lumenal bacteria is held to be important for liberation of 5 ASA (considered the active moiety of sulphasalazine), these animals may not have been truly germ free. Studies of colonic sulphur metabolism in the DSS model have not been reported.

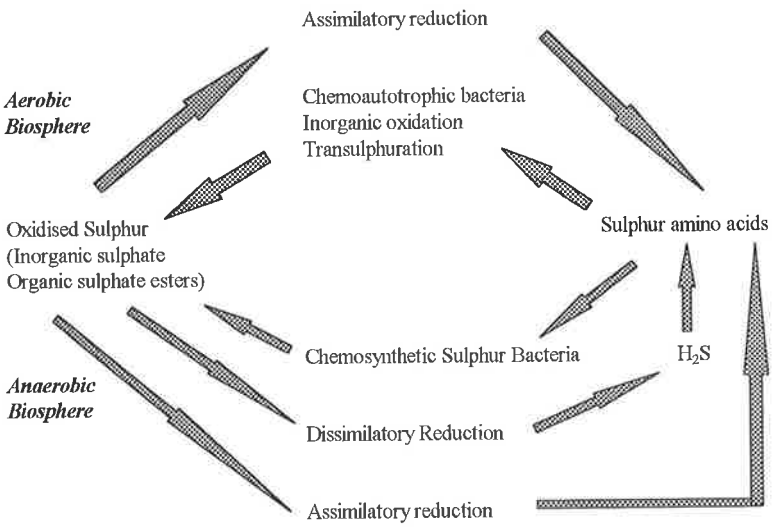
## 2.4 The Sulphur Cycle and Colonic Sulphur Metabolism

The evidence presented thus far suggests that reduced forms of sulphur such as hydrogen sulphide, may be injurious to the colonic mucosa in a manner analagous to that observed in ulcerative colitis and that such a mechanism may be dependent upon the delivery of oxidised forms of sulphur to the colonic lumen. An understanding of colonic sulphur metabolism requires consideration of the role of the sulphur cycle in biology, the importance of sulphate activation in cellular processes, the sources of sulphate for colonic lumenal sulphate reduction and alternative pathways of sulphide generation in the colonic lumen.

### 2.4.1 Overview of the Sulphur Cycle

Sulphur , like carbon and nitrogen, undergoes a complex process of cyclic oxidation and reduction in the global biosphere. In many ways the colonic lumen represents a microcosm of the anaerobic half of this cycle and an understanding of the sulphur cycle in general is helpful to understand the role and metabolic pathways of organic and inorganic sulphur in the colon. It is useful to divide the sulphur cycle into aerobic and anaerobic parts (Figure 2.1). Under aerobic conditions, reduction of oxidised forms of sulphur, notably sulphate, occurs as the assimilatory reduction seen in most bacteria, fungi and all green plants (Widdel 1988). Assimilatory sulphate reduction is that which provides reduced forms of sulphur (including thiol groups and the sulphur amino acids methionine and cysteine) necessary for higher organisms which lack the ability to reduce sulphur in this way (including many eukaryotes and all animals, Schiff 1981).

**Figure 2.1** Overview of the Sulphur Cycle




The oxidative limb of the aerobic part of the sulphur cycle consists of the oxidation of reduced forms of sulphur found in decomposing plant and animal organic matter by the aerobic chemoautotrophic and mixotrophic bacteria (Pfennig 1981), the inorganic oxidation of sulphides by molecular oxygen and transulphuration

reactions such as occurs in mammalian hepatic tissues. The anaerobic part of the cycle is mediated entirely by microorganisms. The reductive limb includes both assimilatory sulphate reduction (as above) and dissimilatory sulphate reduction. This occurs where sulphate acts as an electron acceptor in the process of conservation of energy from organic sources. Reduced sulphur thus formed, notably sulphide, is reoxidised either by escaping to the aerobic environment or by oxidation in the illuminated, anaerobic environment back to sulphate by the phototrophic purple and green sulphur bacteria (Widdel 1988) which use these compounds as photosynthetic electron donors (Schiff 1981).

Sulphate has long been regarded, amongst clinicians at least, as something of an unimportant anionic species, but evolutionally speaking , it has probably been as important as molecular oxygen. In the anoxic environment of the earth early in its evolution, electron acceptors other than oxygen would presumably have been required to support the oxidative processes of early forms of life. Sulphate probably filled this role for some organisms as sulphate reducing bacterial activity has been traced back three billion years (Peck 1966). It has been argued that the progression of the role of sulphate in metabolism from dissimilatory to assimilatory sulphate reduction and thence to predominantly one of sulphate conjugation (ie the place of sulphated glycoaminoglycans and sulphated cerebrosides in tissue structure and differentiation) is strong evidence for the importance of sulphur and sulphate in biology (Tallgren 1980).

### 2.4.2 Cellular Sulphate Metabolism

#### 2.4.2.1 Sulphate Uptake and Activation

All intracellular biochemical processes involving sulphate groups (either assimilatory or dissimilatory sulphate reduction, or sulphoconjugation reactions) require the prior activation of sulphate. This has been extensively studied in a number of organisms including sulphate reducing bacteria, primitive bacteria and animals (Schiff and Saida 1987). Common to all organisms studied is the first step of sulphate activation catalysed by ATP sulphurylase

$$SO_4^{2-} + ATP \rightarrow \text{adenosine-5-phosphosulphate} + PP_i$$

This reaction is thermodynamically unfavourable ($\Delta G = 10.8$ kcal / mol) on its own and requires coupling to hydrolysis of GTP and the splitting of PPi (Leyh 1993). The transfer of the high energy phosphate bond to adenosine-5-phosphosulphate (APS) allows the subsequent reduction of the sulphate moiety by reducing substances such as NADPH in an exergonic fashion, a pathway utilised by many dissimilatory sulphate reducing organisms. APS is an unstable compound and the efficiency of the APS pathway can be increased by further activation by phosphorylation (Tallgren 1980) by a second enzyme, APS kinase,

$$APS + ATP \rightarrow \text{3-phosphoadenosine-5-phosphosulphate} + ADP$$

3-phosphoadenosine-5-phosphosulphate (PAPS) is the source of activated sulphate in all assimilatory sulphate reducers as well as in sulphotransferase (esterification) reactions in all other bacteria, plant and animal cells (Schiff 1981), with the single known exception of ascorbic-O-sulphate as a sulphate donor for steroid sulphation (Lillard 1978). This is despite the fact that in higher animals, no change in redox potential is involved, the sulphate being used exclusively in sulphate conjugation reactions rather than sulphate reduction. Whereas dissimilatory sulphate reduction occurs only via APS as the source of activated sulphate, two recognised pathways of assimilatory sulphate reduction are described. In most assimilatory sulphate reducing photosynthetic organisms including all higher plants and some prokaryotic algae (Tsang and Schiff 1975), APS serves as the sulphate donor in a ferredoxin dependent pathway catalysed by a highly specific APS sulphotransferase. In this pathway, studied most extensively in *Chorella* and other chloroplast containing organisms, APS bound sulphate is transferred to a carrier molecule, glutathione ($G\text{-}S^-$) in some systems studied (Tsang 1978) but almost any thiol carrier may be active (Schiff and Fankhauser 1981), and reduced to a thiosulphate. The carrier bound thiosulphate is reduced by a thiosulphate reductase (ferredoxin:sulphoglutathione oxioreductase) to yield bound sulphide which becomes available for thiol transfer reactions such as that mediated by O-acetyl-serine sulphydrase (Brunold 1976). The second pathway of assimilatory sulphate reduction utilises the more stable form of activated sulphate, PAPS. This pathway

is seen in non photosynthetic organisms such as E. coli and yeasts (Tsang and Schiff 1975, Wilson 1976). In this pathway, which utilises PAPS sulphotransferase the transfer of activate sulphate groups requires thioredoxin and NAD(P)H with the production, as in the APS system, of an enzyme bound thiosulphate (Tsang 1976). The formation of free sulphite requires reduced thioredoxin which is, unlike the situation for APS mediated assimilatory sulphate reduction, unable to be replaced by other thiol groups (Tsang 1976). Finally reduction of free sulphite is carried out by sulphite reductase yielding sulphide (Schiff 1981).

The occurrence of similar sulphate activation processes ie the APS and PAPS pathways in widely diverse organisms would suggest that these organisms would also have specific mechanisms for transport of sulphate into cells and that these may be evolutionally conserved. Sulphate, being a polar, hydrophillic, negatively charged molecule is unable to pass through lipid bilayer membranes by passive diffusion and a transport protein has long been postulated (Cabantchik 1977). Studies of *Salmonella typhimurium* (Dreyfuss 1964), rat ileal brush border membrane vesicle (Lucke 1981) and human erythrocytes (Cabantchick 1977) demonstrate that the process of sulphate uptake is a sodium and energy dependent example of a secondary transport mechanism which is (in *S. typhimurium* at least) independent of the APS and PAPS pathways (Bradfield 1970). The exact mechanism of the inward absorption of sulphate in colonic mucosa that would account for the predominance of sulphate uptake observed autoradiographically (Jennings 1956) has not been elucidated but evidence from permeability studies carried out using isolated guinea pig colonic mucosa suggests that the sulphate pool in colonic mucosa is accessible only from the blood side (Lauterbach 1993).

### 2.4.2.2 The Role Of PAPS In Sulphate Metabolism

PAPS acts as the donor of sulphate groups in a wide variety of reactions according to the general formula

$$R\text{-}OH + PAPS \rightarrow R\text{-}O.SO_3^- + PAP$$

A series of substrate specific enzymes are responsible for this reaction depending on the chemical nature of the R group and include the phenol (aryl), tyrosine ester, alcohol, estrone, steroid and bile salt sulphotransferases (Ramaswamy 1987). In line with the role of sulphation as a major route of detoxification, tissue levels of sulphotransferase are high in the liver of all animals studied (Pacifici 1988, Cappiello 1989). Given the important sulphate transfer reactions which are known to occur in the colonic epithelium, including sulphation of mucus and the sulphoconjugation of potentially harmful phenolic xenobiotics, it is of interest that the levels of both PAPS and sulphotransferase activity in colonic mucosa measured using the model substrate 2-napthol is substantial (Cappiello 1989).

#### 2.4.2.3 Intestinal Mucosal Sulphation in Disease

Two areas of study have dominated the subject of mucosal sulphate handling in disease, sulphation of mucin and the sulphoconjugation of xenobiotics, especially phenolic compounds. Mucus forms an important element in gastrointestinal physiology as a lubricant for lumenal contents and as part of a protective barrier for the mucosa (Filipe 1979). A broad range of lumenal microorganisms are able to use mucus as a fermentable substrate. Sulphation of mucus confers a net negative charge which may contribute to the resistance of sulphated mucin to bacterial glycosidase activity by decreasing enzymic hydrolysis (Mian 1979). Mucin sulphation is important in protecting the gastric mucosa from colonisation by Helicobacter pylorii (Piotrowski 1991, Slomiany 1992) and from the effects of acid in the rabbit oesophagus (Tobey 1986). Sulphated mucus is observed in the presence of bacterial colonisation in normally "sterile" environments such as the stomach (ie in the presence of achlorhydria (Filipe 1979)) and ileal pouches (de Silva 1991a).

It has been long recognised that there is a reduction in the content of sulphated mucin in the colonic mucosa of patients with ulcerative colitis (Allen 1988, Habib 1986, Raouf 1992) and a similar abnormality has recently been described in the mucosa associated with colorectal carcinoma (Cope 1991). This could be due to an

increased susceptibility of such mucosa to desulphation by sulphatases. The human colonic mucosa contains very little sulphatase activity (Rhodes 1985) although a wide variety of lumenal organisms are known to secrete enzymes with sulphatase activity including *Bacteroides* (Tsai 1991), and *Klebsiella* species (Dodgson 1984). Bacterial sulphatases are important aids to the efficient fermentation of complex carbohydrates; following desulphation, such compounds are rendered more susceptible to glycosidase activity (Mian 1979, Tsai 1992). Significant sulphatase activity exists in human faeces (Rhodes 1985, Tsai 1995), with an alkaline pH optimum suggestive of bacterial origin (Rhodes 1985). Conflicting evidence has been provided as to whether a difference exists between control and colitic patients with respect to the level of sulphatase activity in stool (Rhodes 1985, Tsai 1995). Such an abnormality may be of considerable importance as any increase in mucin desulphation in the colonic lumen may increase sulphate available for dissimilatory sulphate reduction and production of hydrogen sulphide. Increased levels of other toxic compounds including lithocolic acid (Cowan 1975) and desulphated phenols (Rowland 1988) may also ensue. Recent work would suggest that whilst sulphate uptake in colitic mucosa is normal, intracellular sulphation is diminished (Raouf 1992) suggesting that the reduction in sulphated mucus observed is not due to an increased susceptibility to desulphation. Interestingly, this abnormality was not observed in Crohn's disease. That there is an abnormality of sulphotransfer reactions in the colonic mucosa of patients with ulcerative colitis is further supported by the observations of Ramakrishna (1991) regarding mucosal sulphoconjugation of the phenolic xenobiotic, paracetamol. Significant reductions in the ability of colitic patients to sulphate this compound were observed compared to controls when using a rectal dialysis technique, but similar reductions were not observed in patients with Crohn's colitis.

### 2.4.4 Sources Of Oxidised Sulphur in the Colon

In higher animals, dietary supply of sulphur for organic use is derived from two sources, dietary inorganic sulphate and the sulphur containing amino acids, cystine, cysteine and methionine and taurine. Dietary inorganic sulphate varies with diet

with certain foodstuffs, especially some fermented beverages, brassicas, some commercial breads and dried fruits having high sulphate contents (Florin 1993). It has been estimated that daily inorganic sulphate in humans may range between 1.5 and 16 mmol/day (Florin 1991b). Dietary intakes of sulphur amino acids vary with dietary protein intake and although studies of rats fed low dietary intakes of organic sulphur (methionine) demonstrated that plasma levels of sulphate may be reduced, hepatic activated sulphate (PAPS) supply was only affected at extremely low levels of methionine deprivation (Rozman 1992). Urinary excretion of inorganic sulphate has been shown to be increased by elevated dietary protein concentration (Lutz 1981).

Inorganic sulphate has long been recognised for its cathartic properties and is extensively used in large doses as a mechanical bowel preparation. Despite this, there is abundant evidence to suggest that inorganic sulphate is absorbed by the small bowel. Sixty percent of an intragastrically administered dose of $^{35}S$ sodium sulphate given to baby pigs can be recovered in the urine at 96 hours (Kulwich 1958) and a similar amount following oral administration to adult swine (Berry 1969). Human studies have shown that serum sulphate rises transiently with oral loading and that this load is excreted rapidly in the urine (Morris 1983). The ileostomy balance study by Florin (1991b) suggested that absorption is occurring in the small intestine in the human with a linear absorption of sulphate in the small bowel up to approximately 7 mmol/day. This study also reported colonic absorption of sulphate of approximately 10 mmol/day. This conclusion was reached only after assuming no sulphate metabolism in the colon (SRB were shown to be absent) and that the sulphate handling in small bowel in patients in whom colonic sulphate absorption was measured was the same as ileostomates. In this study all subjects except one had ulcerative colitis. The assumptions made may not be valid as fluid and electrolyte handling in the small bowel is altered by adaptation following proctocolectomy and ileostomy (Hill 1983) and because ulcerative colitis is a condition with abnormalities of mucosal sulphate transfer (see 2.4.2.3). The assessment as to whether these patients had SRB in their faeces seems to have been

performed before the study and not following. This may be important as it is well known that SRB activity may only become detectable in faeces after a sulphate load (Christl 1992). Recent work in a rat model has suggested that sulphate handling in the small bowel may be segmental with jejunal secretion of free sulphate quite separate from the absorption of sulphate in the form of bile salt sulphoesters in the ileum (Prof. Alison Stephens, unpublished observations).

The other source of sulphate for man and higher animals is that derived from sulphur amino acids, particularly methionine and cysteine. These amino acids are held to be well absorbed in the proximal small bowel (Matthews 1991). Animal studies (Garcia 1992) demonstrate that the splanchnic circulation extracts sulphur amino acids from the gut which is subsequently removed by the liver (measured as arterio-venous (A.V.) difference). Concomitantly, significant amounts of sulphate are secreted from the liver. This sulphate is presumably derived from the trans-sulphuration of the methionine metabolite, L-homocysteine, followed by mitochondrial oxidation of the resulting cysteine (Tallgren 1980).

Sulphate handling in the colon requires separate consideration. The A.V. difference for inorganic sulphate in the rat splanchnic circulation indicates that considerable sulphate excretion occurs in the gut as a whole (Garcia 1992). Autoradiographic studies from the 1950s have demonstrated that the colon efficiently extracts sulphate from the splanchnic circulation (Jennings 1956) suggesting that the colon is an important site of sulphur (sulphate) excretion. Similarly, although the colonic mucosal levels of methionine are low compared to other amino acids (Ahlman 1993), there is a net active extraction of methionine from the circulation by the splanchnic organs (Lund 1986 ). As well as the inorganic sulphate of dietary origin which escapes absorption in the small intestine, a further source of inorganic sulphate to the colonic lumen is that derived from desulphation of complex molecules such as steroid sulphate conjugates, sulphated mucus and bile salts by faecal bacterial sulphatases in the colonic lumen (see 2.4.2.3). The quantitative contribution of bound sulphate to the lumenal supply of sulphate in the colon is

unclear. There are no reliable estimates of the amount of mucus secreted by the gastrointestinal tract in animals or humans. Balance studies of ileostomates have suggested that between 0.96 and 2.6 mmol sulphate/day may be excreted in ileostomy effluent on diets containing between 2.1 and 15.6 mmol free sulphate/day (Florin 1991b). This may provide an estimate of the sulphate entering the colon but takes no account of sulphate derived from secreted colonic mucus. That sulphate in such bound form is important to the availability of free inorganic sulphate in the colonic lumen is evidenced by the observation of increased sulphate reduction rates, sulphidogenesis and viable SRB counts in a three stage continuous culture system modelling the human colon following the addition of bound sulphate in the form of sulphated mucin (Gibson 1988c). Free faecal sulphate content has been estimated at 0.58 μmol/gm wet weight stool and bound sulphate at 0.81 μmol/gm wet weight stool (Pitcher 1995b).

Ileostomy studies reveal that significant amounts (0.5–4.0 gm/day) of protein nitrogen exit the small bowel (Smiddy 1960, Nuguid 1961). The composition of these losses has been examined in further studies of nitrogen losses in ileostomy effluent in patients on varying levels of nitrogen intake (Chacko 1988). On a low protein (0.17 gm N/day) diet the majority of nitrogen excreted consisted of protein and peptides with only approximately 10% accounted for by free amino acids. Analysis of the amino acids excreted showed that only very small amounts of free sulphur amino acid (cysteine and methionine) were excreted even on diets supplemented by greater than 35 gm protein per day (as soy bean). Proteolytic activity in faeces is considerable, however, (MacFarlane 1986a, 1986b), contributed to by activity of both host and bacterial origin (Bohe 1983, MacFarlane 1988). Such proteolysis is important as reaction products, including small peptides and amino acids are increasingly recognised as important fermentable substrates in the left colon. This may be of particular relevance in the setting of ulcerative colitis where an increased supply of lumenal protein (consequent upon mucosal inflammation) may provide an increased supply of (sulphur) amino acids for bacterial fermentation.

### 2.4.4 Sources of Reduced Sulphur in the Colonic Lumen

#### 2.4.4.1 Dissimilatory Sulphate Reduction and Sulphate Reducing Bacteria

Dissimilatory sulphate reduction occurs in the human colon as a consequence of the activities of sulphate reducing bacteria (SRB). SRB are phylogenetically "old" organisms, with studies of isotopic sulphur in minerals suggesting their activity up to three million years ago (Peck 1966). Originally grown in the laboratory by Beijerinck (1895), these organisms have proved a diverse group in many respects. They have been characterised by (1) the requirement for energy dependent activation of inorganic sulphate with ATP producing adenosinephosphosulphate (APS)and (2) the utilisation of such activated sulphate as an electron acceptor in the conservation of energy (dissimilatory sulphate reduction) (Pfennig 1984). Dissimilatory sulphate reduction by such organisms results in the generation of a variety of sulphur compounds of varying oxidation states, although the exact pathways are variable and incompletely understood. Common to most organisms studied however, is the generation of sulphide (Gibson 1990a) which at physiological pH exists predominantly as $HS^-$ anion ($pK_{a1}$ ($H_2S$) = 7.04). Classification of SRB has been difficult as morphologically, their appearances are diverse with rod, coccal, vibrio and filamentous forms recognised. Substrate studies identify two very broad groups of SRB; those that oxidise even numbered fatty acids to acetate and those that exhibit the ability to further metabolise such fatty acids past acetate to $CO_2$.(Pfennig 1984, Gibson 1990a). Sulphate reducing bacteria are widely distributed in nature, being an important link in the atmospheric sulphur cycle (Widdel 1988) and important for their economic impact in areas as diverse as petroleum distillation, paper manufacture and pipeline maintenance (Pfennig 1984).They are particularly important for their role in the decomposition of organic matter, returning reduced forms of sulphur to the aerobic biosphere (see 2.4.1). In contrast to nitrate reducing bacteria, many of which are facultative anaerobes, all SRB are strict anaerobes. Furthermore, they require extremely low redox potentials (of the order zero to –100mV) for growth in pure culture (Widdel 1977). The human colon offers such an environment and indeed, the presence and activity of SRB in the faeces of normal human subjects has been well documented

(Beerens 1977, Gibson 1988a, Gibson 1988b, Pochart 1992). Carriage of SRB in the colon of normal healthy individuals is common with studies reporting carriage in 70–100% of individuals consuming a Western diet (Pochart 1992, Gibson 1990b, Pitcher 1995b). A wide variety of different species can be isolated from stool but the commonest are *Desulfovibrio, Desulfobacter* and *Desulfomonas* species (Gibson 1988b, 1991). Of these *Desulfovibrio* are the commonest organism isolated ,being isolated from 66% of healthy control faeces in one study (Gibson 1991). SRB distribution within the colon is variable. In an autopsy study carried out on two sudden death victims within three hours of death, MacFarlane et al (1992) reported that SRB counts in one individual whose stool was non methanogenic ranged between 2 and $8 \times 10^9$ in different parts of the colon but with highest counts in the rectum. Stool sulphide was highest in the rectosigmoid region (2.4 mmol/kg wet weight stool). The other individual studied did not harbour detectable SRB in the colon.

Better studied in humans has been the role of SRB in the fermentative processes in the colon particularly their role in the colonic "hydrogen sink". Fermentation of carbohydrate in the colon is associated with the generation of considerable amounts of hydrogen through the oxidation of pyruvate, formate or reduced pyridine nucleotides ($FADH^+$, $NADH^+$) (Gibson 1993). The accumulation of hydrogen is energetically unfavourable to the re-oxidation of pyridine nucleotides, impairing the capacity of glycolytic and oxidative phosphorylation pathways. A mechanism whereby the colon is able to keep $H_2$ partial pressures low can therefore be seen as a functional advantage in terms of fermentative efficiency. Three pathways of hydrogen disposal are recognised in the colon; per anum, via breath and through microbial utilisation (Cummings 1991). The relative roles of each varies quantitatively depending on $H_2$ production rates, fermentable substrate present and type of colonic flora present (Christl 1992b). Certain bacteria in the colon use $H_2$ as an electron donor in further metabolic activity including dissimilatory nitrate reducing bacteria (Allison 1988), methanogenic (Miller 1982, Strocchi 1991), acetogenic (Lajoie 1988) and sulphate reducing bacteria. The reason for the

observation that only a percentage of any given population (ranging from 25% in orientals (Pitt 1980) to 84% in rural black South Africans (Segal 1988)) excrete methane in breath has proved difficult to ascertain. Gibson (1988a, 1988b) found that SRB successfully outcompete methanogenic bacteria for available $H_2$ in the presence of sulphate *in vitro* and postulated that the presence of SRB and available sulphate determined the presence or absence of methanogenesis in the colon and hence methane in breath. This was supported by Christl (1992a), who studied six methanogenic individuals on a low sulphate diet, all of who had no detectable SRB in stool. The addition of sulphate (15 mmol/day) to their diet led to the development of significant SRB counts, increased sulphidogenesis and abolished methanogenesis in 3 individuals. In the three other patients studied, methanogenesis was unaffected by the addition of sulphate to the diet and none had SRB detectable in faeces. That sulphidogenesis should be favoured over methanogenesis is supported by the greater affinity of SRB for $H_2$ than methanogens ($H_2$ $K_S$ for *Desulfovibrio vulgaris* = 1μM, *Methanobrevibacter smithii* = 6 μM) (Kristjansson 1982) although this has been questioned (Strocchi 1994). Gibson (1993) has pointed out that the energetics of hydrogen oxidation theoretically favour sulphate reduction.($\Delta$Go = –152.2 kJ/mol) over methanogenesis ($\Delta$Go = –131 kJ/mol) (Thauer 1977). In direct contrast are the findings of Strocchi (1991, 1994) who performed very similar studies to Gibson (1988a) and found that coincubation of methanogenic and nonmethanogenic faecal slurries inhibited the activity of SRB (measured as sulphide production) even in the presence of 20 mM sodium sulphate. These findings have been received some support from Florin (1991a) and Tangermann (1994) who found that faecal sulphide levels were similar irrespective of methanogenic capacity. The reason for these diametrically opposed opinions remains unclear.

### 2.4.4.2 Protein Fermentation

Whilst dissimilatory sulphate reduction by SRB is undoubtedly an important source of sulphide in the colonic lumen, fermentative activity of other bacteria is capable of producing reduced sulphur compounds. A wide range of organisms are capable of

the fermentation of the sulphur amino acids with the production of volatile thiols including $H_2S$, methanthiol and dimethyl sulphide (Kadota 1972). *In vitro* studies using incubation of faeces from healthy individuals have demonstrated significant sulphidogenesis using cysteine as a sulphur source (Florin 1991, Tangerman 1994). The importance of these findings lies with the increased levels of lumenal protein consequent upon mucosal inflammation in colitis. It has been suggested that lumenal protein / amino acid fermentation is the major source of lumenal sulphide in colitis (Florin 1995). Experiments performed by Duncan et al (1990), however, found that fermentative production of mercaptoacetate, a compound known to inhibit fatty acid oxidation in the liver (Bauche 1983) and colonocytes (Roediger 1990), is not increased in anaerobic batch culture of faecal homogenates from patients with ulcerative colitis compared to controls.

### 2.4.5 Sulphate Reduction and Ulcerative Colitis

The notion that sulphate reducing bacteria (SRB) may be aetiologically important in ulcerative colitis has received some attention. Florin (1990) reported SRB carriage and faecal sulphide production in 24 patients with ulcerative colitis and compared this with 37 controls. SRB carriage in stool was significantly higher in patients with U.C. compared to controls (96% cf 73%) and faecal sulphide levels were significantly higher in patients compared to controls (0.22 vs 0.13 μmol/gm wet weight faeces). Sulphide production levels correlated with disease activity. A similar SRB carriage rate in patients with ulcerative colitis has been reported by Pitcher (1995b). In this study, stool sulphide was not elevated in patients with colitis, however in a subsequent study (Pitcher 1995c) stool sulphide was reportedly elevated in patients with colitis who were not taking salicylate medication when compared to healthy controls. Salicylates may be important as *in vitro* studies have shown that they may inhibit sulphidogenesis in stool (Pitcher 1995c) although conflicting evidence regarding this has been provided (Gibson 1991). Whilst this suggests an important role for SRB in U.C., these findings may simply represent an epiphenomenon, as work from the same group (Gibson 1991) has suggested that at least *in vitro*, the more dilute faeces seen in colitis provides a

culture medium which selects in favour of SRB, particularly the isolate most commonly encountered, *Desulfovibrio desulfuricans*. Unfortunately again, no data is available regarding disease controls (other diarrhoeal states) to clarify the disease specificity of these findings. Colonic mucosal injury, including superficial ulceration, goblet cell apoptosis and crypt separation has been demonstrated in the mucosa of a vascularised, isolated loop of rat colon exposed to a $H_2S$ containing perfusate (Aslam 1992). Clinical trials using oral tobramycin have suggested that this agent is effective in treating acute relapses of ulcerative colitis when used in conjunction with conventional therapy (Burke 1990). Although this agent is not widely used in the treatment of U.C., the finding that aminoglycosides are active against SRB *in vitro* (Pitcher 1994) may offer an explanation for these observations. A further difference in colonic sulphur metabolism between control and colitic patients has been described by Tsai (1992). Faecal sulphatase activity, measured using a $^{35}S$ labelled human colonic mucin as substrate was significantly elevated in faecal extracts from patients with ulcerative colitis compared to controls and disease controls (Crohn's disease). Faecal sulphatase activity correlated with disease activity. It was hypothesised that such increased desulphation may contribute to the colitic process by a reduction in the protective role of mucin. Such findings are compatible however with an alternative hypothesis, ie that such increased activity provides an increase in lumenal sulphate for dissimilatory sulphate reduction in the colonic lumen by colonic microflora. Sulphatase inhibitors such as bismuth salts have been shown to favourably influence steroid resistant ulcerative colitis (Ryder 1990).

## 2.5 Toxicology Of Sulphide

### 2.5.1 Exposure Studies

Hydrogen sulphide is widely recognised as a highly toxic compound and several excellent reviews of the toxicology of sulphide have been published (Beauchamp 1984, Reiffenstein 1992). Studies of the toxicity of sulphide have focused on the lethal and sublethal effects of controlled acute exposure in animals and the effects of uncontrolled exposure to humans in the setting of industrial accidents. The

observed $LD_{50}$ for sulphide has varied between studies and species. Four hour inhalation studies suggest that the $LD_{50}$ for rats is 444 ppm (Tansy 1981). It is lower in canaries (< 100 ppm) and guinea pigs (Reiffenstein 1992) who, as obligate nose breathers may be more susceptible to sulphide due to local nasopharyngeal oedema associated with sulphide exposure. Exposure at lower levels (100–200 ppm) was first observed to stimulate respiration by Haggard (1922), a feature which has subsequently been demonstrated to be mediated through the carotid body chemoreceptors. At higher levels of exposure, however, this hyperpnoea gives way to a centrally mediated depression of respiration (Reiffenstein 1984), often regarded as the cause of death in acute sulphide exposure. Exposure of Fischer rats to inhaled $H_2S$ (400 ppm) for 4 hours produces severe exfoliation of ciliated but not of squamous nasopharyngeal cells (Lopez 1988a). Similar exposure conditions only produced significant interstitial pulmonary oedema in animals treated with 615 $mg/m^3$ (approximately 440 ppm ie around the reported $LD_{50}$), a finding that the authors felt was similar to the effects of other irritant gases such as nitrogen dioxide, ozone and sulphur dioxide, with the exception that Type 1 pneumocytes were unaffected (Lopez 1988b). Such changes may be due to the functional cellular hypoxia believed to be responsible for the toxic effects of sulphide due to the inhibition electron transport chain enzymes (Khan 1984). Neurophysiologic effects of sulphide exposure have been studied and include changes in brainstem neurotransmitter concentrations after intraperitoneal NaHS (Kombian 1988), abolition of tetrodotoxin sensitive, sodium channel derived changes in transmembrane potential in mouse neuroblastoma cells (Warenycia 1989) and morphologic changes in neonatal Sprague Dawley rat neuronal populations (Roth 1984). Intravenous $Na_2S$ administration in rats significantly reduces hepatic bile flow (Sava 1980).

### 2.5.2 Effects On Electron Transport

*In vitro* studies of sulphide effect on cytochrome c oxidase have suggested an inhibitory effect on the terminal enzyme of the electron transport chain cytochrome c oxidase (Petersen 1977, Nicholls 1975). This has been confirmed in inhalational

experiments in rats, in which lung mitochondria exhibit a specific reduction in cytochrome c oxidase activity (Khan 1989). This interaction seems to be substrate (ie oxygen or ferrocytochrome c) noncompetitive (Petersen 1977, Wilms 1980). Sulphide has also been shown to inhibit cytochrome c by reduction from its ferric to ferrous form (Wilson 1978). Other biologic activities include persulphide formation in biologically important molecules (see 2.6.3) and the formation of methaemoglobin and sulphmethaemoglobin (Khan 1989).

### 2.5.3 Immunologic Effects Of Sulphide

Largely as a result of the immediate and often profound toxicity of hydrogen sulphide, little attention has been paid to the possible effects of sulphide species on aspects of immunologic function. This is of great importance as it is well established that the immune system plays an important role in the perpetuation, if not the initiation, of mucosal inflammation seen in inflammatory bowel diseases. Nevertheless, some evidence exists to suggest that sulphide may influence several facets of immune activity.

Oxidative injury to the mucosa by reactive oxygen species (ROS) has been recognised for some time. The levels of end products of lipid peroxidation, malondialdehyde (MDA) (Bhaskar 1995) and breath ethane (Sedghi 1994) are elevated in patients with ulcerative colitis and correlate with disease activity. Levels of the antioxidant glutathione are reduced in ulcerative colitis (Fields 1994) in both colonic tissue and blood suggesting active oxidative injury. Oxidative injury to protein (Lih-Brody 1994) has also been demonstrated in ulcerative colitis. ROS are products of activated phagocytes, particularly neutrophil polymorphs (PMN) (Weiss 1989). The effects of one ROS, hydrogen peroxide, as measured by the ability to kill E. coli K12 strain K37 *in vitro*, have been shown to be potentiated by the presence of either cysteine (Berglin 1982) or hydrogen sulphide (Berglin 1985). The former effect was shown to be due to the fermentation of cysteine to hydrogen sulphide by the organism studied. As the generation and action of ROS are held to

be non specific effects, sulphide in the colonic lumen may possibly potentiate the "innocent bystander" effect of ROS on colonic mucosa in colitis.

The effect of sulphide on a number of serum proteins important in immune activity has been studied (Granlund-Estedt 1991). No effect on the activities of IgG, C1q, C1r, C1s, C4 or C5 were demonstrated by SDS-PAGE and immunoblotting, but sulphide (2 mM) significantly reduced C3 activity. This was shown to be due to cleavage of an intrachain disulphide bond within the C3b α chain, liberating the C3bi fragment of C3b. C3 is an important opsonin for phagocytic cell activity. This finding is of importance as the C terminal fragment of C3b (containing C3bi) is thought to be responsible for binding to the C3b receptor of polymorphonuclear leukocytes (Wright 1987) and thus sulphide may reduce the capacity of PMN to phagocytose opsonised bacteria. Subsequent studies (Granlund-Estedt 1993) have confirmed that the bactericidal effect of PMN against encapsulated group B Streptococcus is reduced by coincubation with 2 mM sulphide.

### 2.5.4 Mucosal Effects Of Sulphide

Although little studied in the colon, several reports exist of possible deleterious effects of hydrogen sulphide on oral mucosal function. Sulphide is a well recognised by product of bacterial fermentation in gingival pockets, achieving levels of 2.0 mM (Persson 1992). Such levels of sulphide have been shown to increase permeability of porcine sublingual mucosa to [$^{35}$S]-$Na_2SO_4$ by approximately 75% (Ng 1984) in a dose dependent manner. Volatile thiols including $H_2S$ inhibit gingival fibroblast collagen synthesis and increase its degradation (due to an increase in collagen solubility consequent upon sulphide exposure) (Johnson 1992). The effects of sulphide on colonic epithelial intermediary metabolism have already been discussed. *In vivo* perfusion of vascularised rat colon with sulphide (0.2–1.0 mM) produced colonic epithelial injury as evidenced by histologic changes including epithelial cell apoptosis, surface goblet cell loss and superficial ulceration as well as increased perfusate carbohydrate and DNA content (Aslam 1992).

## 2.6 Sulphide Excretion And Metabolism

Broadly speaking, four significant routes of sulphide detoxification have been recognised, oxidation to sulphate, covalent binding to disulphide and metallo-containing proteins , methylation and possibly cyano-sulphur transfer reactions. Two of these (oxidation and covalent binding) were recognised early as a result of the experimental work described below although the relative importance importance of each was disputed.

### 2.6.1 Studies Of Parenterally Administered Sulphide

The almost complete absence of human studies on the detoxification of sulphide means that the available evidence comes from animal studies, many of which were performed over 30 or 40 years ago. Whole animal autoradiographs of young rats following intraperitoneal administration of sodium [$^{35}$S] sulphide demonstrate rapid clearance of radioactivity from the blood with substantial uptake of tracer by the liver, gastrointestinal tract, bone and brain (Curtis 1972). Excretion via the lung is minimal with less than 0.5 % of an intravenous dose of $^{35}$S sulphide being recovered in dogs breath (Gunina 1957) and no sulphide detected in end tidal mouse breath after intraperitoneal sulphide administration (Susman 1978). Very small amounts of intravenously administered $^{35}$S labelled sulphide (< 5%) are detectable in the bile of rats even six hours after dosing (Curtis 1972) with approximately 5% of such a label being detectable in the faeces over 24 hours (Curtis 1972, Dziewiatkowski 1945). By far the majority of an intravenously (90%), intraperitoneally (90%) or orally (approx 65%) administered dose of sulphide is recoverable in the urine as either inorganic or ethereal (bound) sulphate with only 10.7% of intravenously administered $^{35}$S remaining in blood 5 minutes after administration (Curtis 1972).

### 2.6.2 Oxidation

The oxidation of sulphide to sulphate requires the transfer of eight electrons and is an example of an element passing from its most reduced state (–2) to its most oxidised (+6). The mechanism by which sulphide is oxidised to sulphate has been

studied predominantly in hepatic tissues. Such oxidation has been shown to be dependent on an intact mitochondrial fraction (Bartholemew 1980, Vetter 1984), require oxygen (Baxter 1958a) and to be linked to respiration in protozoans, fish and mammals (Vetter 1984). As early as 1945 a sulphide oxidase was proposed to act to produce thiosulphate as an intermediate in the oxidation of sulphide in hepatic tissues (Der Garabedian 1945). Subsequent studies have suggested two separate oxidation pathways to thiosulphate, one enzymic (heat labile, saturable and pH dependent) and one non enzymic (heat stable and non saturable (Baxter 1958a). The enzymic activity was associated with the mitochondrial fraction (Baxter 1958a). Sorbo (1960) found that haem catalysed sulphide oxidation and suggested that this was the nonenzymatic system described by Baxter (1958b). It has been suggested that, in the presence of haem, oxidation of sulphide is carried out within mitochondria through the generation of inorganic polysulphides (Baxter 1958a, Sorbo 1960) followed by oxidation to thiosulphate in the presence of oxygen (Bartholemew 1980, Hayden 1984). Subsequent oxidation of thiosulphate in rat liver mitochondria would seem to be glutathione dependent (Bartholemew 1980). Based on studies of cyanide poisoned mitochondria from some marine organisms tolerant of high sulphide environments such as the Californian killifish (*Fundulus parvipinnis*) it seems that mitochondrial sulphide oxidation occurs in the mitochondrial intermembrane space (Vetter 1989). Mitochondrial oxidation of sulphide occurs only at low concentrations (< 60 μM) above which toxic effects on electron transport inhibit such mechanisms (Bartholemew 1980).

The major site of sulphide oxidation has been the subject of study. The *in vitro* observations of Curtis et al (1972) using rats showed that there was rapid removal of radiolabelled sulphide from blood (11% remaining at 5 minutes). A small amount of the label was present as protein bound persulphide but the majority could be identified as [$^{35}$S]sulphate. Subsequent studies (Bartholemew 1980) using isolated perfused rat livers, kidneys and lung showed that the liver was a major site of sulphide uptake and oxidation, whereas the kidney was less important. Negligible sulphide oxidation was seen in the isolated perfused rat lung. These findings are

supported by the observation in a variety of mammalian tissues that sulphite oxidase activity is greatest in liver, kidney and myocardial tissues and low in brain, testis and lung (Cabre 1990, Beck-Speier 1985) without significant differences between species. Sulphite oxidase has been reported to be plentiful or at least not rate limiting in the colonic mucosa of rats (Khalil 1995).

The colon represents the greatest single site of sulphide exposure in the human (Roediger 1993a) and represents an ideal site for entry given the large area of exposed mucous membrane.The extent to which sulphide may be oxidised in the colonic lumen or mucosa has never been assessed. Nevertheless, it is unlikely that sulphide is significantly detoxified by this route in the colonic lumen as it is highly anaerobic and exhibits a strongly reducing environment ($\Delta Eo = -300$ mV). The distribution of the enzymes of sulphide oxidation in the colonic mucosa is unclear but Roediger (1993a) found that sulphide diminished n-butyrate oxidation by colonic epithelial cells despite a highly aerobic environment, suggesting indirectly that oxidative processes against thiols were not a major protective pathway in such cells.

### 2.6.3 Covalent Binding

Sulphide combines with a number of essential and non essential proteins such as haem (Curtis 1972, Sorbo 1960), ferritin (Baxter 1958 b) and albumin (Curtis 1972); this perhaps can be regarded as a form of detoxification. Many of these *in vitro* studies were performed using sulphide concentrations which have subsequently been shown to inhibit other forms of sulphide detoxification (ie mitochondrial oxidation, Vetter 1984) and may not be of physiological relevance. The mechanism by which sulphide exerts these effects may be due to covalent binding to alkaline metals contained within these molecules or to the reduction of disulphide bridges altering protein configuration (Beauchamp 1984). Conversely, the rapid binding of sulphide to such species has been invoked in the mechanism of sulphide toxicity including its affinity for cytochrome c oxidase, inhibiting electron transport in the terminal phase of oxidative phosphorylation (Nicholls 1975,

Peterson 1977, see 2.5.2). Finally it is likely that the binding of free sulphide to lumenal cations ($Fe^{++}$, $Cu^{++}$, $Zn^{++}$) and to lumenal proteins is a significant pathway of sulphide detoxification in the colon as the majority of sulphide in faeces is present as the bound or "acid labile" fraction (Florin 1991a, Tangerman 1994) and the addition of free sulphide to faecal slurries results in significant losses of free sulphide (approx. 85%) when assayed (Florin 1991a).

### 2.6.4 Sulfane Sulphur Transfer Reactions

Rhodanese catalyses a sulfurtransferase reaction between a thiophillic anion such as cyanide, and a sulfane such as thiosulphate yielding a thiolated anion such as thiocyanate and is found distributed widely in nature (Dudeck 1980). Relative tissue distributions vary between species with highest levels in the brains and colons of dogs and the fermentative organs of ruminants (Aminlari 1991). Colonic mucosal levels in rats have recently been reported as being approximately 20% of hepatic levels (Khalil 1995). Colonic mucosal rhodanese activity in humans is yet to be reported. It will be recalled that one suggested intermediate in sulphide oxidation was thiosulphate. One of the few well established associated features of ulcerative colitis which has never been adequately explained is the clear association with non smoking of cigarettes (Calkins 1989). That sulphide may be detoxified by the colonic epithelium by the action of rhodanese through cyano group transfer may provide an explanation for this apparent protective effect of smoking, cyanide being a component of cigarette smoke which can be extracted by the colonic mucosa (Clemedson 1960).

### 2.6.5 Methylation

The final recognised pathway of sulphide detoxification is methylation. The methyltransferases are a diverse group of enzymes which can be divided into O-methyltransferases (eg catechol-O and phenol-O methyltransferase), N-methyltransferases (eg Histamine-N and a variety of mRNA and DNA methyltransferases) and those that methylate sulphhydryl groups, the S-methyltransferases. The methyl group donor for all methyltransferase reactions is

the "activated" form of methionine, S-adenosyl-methionine. The S-methyltransferases can be further divided into two forms , thiol methyltransferase (TMT) and thiopurine methyltransferase (TPMT). The latter is the agent responsible at least in part for the low oral bioavailability of a number of cyclic sulphydryl compounds including 6 mercaptopurine and propylthiouracil (Pacifici 1993)and is found in the cytosol. Thiol methyltransferase is microsomal in origin and responsible for the methylation of alkyl sulphydrhyl groups. It is widely distributed in both foetal and adult human tissues (Pacifici 1991) with highest activities in the liver and intestine. Alone amongst drug metabolising enzymes, thiol methyltransferase exhibits significantly higher activity in the colon compared to the small intestine (Pacifici 1993). Furthermore, these authors have demonstrated an aboral gradient in the activity of this enzyme in the human intestine, with higher activity in the transverse (1791 pmol/min/gm protein) and descending colon (964 pmol/min/gm protein pmol/min/gm protein) than more proximally (786 pmol/min/gm protein) . Although rectal values were lower (687 pmol/min/gm protein) than those seen in the transverse and descending colon, only two cases were studied.

The thiol-methylation pathway was first proposed to be important in the colon by Weisiger (1980). The principle product of sulphide methylation, methanthiol, is itself a substrate for methylation by the same process. As the $K_m$ for methylation of $H_2S$ in rat livers is 64 μM ($V_{max}$ 7.8 nmol/min/mgm) and that for methylation of methanthiol = 240 μM ($V_{max}$ 0.9 nmol/min/mgm) (Weisiger 1980) it has been suggested that relatively little methanthiol would be methylated to dimethyl sulphide. Methanthiol has been shown to be less toxic than the parent $H_2S$ to colonic epithelial cells (Roediger 1993b). The levels of thiopurine and thiol methyl transferases in human red blood cells have been demonstrated to be under a clear genetic control , with the calculated gene frequency of the putative gene for high and low TMT levels, $TMT^H$ and $TMT^L$, have been estimated at 0.42 and 0.58 respectively (Price 1989). It has been suggested that this genetic polymorphism and the ensuing TMT activity may be associated with disease states such as Parkinson's

disease (Waring 1989). The influence of genotype seems to be exhibited across tissue types for TPMT (Weinshilboum 1992) but whether this is so for colonic mucosal TMT activity is unclear as the only study to report both values did not correlate the two (Babidge 1995). The role of a genetic determinant in lumenal sulphide detoxification may be important, given the well demonstrated genetic influence in the pathogenesis of ulcerative colitis (Satsangi 1994).

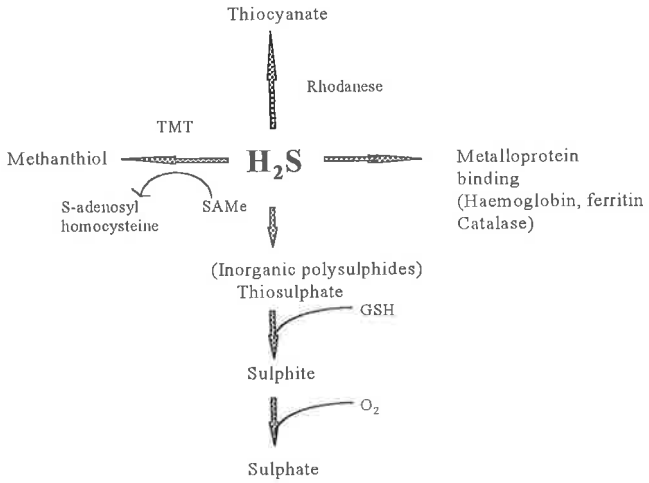



**Figure 2.2.** Pathways of sulphide detoxification.

## 2.7 Assay Methodologies

### 2.7.1 Sulphide

The measurement of sulphide species has long been the subject of considerable interest and difficulties. Hydrogen sulphide is a common byproduct of chemical and textile manufacture (Beauchamp 1984) and accordingly its measurement is an issue of major occupational safety importance. Sulphur containing compounds including $H_2S$ are major environmental pollutants derived from burning of hydrocarbons and their measurement is thus a public health and environmental issue. Several problems of sulphide measurement , particularly as they relate to biological samples, merit

consideration. Hydrogen sulphide (and other organic thiols) are volatile compounds requiring careful handling to avoid sample loss. It is also subject to rapid oxidation by atmospheric oxygen (Cline 1969). This has been overcome through the use of sample pretreatment with zinc acetate producing an insoluble zinc sulphide precipitate (Pomeroy 1957). Hydrogen sulphide is later evolved by sample acidification. As sulphide is present in stool both free ($H_2S$) and bound form (mainly as insoluble cationic complexes with $Fe^{++}$, $Zn^{++}$ and $Ca^{++}$) (Florin 1991a) this method will not allow measurement of the biologically active compartment, namely, free sulphide. It has been, however, widely used for the measurement of total sulphide concentrations in sewage water (Pomeroy 1957), rumen fluid (Savage 1990) and faeces (Florin 1991b). No report exists as far as this author is aware of attempts to overcome the problem of sample volatility by *in vivo* techniques.

Two main considerations apply to sample analysis, that of sample extraction and that of detection. Gas chromatography (GC) has been extensively used as a method of extraction for the estimation of sulphide concentrations in atmospheric samples (Bruner 1975) and natural waters (Cutter 1987) using a variety of detection techniques (see below). Tangerman (1994) has reported the application of an advanced GC technique to the measurement of sulphide in faeces. GC may not be ideal for application to analysis of stool because of the volatility problems alluded to above. Significantly, Mangani (1983) reported that several low molecular weight hydrocarbons including methane closely co-elute with $H_2S$ using GC. Cutter (1987) attempted to overcome these problems using a liquid nitrogen trap to separate interfering substances prior to GC but this is cumbersome, expensive and not validated in more complex biological samples. Ion chromatography of sulphide is difficult as only alkaline mobile phases can be used ($pKa_1 = 6.99$) with weak retention on anion exchange columns (Haddad 1988). Liquid chromatographic methods used for sulphide determination in biological samples have centred around reversed phase high performance liquid chromatography (HPLC) (Savage 1990,

Mitchell 1993) which offer the advantages of being a widely available and familiar technique in most laboratories.

Barium sulphate turbidimetry (Treiber 1952) was amongst the first detection methods used to measure sulphide concentrations in natural waters but the turbidity of biological samples makes this inappropriate. Iodine reacts with sulphide in aqueous acidic solution forming the basis of a titrimetric method for aliphatic sulphide determination (Danehy 1974). Both methods lack sensitivity or have significant interferences (Savage 1990). The methylene blue reaction whereby sulphide is acid incorporated into methylene blue by two equivalents of N,N-dimethyl-p-phenylenediamine (DPD) was first described in 1883 by Fischer. Methylene blue has the virtue of light absorption at 670 nm making spectrophotometric detection a simple option. It is considered sulphide specific (Haddad 1988, Savage 1990, Mitchell 1993) and sensitivity is in the low micromole range (Mitchell 1993, Florin 1991a). This direct spectrophotometric assay is troubled by several interfering substances including thiocyanate, sulphite and ferrocyanide (Haddad 1988) and by turbidity of biological samples producing falsely high blank readings (Strochhi 1992). The former problem can be overcome by the use of HPLC following pre-column derivatisation. Gibson (1988b) attempted to overcome the problem of turbidity by sintered glass filtration of faecal slurries prior to derivatisation. Likewise, Florin (1991a) centrifuged derivatised specimens of faeces prior to spectrophotometric analysis and included a faecal blank to allow for the effects of faecal pigments. Strocchi (1992) has further modified this technique to include correction for variable within run recovery of sulphide. Pre-column derivatisation using o-phthalaldehyde and 2-amino ethanol followed by HPLC with flourometric detection has been described for the measurement of organic thiols such as glutathione, cysteine and methanethiol but not applied to $H_2S$ (Mopper 1984). Mass spectroscopy has been used as a detection method for liquid sample headspace sulphide determination but has not been applied to biological samples (Jacobsson 1989). Finally an extractive alkylation method using pentaflourobenzyl bromide as alkylating agent and tetradecyl-

dimethyl-benzylammonium as phase transfer catalyst has been described (Kage 1988) which whilst reducing the problem of interferences remains expensive and demanding (Savage 1990).

### 2.7.2 Sulphate

The role of inorganic sulphate in health and disease has received scant attention in the literature with the exception of its role in chronic renal failure (Tallgren 1980, Marangella 1991). However a growing appreciation of the role in the detoxification of a variety of drugs (Jakoby 1990, Weinshilboum 1992), the importance of sulphate activation in a number of important biologic processes (Liau 1976, Schiff 1987, Leyh 1993), and the interest in the role of sulphate reducing bacteria in natural waters (Cline 1969, Marko-Varga1984), in industry (Beauchamp 1984) and the colonic milieu have generated some interest in the development of appropriate assays for inorganic sulphate in a number of different organic settings. Dominant amongst these are various assays based on precipitation of barium sulphate using various barium salts and the use of ion chromatography (see below). Inorganic sulphate has been measured using indirect atomic absorption spectroscopy (Michalk 1980) and ultra violet detection (Koopman 1985)but these methods have been little used.

#### 2.7.2.1 Barium Precipitation Methods

These methods rely on the precipitation of insoluble $BaSO_4$ following the addition of soluble Ba salts such as barium chloride or chloranilde to sample solutions. Detection of precipitated sulphate has been by a variety of methods including colorimetry (Hakkinen 1959), turbidimetry (Berglund 1969), centrifugal analysis (Pascoe 1984) and $\gamma$ emission spectrophotometry (through the addition of $^{133}Ba$) (Miller 1961, Cole 1979). These methods have been applied to a number of different organic matrices including plasma (Miller 1961, Pascoe 1984), serum (Cole 1979), urine (Cocchetto 1981), but not to the study of faecal sulphate. Several problems have been identified with the use of barium based methods. Incomplete recovery of sulphate has been reported at sulphate levels less than

300 μmol/L (Tallgren 1980). Furthermore, interferences with other anions, particularly phosphate, have been reported. Cole (1979) using a $^{133}Ba$ labelling method to measure serum sulphate, found that phosphate concentrations above 2.5 mmol/L significantly impaired $BaSO_4$ precipitation, although this has been challenged by Pascoe (1984). Nevertheless, stool water as obtained by *in vivo* dialysis of faeces has phosphate concentrations of approximately 2–3 mmol/L raising concerns that this methodology may not be appropriate for stool sulphate measurement.

### 2.7.2.2 Ion Chromatography

Ion exchange chromatography is well known for its ability to separate ionic species but until the mid 1970s had been plagued by difficulties with low "signal to noise" due to interferences from background ionic species in the eluent solution. The development of "suppressed" chromatographic methods using suitably designed "suppression" or "stripper" columns in addition to a separator column (Small 1975) has led to the development of several ion chromatographic methods for the determination of inorganic sulphate using conductivity (de Yong 1983, Cole 1981, Rozmann 1992a, Reiter 1987) or refractive index detection (Buytenhuys 1981) in a variety of biological samples including serum, urine, saliva and amniotic fluid. More recently, such methodology has been applied to the measurement of free and bound (following acid hydrolysis of organic sulphate esters) sulphate (Florin 1991b).

## 2.8 Conclusions

From the foregoing reviews the following salient points may be drawn. Reduced sulphur compounds impair colonocyte n-butyrate oxidation in a manner analagous to that seen in ulcerative colitis. The levels of such compounds are increased in at least one animal model of colitis, a model in which the delivery of sulphate to the colon and the presence of an intact anaerobic microflora are required. In human studies, organisms capable of dissimilatory sulphate reduction (and subsequently sulphide production) are found in the human colon and are present in increased numbers in the colitic colon. Sulphatase activity (which may increase the availability

of sulphate in the colonic lumen) is increased in the stool of patients of ulcerative colitis. Finally, stool sulphide may be elevated in patients with ulcerative colitis, although this has been disputed. Taken together, these observations serve to suggest that an abnormality of colonic sulphur metabolism may be of aetiologic importance in ulcerative colitis. The studies that follow aim to further explore the role that reduced sulphur compounds, particularly sulphide, may play in the aetiology of ulcerative colitis.

# Chapter 3

# The Effect Of Lumenal Sulphide On The Colonic Epithelium Of The Rat

## 3.1 Introduction

While several lines of evidence support the hypothesis that reducing sulphur compounds are important in the aetiology of the metabolic abnormality of ulcerative colitis, those studies that have directly examined colonic epithelial injury by sulphide (Roediger 1993a,b) have potentially exposed the entire colonocyte to this agent. This may be important, as it might be expected that the lumenal aspect of the colonocyte would be the only part of the cell exposed to lumenal sulphide. The development of an appropriate animal model in which a direct link between lumenal sulphide and mucosal inflammation could be demonstrated, would provide compelling evidence for such a hypothesis. To date, no *in vivo* experiments assessing the effect of sulphide on the colonic mucosa have been reported with the single exception of that of Aslam (1992). Animal models of colitis have largely focused on immunologic effectors as the injurious agent. Roediger (1986b) has unsuccessfully attempted to reproduce the abnormality of n-butyrate oxidation consistently observed in ulcerative colitis in an animal model. Accordingly, it seemed appropriate to attempt to develop an animal model of ulcerative colitis by the intralumenal administration of sulphide. The aims of these studies were to assess the effect of lumenal sulphide in varying concentration on the metabolic welfare of the rat colonic epithelium *in vivo* and to assess whether any mucosal metabolic derangement so produced may be manifest as mucosal inflammation.

## 3.2 Materials and Methods

### 3.2.1 Reagents

All chemicals were of analytic grade and obtained from Ajax Chemical Co. (Sydney) with the exceptions of: [1-$^{14}$C] n-butyrate, (740 Mbq / mmol), [6-$^{14}$C] D-glucose (2.1 Gbq/mmol) from New England Nuclear (Boston), acetoacetic acid (lithium salt), L(+) lactic acid (lithium salt), DL-dithiothreitol and bovine serum albumin (type 5) from Sigma Chemical Co. (St Louis, Missouri) and sodium

n-butyrate (unlabelled) from B.D.H. (Poole, England). Lactate dehydrogenase, 3-hydroxybutyrate dehydrogenase, NAD and NADH were obtained from Boehringer Mannheim (West Germany). Scintillation fluid comprised 60 gm napthalene (scintillation grade, United Technologies Packard, Illinois), 5.5 gm permablend (scintillation grade, Canberra Packard, Mt Waverly, Victoria) in 600 mL toluene and 400 mL methoxyethanol (scintillation grade, B.D.H., Poole, England). Ultrapure water (MilliQ Plus 185 Ultrapure Water System, conductance > 18 MΩcm) was used for all experiments. Krebs Henseleit saline was prepared by mixing the following stock solutions: 18% NaCl (50 mL), 1.15% KCL (40 mL), 2.1% $KH_2PO_4$ (10 mL), 3.82% $MgSO_4$ (10 mL), 1.3% $NaHCO_3$ (180 mL), 1.62% CaCl (6 mL), and MilliQ water (935 mL). Calcium free KH was prepared as above but with the omission of CaCl.

### 3.2.2 Age, Sex and Fed State of Animals

Adult Sprague Dawley rats were used for all experiments. It has been shown that metabolic performance of isolated colonocytes, as measured by $O_2$ consumption, is influenced by the age of animal, with highest oxygen consumption being noted in rats of approximately 150 gm and a reduction of approximately 30% being noted over the subsequent 14 days (Roediger 1979a). To overcome this variability, adult male rats of 200–300 gm at the time of cell harvest were used. Due to the observation that fasting influences rat jejunal enterocyte glucose utilisation (Hanson 1978) and that cell harvest and $O_2$ consumption are reduced by fasting (Roediger 1979a,b), isolated cells were collected from animals in the fed state. To obviate any diurnal influence on colonocyte metabolism, all experiments were performed in the morning.

### 3.2.3 Animal Housing

All animals were obtained from the University of Adelaide Central Animal House. They were housed in communal cages (≤ 5 animals per cage) in the University of Adelaide Animal House at The Queen Elizabeth Hospital until operation after which they were housed in separate rack mounted cages. The rodent room in which

they were housed was subject to a 14 / 10 hour light / dark cycle and maintained at 21°C. Animals were fed a commercially available rat chow (Joint Stock Ration, Adelaide Milling Industries, Adelaide) and unfiltered tap water, both allowed adlibitum. As a rule, instillation of test solutions did not begin for approximately one week following surgery, allowing recovery of normal stooling patterns, healing of all wounds and acclimatisation to single cage accommodation.

### 3.2.4 Operative Technique

Animals were anaesthetised using intraperitoneal Nembutal (pentobarbitone, 50 mg/kg). The abdomen was clipped and the animal taped on a board under a lamp to keep the immediate vicinity at a temperature of 30–32°C. The abdomen was prepared with aqueous chlorhexidine but sterile drapes were not used. Supplemental oxygen was not used. A midline incision no longer than 2 cm was made and the caecum delivered onto a moist gauze swab on the animal's abdominal wall. Exteriorised loops of bowel were kept moist with periodic normal saline irrigation. The caeco-colic junction was identified and the proximal colon mesentery transilluminated to demonstrate the regional vascular supply. A mesenteric window was made avoiding vessels (usually at the level where the terminal branches of the ileocolic artery separate to join the marginal vessels), the colon clipped with an artery forcep and divided on the proximal side of this clip. A colotomy was then created in the distal colon 2.0 cm from the divided end, any contained stool wiped clear with a gauze swab and the proximal colon anastomosed end to side to the distal colon using interrupted 3/0 Vicryl (Ethicon) sutures. A representation of this arrangement is given in Figure 3.1. A stomal trephine was created on the left side of the animals abdominal wall by excising a small disc of skin and passing the tip of a pair of iris scissors through the abdominal wall. The tip of the clip attached to the distal colon (now the defunctioned limb of the Roux-en-Y construct) was passed out through the trephine and the colon grasped. The midline wound was closed with 3/0 Vicryl in continuous fashion and the stoma matured after excision of any crushed tissue. All animals received 3–5 mL normal saline intraperitoneally during the procedure to account for evaporative losses.

Local anaesthesia or post operative narcotic analgesia were not used. Animals were recovered under an incandescent lamp in a 10 L plastic bin (ambient temperature 32°C) until mobile when they were returned to the rodent room in single cages. All animals were allowed water immediately and food by the evening of the operation. Recovery endpoints are outlined above.

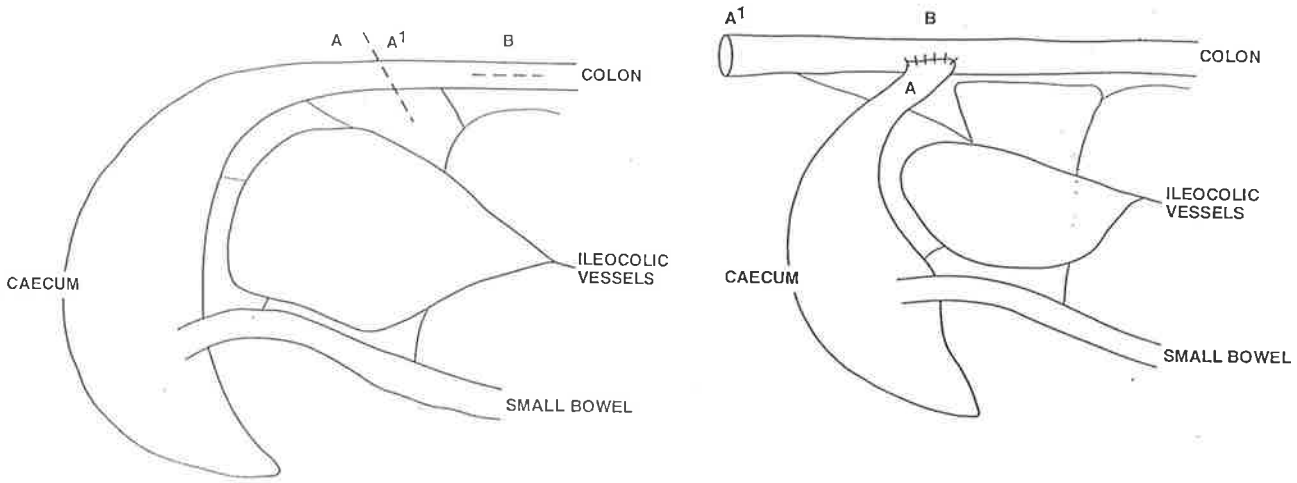



**Figure 3.1** Diagrammatic representation of the Roux-en-Y antegrade colostomy constructed to allow access to the "in flow" bowel.

### 3.2.5 Experimental Protocol – Acute Experiments

Test solutions, comprising 10, 20 or 30 mM NaHS, made up to osmolarity of 150 milliosmoles/L with NaCl, and 150 mM NaCl control solution (pH adjusted to 8.5 to match that of sulphide solutions) were made up fresh daily and kept at 4°C between instillations The mean loss of sulphide stored in this way was <10% between instillations on any given day (3 observations). Two mL of test solution

were instilled via the colostomy twice daily for four days using a soft rubber tipped catheter. The catheter had a graduated tip to allow the solution to be introduced at the same level (2.0 cm from mucocutaneous junction) on each occasion. On the morning of day five, animals were sacrificed by stunning/cervical dislocation and their colons rapidly harvested from the rectal ampulla to one cm distal to the colo-colostomy. Histological and metabolic studies were performed on separate animals.

For histological studies, 0.5 cm circumferential samples were taken one cm from the proximal and distal ends of 12 resected colons. These were fixed in 10% buffered formalin prior to routine sectioning and staining with haematoxylin and eosin in the Department of Histopathology at The Queen Elizabeth Hospital. Slides were coded and histologic appearances scored by a pathologist experienced in colorectal disease (blind to treatment group) using the mean score from three sections at each location (proximal or distal) based on the following scoring system; 0, normal appearances; 1, focal polymorphonuclear infiltrate; 2, polymorphonuclear infiltrate, gland drop-out and/or crypt abscess; 3 mucosal ulceration (Onderdonk 1978). In addition, proximal and distal specimens from two control and two 10 mM NaHS treated animals were collected and submitted for transmission electron microscopy.

### 3.2.6 Experimental Protocol – Chronic Experiments

Experiments to assess the effect of a more prolonged exposure of the colonic epithelium to sulphide were undertaken. Operative technique, animal housing and instillation technique were identical for these experiments with the exception that only 10 mM NaHS was tested against saline controls. Two animals were used for each of six experiments in each group. Instillations were carried out twice daily for 90 days at which time animals were sacrificed, proximal and distal specimens taken for routine haematoxylin and eosin staining (as above) and metabolic studies carried out as below. Histologic and metabolic studies were performed on the same animals. The effect of chronic sulphide instillation on mucosal sulphomucin content was assessed following alcian blue staining of 5 micron thickness sections of the

blocks produced from proximal and distal colon from six saline control treated and nine 10 mM NaHS treated animals. Staining was performed at pH = 1.0 and blotted rather than rinsing in water to avoid staining of sialic acid residues and to preferentially stain sulphated glycoproteins only (Lev 1964). The number of alcian blue positive cells in the top half of the crypt (as measured in microns with a graticule on the subjective lens) in ten well oriented crypts from each of three sections for each proximal and distal specimen was counted using an Olympus binocular light microscope at a magnification of 400×. The top half of the crypt was assessed because staining in the lower half of the crypt is often diffuse and difficult to assess accurately and, at least in human tissue, regional differences exist in the level of the crypt at which sulphomucin content is greatest (Filipe 1979). The results were expressed as the number of alcian blue positive cells per mm crypt mucosal length.

### 3.2.7 Metabolic Studies

Metabolic performance of mucosal epithelial cells was measured by $^{14}CO_2$, ketone body and lactate production of isolated colonic epithelial cell suspensions as previously described (Roediger 1979b). The colons of three (acute experiments) or two (chronic experiments) rats were used for each experiment with 6 experiments performed in each test group. The harvested colons were flushed with normal saline, everted, distended with calcium free Krebs Henseleit saline containing 0.25% bovine serum albumin (Ca free KH-BSA) and ligated. These colonic sacs were incubated in Ca free KH-BSA containing 10 mM EDTA for 20 minutes. Mucolytics were not used (see below). Colons were removed, rinsed in Ca free KH-BSA and an isolated colonic epithelial cell suspension prepared by vigorous stirring. The suspension was centrifuged (1250 rpm, 2 minutes) and the cell pellet resuspended in ordinary Krebs Henseleit saline containing 2.5 % BSA. One mL of cell suspension was placed in the outer portion of conical glass flasks equipped with a glass centre well and a rubber seal, in an atmosphere 19:1 $O_2$ : $CO_2$ and incubated with either 5 mM [6-$^{14}$C] n-butyrate or 5 mM [1-$^{14}$C] glucose in Krebs Henseleit saline containing 2.5% BSA (final volume 2.0 mL) for 40 minutes at

37°C in a shaking water bath. Time between mechanical disaggregation and the start of incubation was approximately 5 minutes. Incubations were terminated by the addition of 0.5 mL 10 % perchloric acid (through the rubber seal) to the outer portion of the flask and $CO_2$ collected by the addition of 0.5 mL 2 M NaOH to the centre well and shaking flasks on ice for 90 minutes. $^{14}CO_2$ production was measured radiometrically by counting activity in 0.1 mL of recovered central well NaOH solution and corrected for background $^{14}CO_2$ by counting activity from NaOH solution from flasks incubated without cell suspension. The specific activity was derived by counting the activity in a 0.1 mL sample of a solution of 2 mM n-butyrate made from the labelled solution used for incubations and representing the activity associated with 0.2 μmol n-butyrate. $^{14}CO_2$ production was then calculated according to the formula

$$R = (C_s/C_{sa})x(1/W)x(1/T)$$

Where R = rate of $^{14}CO_2$ production in μmol of metabolite produced/minute/gm dry weight of cell suspension

$C_s$ = activity (cpm) of 0.1 mL sample NaOH aliquot

$C_{sa}$ = activity (cpm) of 0.1 mL specific activity sample

W = dry weight (gm) of one mL of cell suspension

T = incubation time in min (= 40)

Ketone body (acetoacetate and β-hydroxybutyate) formation from cell suspensions incubated in the presence of n-butyrate, were measured spectrophotometrically according to the methods described by Williamson (1974) and Melanby (1974) using β hydroxy-butyrate dehydrogenase. Lactate production in those cell suspensions incubated in the presence of glucose was measured using the method of Gutmann (1974) using lactate dehydrogenase. The perchloric acid induced protein precipitate of the lateral well was removed by centrifugation (3000 rpm, 5 minutes), the supernatant neutralised using 20% KOH to pH 7 in the presence of universal indicator. Potassium perchlorate precipitate was removed by further centrifugation (3000 rpm, 5 minutes). For ketone body estimation, 0.5 mL, and for lactate, 0.1 mL of supernatant was added to appropriate buffer to a final volume of

2.0 mL in cuvettes and absorbance read at 21°C before and at timed intervals following the addition of enzyme The reaction was considered to have reached completion when absorbance was steady on two successive readings, almost always within 40 minutes. Metabolite formation was then calculated using the formula

$$R = \Delta A \times 1/W \times 1/T \times F_v/S_v \times \varepsilon_o$$

Where $\Delta A$ = change in absorbance following enzyme addition

W = dry weight (gm) of one mL cell suspension

T = incubation time (= 40)

$F_v$ = volume of incubation mix (= 2.0 mL)

$S_v$ = volume of supernatant assayed (= 0.1 mL)

εo = extinction coefficient for NAD, NADH

All metabolite values were corrected for background counts with measurements made on flasks incubated in the absence of cells. Lactate and acetoacetate (2 mM) (but not hydroxy-butyrate) standards were run concurrently with experiments. Within run variability was assessed on 8 replicates of 2 mM standard solutions of lactate and acetoacetate. Between run variability was assessed comparing the standards from each experiment as they were performed. Results are expressed as mean (SEM) in μmol of metabolite produced/minute/gm dry weight of cell suspension (obtained by drying one mL of cell suspension to constancy at 80°C).

### 3.2.8 Use of Mucolytics

Isolated colonocyte suspensions tend to clump on standing due at least in part to the presence of mucus. This can make the pippetting of a given volume subject to greater variability. Furthermore, it has been shown that mucolytics improve $O_2$ consumption (Roediger 1979b), either by allowing better egress of $O_2$ through the clumped suspension or by reducing the dry weight of the cell suspension (mucus being dispersed and removed following centrifugation) which produces a corresponding increase in the calculated $O_2$ consumption per gm dry weight of cells. The most effective mucolytic examined in this setting is DL-dithiothreitol (DTT). This agent is used as a protective agent for thiol (-SH) groups. As any putative action of sulphide against colonocytes at physiological pH may involve

activity directed against thiol groups, no DTT was used in the experiments described. To assess variability in pippetting, the first and last one mL samples pippetted were used to estimate dry weight of cells. The difference between these paired estimations was never more than 5% and averaged 1.1%.

### 3.2.9 Cell Harvest

Adequacy of cell harvest following the described methodology has been demonstrated previously through histologic assessment of the colons following preparation (Roediger 1979b). The technique is, not surprisingly, operator dependent and thus has been again assessed (Figure 3.2). The lamina propria is seen to be intact as is the basement membrane with almost complete depletion of epithelial cell elements. The ability to achieve these appearances did not differ between control and treated groups. The viability of harvested cells was assessed by trypan blue dye exclusion. Fifty microlitres of 0.5% trypan blue in 150 mM NaCl was added to 0.5 mL of cell suspension and examined using light microscopy. Fifty free cells (as opposed to those present within intact crypts which are extremely difficult to count) were counted at the edge of the cover slide and the number showing nuclear staining with the trypan blue dye expressed as a percentage. This will over estimate the percentage of cells considered non viable within the suspension as most cells that do stain are located peripherally on the slide as isolated cells.

### 3.2.10 Infiltration Via Antegrade Stoma

To demonstrate that instilled sulphide did indeed bathe the colon to be harvested for cell studies (ie that distal to the colocolostomy), contrast radiologic examination of anaesthetised animals was undertaken. Varying volumes of dilute gastrograffin (0.5–2.0 mL) were introduced per stoma using the rubber tipped catheter to be used for sulphide instillation and contrast passage through the colon observed (Figure 3.3). The levels of total sulphide achieved in the colonic lumen following sulphide instillation were measured in three control and three 10 mM NaHS treated rats by collecting stool one hour after last test solution instillation (ie on day four of

protocol) and creating a 5% wt:wt zinc acetate slurry. Total stool sulphide was measured on this zinc acetate slurry as described in Chapter 5 and expressed in μmol/gm wet weight stool.

### 3.2.11 Concentrations of Sulphide Used For Animal Experiments

From previous work (Roediger 1993a), it was known that isolated rat colonocyte butyrate oxidation is inhibited by sulphide concentrations of 0.5–5 mM. The reported concentration of free sulphide in human faeces measured *in vitro* has been reported to be of the order 0.17 μmol per gram wet weight of faeces and total acid volatile sulphide 0.6 μmol per gram wet weight of faeces (Florin 1991) (ie of the order 0.5–1.0 mM) but whether this is an accurate reflection of the state of affairs *in vivo* is unknown. Furthermore, the addition of free sulphide to faeces is known to result in a significant reduction in the amount of sulphide remaining as the free species (Florin 1991), large amounts being irreversibly bound to divalent cations in stool such as $Fe^{++}$, $Ca^{++}$ and $Zn^{++}$. As one of the stated aims of these experiments was to attempt to induce acute inflammatory changes in the colonic mucosa and as a result of the measurements of stool sulphide observed following instillation of 10 mM NaHS (see below), concentrations of 10, 20 and 30 mM NaHS were chosen for instillation.

### 3.2.12 Effect of Sulphide Instillation on Lumenal Butyrate Levels

To ensure that instillation of NaHS test solutions did not reduce lumenal n-butyrate levels (which might result in a condition akin to diversion colitis), the levels of n-butyrate in stool were measured on the morning of day 5 in three control and three 10 mM NaHS treated rats. Freshly voided stool was collected into preweighed containers containing Krebs-Henseleit saline to produce a 20% wt:vol slurry. These were mixed using a vortex mixer to disperse particulate matter and stored at –20°C until analysis. Samples were kindly analysed by Ms Ann McIntyre at The University of Melbourne Department of Medicine using a well established gas chromatographic technique (McIntyre 1991).

### 3.2.13 Statistical Analysis

All data was collected and stored on a computerised database (Excel version 5.0, Microsoft Corp., Seattle). Statistical calculations were performed using Instat 2 software (Graphpad, San Diego, California). Comparisons of means in multiple groups was made using one way ANOVA with comparison between groups by the Tukey Kramer post test. Differences in variance between groups was assessed using Bartlett's test and if variance was found to be unequal between groups, data was transformed ($Y = \log Y$ or $Y = 1/Y$) to correct this before analysis. Comparisons between two groups with equal variance was compared using the unpaired Students t test. Statistical significance was inferred if the calculated p value was $< 0.05$.

## 3.3 Results

### 3.3.1 General Comments

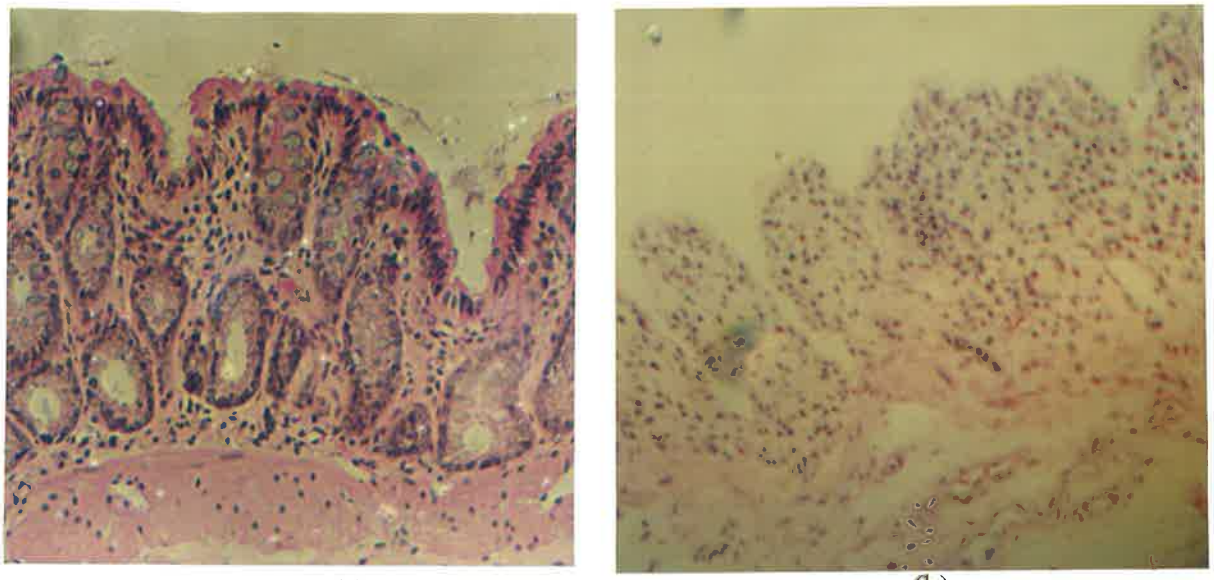
Operative mortality following the creation of the Roux-en-Y colostomy was minimal. Sixteen rats succumbed in the immediate post operative phase whilst experience was gained with the use of intraperitoneal Nembutal, the creation of the stoma arrangement in the rat and learning the techniques of colonocyte harvest. All these deaths occurred without the animal regaining consciousness following anaesthetic and were attributed to excessive barbiturate. No perioperative deaths were encountered during the time that the experiments reported herein were performed. No untoward septic complications such as anastomotic dehiscence, intra abdominal abscess or wound infection were encountered in any animal operated upon (and subsequently sacrificed). All animals regained preoperative weight within one week of operation. Instillation of two mL of gastrografffin reproducibly resulted in contrast filling the distal colon, even beyond faecal pellets (Figure 3.3) whereas one mL often resulted in contrast reflux into the proximal colon and caecum. All animals tolerated the instillation of test solutions well with no animal losing weight over the four days of treatment. No animal developed diarrhoea, bloody or otherwise, and all animals fed and preened as usual. Mean (SEM) colonic faecal n-butyrate concentrations did not differ significantly between

control and 10 mM NaHS treated animals (control = 18.7 (3.1) μmol/gm wet weight stool, 10 mM NaHS treated = 16.3 (4.1) μmol/gm wet weight stool; $t = 0.46$, $p = 0.66$, unpaired t test). Mean total sulphide in stool taken one hour after instillation in control rats was 0.78 μmol/gm wet weight and 2.36 μmol/gm wet weight in 10 mM NaHS treated rats.

### 3.3.2 Histologic Studies

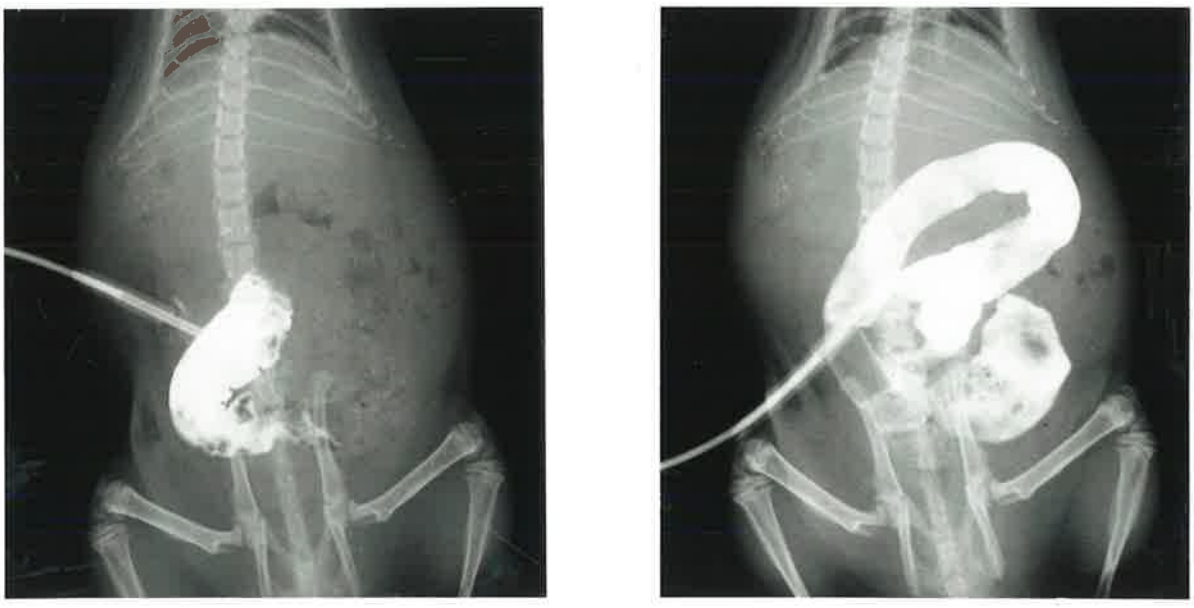
#### 3.3.2.1 Acute Experiments

At sacrifice, no animal exhibited macroscopic evidence of colitis manifest by visible inflammatory changes, ulceration or wall thickening; indeed there was no apparent difference between control and treated animals. While no attempt was made to formally score this, the impression was borne out by the results of histologic assessment. No animal, treated or control was scored higher than 0, ie no instance of focal neutrophillic infiltration or more marked inflammatory change were observed irrespective of sulphide concentration used. Representative sections from the proximal and distal colon of a control and 10 mM treated animal are shown (Figure 3.4, 3.5). Electron microscopy of proximal and distal colonic sections revealed no difference between control and 10 mM NaHS treated animals with respect to intercellular or organelle appearances. In particular there was no evidence of intercellular gap widening, cytoplasmic vacuolisation or cellular microvilli shortening as has been described in ulcerative colitis.



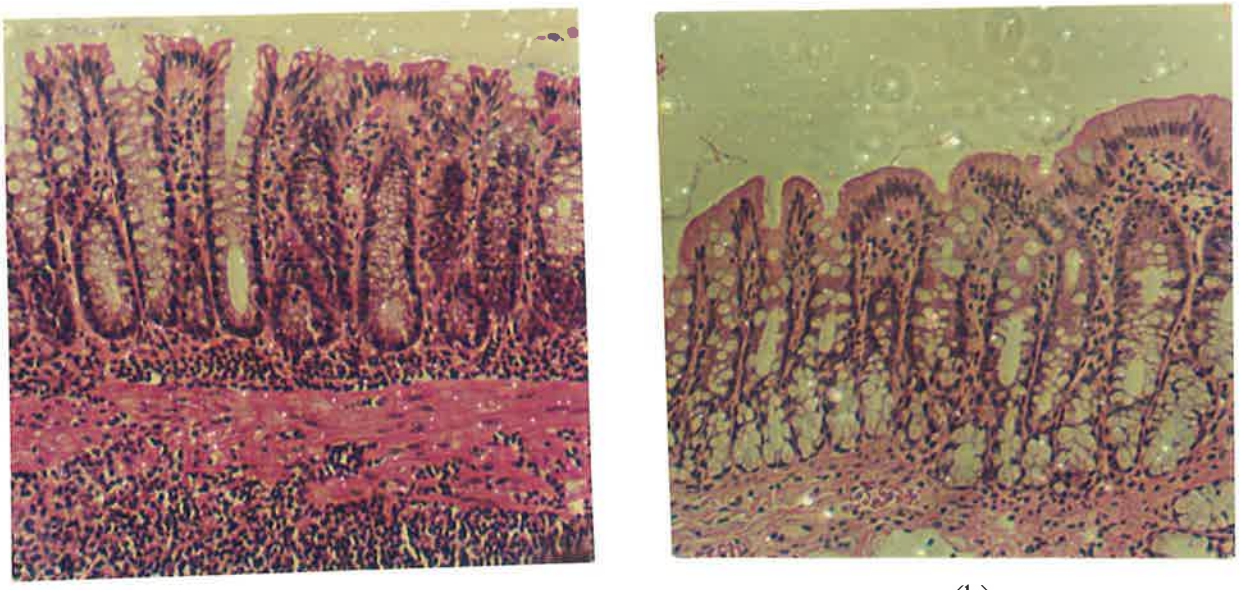
(a) (b)

**Figure 3.2** Histologic assessment of rat colonic mucosa (a) before and (b) after cell harvest. Sections are taken from the same segment of colon in two separate animals. Following harvest, the lamina propria is seen to be intact with extensive areas of de-epithelialised mucosa present. Magnification x 150.



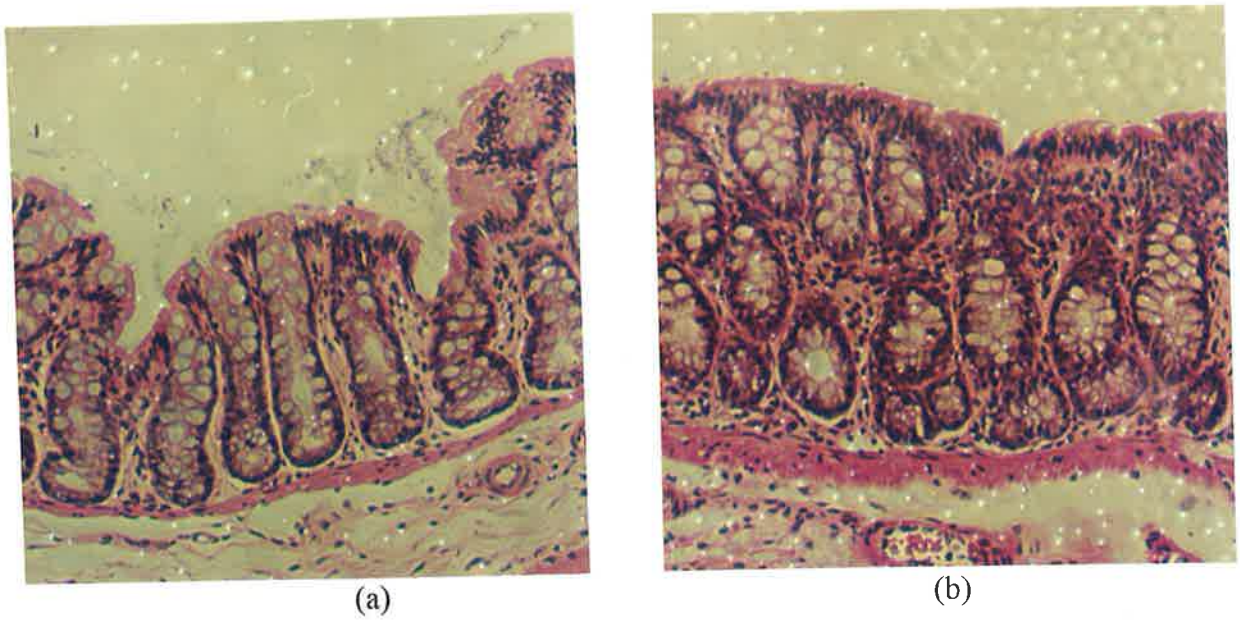
(a) (b)

**Figure 3.3** Radiologic assessment of instillation of dilute Gastrografin via antegrade Roux-en-Y colostomy. (a) 1.0 mL, (b) 2.0 mL



(a) (b)

**Figure 3.4** Histologic assessment of proximal rat colonic mucosa following four days instillation with (a) control saline or (b) 10 mM NaHS. Magnification x 150



(a) (b)

**Figure 3.5** Histologic assessment of distal rat colonic mucosa following four days instillation with (a) control saline or (b) 10 mM NaHS. Magnification x 150

### 3.3.2.2 Chronic Experiments

Similarly, animals treated with 10 mM NaHS for 90 days experienced no significant symptoms which could be related to the development of a colitic illness. Mucosal histology revealed no inflammatory response (histology score = 0) in all but two distal colonic sections. A focal neutrophillic infiltrate was recorded in distal colonic sections from one control and one 10 mM NaHS treated animal. Focal neutrophillic infiltrates were commonly seen in proximal colonic sections from 10 mM NaHS treated animals (mean score of three sections for 9 proximal colons examined = 0.78) but such findings were also common in control animals (mean score = 1.0) suggesting that such minor infiltrates may be a normal finding in the proximal rat colon following the creation of a Roux-en-Y colostomy. No sulphide treated animal had evidence of crypt abscess, mucosal ulceration or gland dropout (ie no section scored 2 or 3) nor was any evidence of crypt glandular architectural distortion observed following 90 days 10 mM NaHS exposure.

Alcian blue (pH 1.0) stained sections were scored for sulphomucin content. In neither proximal nor distal sections was there a significant difference in the number of cells staining positively / mm crypt mucosal length in the top half of the crypt (proximal colon, control = 89.2 (13.8), treated = 104.8 (9.0); t =0.99, p = 0.34, distal colon, control = 91.0 (17.0), treated = 95.4 (9.2); t = 0.25, p = 0.81).

### 3.3.3 Metabolic studies

### 3.3.3.1 Acute Experiments

The viability of harvested cells as measured by trypan blue exclusion did not differ significantly between control and treated animals (mean values (SEM),control 88.3% (2.1), 10 mM group 89.0% (2.9), 20 mM group 83.3% (3.3), 30 mM group 86.0% (1.9): p = 0.34 one way ANOVA). The mean (SEM) dry weight of cells harvested was not significantly different between groups (control 3.1 mg (0.2), 10 mM group 5.3 mg (0.9), 20 mM group 4.4 mg (0.8), 30 mM group 5.8 mg (0.9): p = 0.10 one way ANOVA). Within run coefficient of variation for enzymatic assays was 0.3% for 2.0 mM acetoacetate standard and 5.4% for lactate

(2.0 mM). Between run variability of acetoacetate standards was 5.9% (n = 11) and that for lactate standards was 6.3% (n = 20).

Oxidation of n-butyrate, as measured by $^{14}CO_2$ production, in incubations carried out in the presence of 5 mM [1-$^{14}$C] n-butyrate was significantly reduced by the four day pretreatment with NaHS irrespective of the concentration used (Table 3.1, Figure 3.6). $^{14}CO_2$ production was reduced from a mean of 1.34 µmol/min/gm dry wt cells in the control group to 0.87 µmol/min/gm dry wt cells in the 10 mM group. This amounted to a mean reduction in $^{14}CO_2$ production from n-butyrate of 33% in treated animals (when taken as a group) compared to controls. No significant difference was found between the values observed in the three treated groups (Tukey Kramer $p > 0.05$).

Oxidation of glucose, again as measured by $^{14}CO_2$ production from 5 mM [6-$^{14}$C] labelled glucose was also significantly reduced in all treated groups compared to control (Table 3.1, Figure 3.7). Mean (SEM) control values were 0.65 (0.05) µmol/min/gm dry weight cells and this value was reduced to 0.48 (0.04) µmol/min/gm dry weight cells after 4 days pretreatment *in vivo* with 10 mM NaHS ($p < 0.01$ one way ANOVA) as described. Again, there was no significant difference between 10, 20 and 30 mM NaHS treatments (Tukey Kramer $p > 0.05$). When taken as a group, the mean reduction in $^{14}CO_2$ observed in experiments conducted on the colons from treated animals was 26.6% compared to control values.

Ketone body formation was significantly affected in a manner reflecting the results above. Acetoacetate production in those incubations carried out with 5 mM [1-$^{14}$C] n-butyrate fell from 1.71 (0.21) to 1.12 (0.14) µmol/min/gm dry weight cells in the 10 mM NaHS treated group, again with no evidence of a dose response effect (Table 3.1, Figure 3.8). β-hydroxy-butyrate production fell from a control value of 0.29 (0.03) to 0.19 (0.03) µmol/min/gm dry weight cells in the 10 mM NaHS treated group ($p < 0.001$ one way ANOVA). The acetoacetate: β-hydroxy-

butyrate ratio was similar in control (5.95:1), 10 mM treated (5.97:1), and 20 mM treated (5.85:1) groups, but was higher in the 30 mM treated group (7.73 :1). Assessment of the glycolytic pathway by measurement of lactogenesis in incubations performed with 5 mM [6-$^{14}$C] glucose revealed no significant difference between control and treated groups ($p = 0.87$ one way ANOVA, Figure 3.9).

### 3.3.3.2 Chronic Experiments

Although six experiments were undertaken both control and 10 mM NaHS treated groups, viability of the cell suspension in one control experiment was of the order 50%, and the observed $^{14}CO_2$ production was commensurate with a cell suspension containing a majority of non viable cells. As the protocol required three months instillation, this experiment was not repeated, leaving 5 control and 6 treated observations. Mean (SEM) cell harvest did not differ between groups treated with control saline (control = 2.6 (0.32) mg, treated = 2.70 (0.23).mg; $t = 0.31$, $p = 0.76$, unpaired t test).

The changes observed in metabolic performance of isolated colonic epithelial cell suspensions derived from rats treated with twice daily instillation of 10 mM NaHS for ninety days closely mirrored those seen in the acute experiments described above. $^{14}CO_2$ production in incubations containing [1-$^{14}$C] n-butyrate was significantly reduced by sulphide treatment compared to control values (control = 1.19 (0.06) µmol/min/gm dry weight cells, treated = 0.69 (0.06), $t = 5.68$, $p < 0.001$, unpaired t test). This represented a 42.5% reduction of control values. $^{14}CO_2$ production from [6-$^{14}$C ] glucose was also significantly reduced in the treated group compared to control values (control = 0.59 (0.05) µmol/min/gm dry weight, treated = 0.39 (0.05); $t = 2.70$, $p = 0.02$, unpaired t test), representing a lesser reduction of control values (34.6%) when compared to the effect on butyrate oxidation. β-hydroxybutyrate production did not differ significantly between groups (Table 3.2) but the production of the quantitatively more important ketone body, acetoacetate, was reduced in the sulphide treated group ($p = 0.02$, Table 3.2). As in the acute experiments, *in vivo* sulphide treatment did not influence lactogenesis in isolated cell suspensions when compared to controls (Table 3.2).

**Table 3.1** $^{14}CO_2$, ketone body and lactate production (mean ± SEM in μmol/min/gm dry weight cell suspension) in isolated rat colonic epithelial cell suspensions following *in vivo* sulphide exposure (acute experiments).

| | **Control** | **10 mM** | **20 mM** | **30 mM** |
|---|---|---|---|---|
| **Substrate** | n = 6 | n = 6 | n = 6 | n = 6 |
| n-Butyrate (5 mM) | | | | |
| $^{14}CO_2$ | 1.34±0.06 | 0.87±0.04 [a] | 0.89±0.06 [a,c] | 0.83±0.05 [a,c] |
| Acetoacetate | 1.71±0.21 | 1.12±0.14 [b] | 1.02±0.09 [b,c] | 0.98±0.04 [b,c] |
| Glucose (5 mM) | | | | |
| $^{14}CO_2$ | 0.66±0.05 | 0.48±0.04 [b] | 0.46±0.03 [b,c] | 0.43±0.03 [b,c] |
| Lactate | 4.27±0.43 | 4.23±0.14 [d] | 4.07±0.74 [d] | 3.86±0.41 [d] |

[a] $p < 0.001$ versus control, (ANOVA), [b] $p < 0.01$ versus control (ANOVA), [c] p = n.s. versus 10 mM group (Tukey Kramer test), [d] p = n.s. versus control (ANOVA)

**Figure 3.6** $^{14}CO_2$ production from rat colonic epithelial cell suspensions incubated in the presence of 5 mM [1-$^{14}$C] labelled n-butyrate following 4 days *in vivo* sulphide exposure at concentrations shown. (See Table 3.1 for mean (SEM) values and statistical comparisons.)

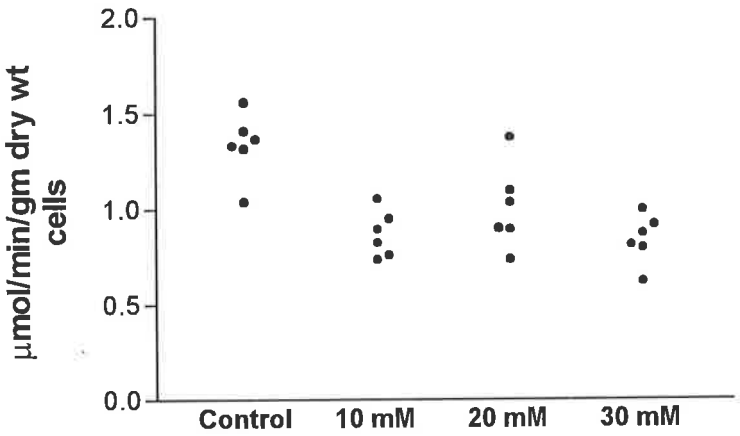



**Figure 3.7** $^{14}CO_2$ production from rat colonic epithelial cell suspensions incubated in the presence of 5 mM [6-$^{14}$C] labelled glucose following 4 days *in vivo* sulphide exposure at concentrations shown. (See Table 3.1 for mean (SEM) values and statistical comparisons.)

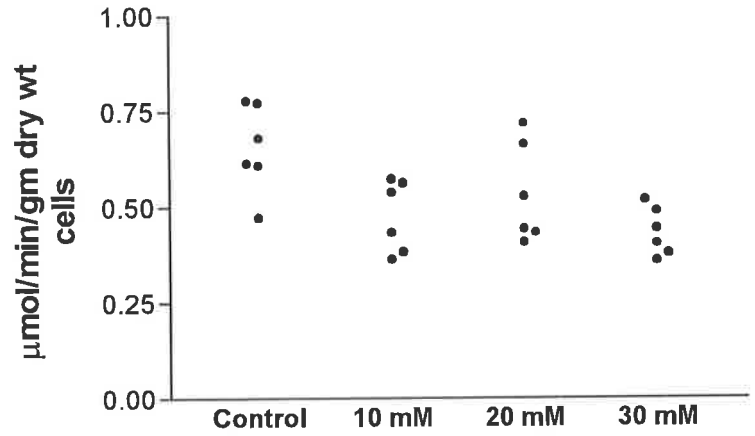


**Figure 3.8** Acetoacetate production from rat colonic epithelial cell suspensions incubated in the presence of 5 mM n-butyrate following 4 days *in vivo* sulphide exposure at concentrations shown. (See Table 3.1 for mean (SEM) values and statistical comparisons.)

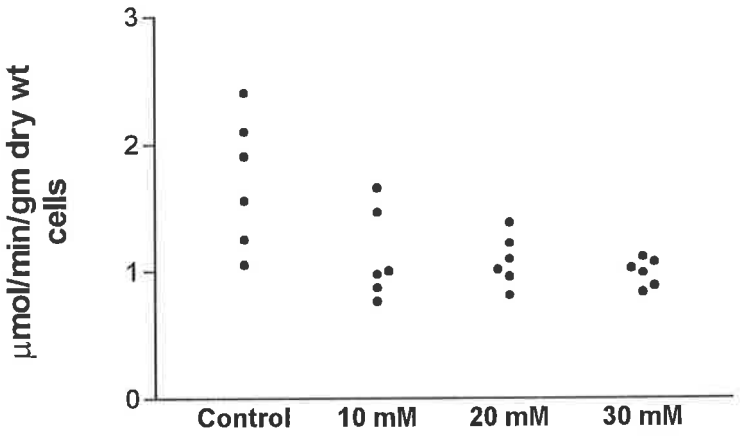



**Figure 3.9** Lactate production from rat colonic epithelial cell suspensions incubated in the presence of 5 mM glucose following 4 days *in vivo* sulphide exposure at concentrations shown. (See Table 3.1 for mean (SEM) values and statistical comparisons.)

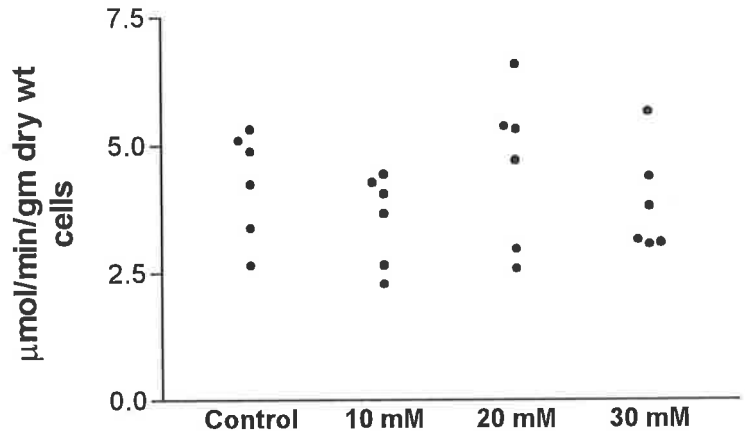


**Table 3.2** $^{14}CO_2$, ketone body and lactate production (mean ± SEM in μmol/min/gm dry weight cell suspension) in rat colonic epithelial cell suspensions following 90 day *in vivo* sulphide exposure (chronic experiments).

| | **Control** | **10 mM NaHS** |
|---|---|---|
| **Substrate** | n = 5 | n = 6 |
| n-Butyrate (5 mM) | | |
| $^{14}CO_2$ | 1.19 ± 0.06 | 0.69±0.06 [a] |
| Acetoacetate | 1.48±0.10 | 0.99 ± 0.14 [b] |
| β-Hydroxybutyrate | 0.17 ± 0.02 | 0.15 ± 0.02 [c] |
| Glucose (5 mM) | | |
| $^{14}CO_2$ | 0.59±0.05 | 0.39 ± 0.05 [b] |
| Lactate | 4.44±0.49 | 4.06 ± 0.43 [c] |

Two sided $p < 0.001$[a], $p < 0.05$[b], p = n.s.[c], compared to control (unpaired t test)

### 3.4 Discussion

The animal model described has been shown to be a reproducible and reliable one for the study of the effects of sulphide on the colonic mucosa *in vivo*. It has allowed the delivery of varying concentrations of the substance under study to the colon under circumstances which overcome the confounding influence of faecal diversion which are associated with isolated loop preparations. That sulphide was delivered to the mucosa under study has been confirmed both radiologically and by direct measurement of stool sulphide. No significant effect on colonic lumenal n-butyrate levels was observed suggesting that if sulphide does influence the resident microflora, it does not significantly affect fermentative production of the preferred fuel for the colonic mucosa. The model is well suited to chronic exposure experiments as the animals tolerate the instillation extremely well once they have become accustomed to being handled (usually after a few days only).

The observation that sulphide treatment *in vivo* diminishes both $^{14}CO_2$ production and ketogenesis from n-butyrate suggests that sulphide must be influencing the β oxidation pathway. Such a metabolic change would be expected to affect both acetoacetate and β-hydroxybutyrate production equally as has been observed. The pattern and degree of reduction of butyrate metabolism observed *in vivo* in this study reflects that seen in previous *in vitro* work (Roediger 1993a,b) and mirrors the pattern and degree of impaired n-butyrate metabolism observed in quiescent colitis (Roediger 1980c, Chapman 1994). However, the finding that glucose oxidation as measured by $^{14}CO_2$ production was also significantly reduced and that lactogenesis (involving the glycolytic pathway) was unaffected, suggests that either Krebs cycle function or (more likely) the electron transport chain is also affected by sulphide treatment using the concentrations used for these studies. This is in keeping with the observation that electron transport chain toxicity mediated by sulphide is not apparent at concentrations below approximately 60 μM (Bartholemew 1980). To differentiate these effects would require measurement of NAD : NADH ratios but was not addressed in this study.

Glucose oxidation has been reported not to be influenced (Chapman 1994, measured radiometrically as $^{14}CO_2$ production) or increased (Roediger 1980c, measured manometrically by $O_2$ consumption) in quiescent colitis and to be significantly increased in active colitis (Roediger 1980c). The observation that glucose oxidation was significantly reduced in both acute and chronic experiments (both of which used at least 10 mM NaHS instillation) suggests that the metabolic abnormality observed in sulphide treated rats may not accurately represent that seen in human ulcerative colitis. It may be argued that lower concentrations would have selectively affected β oxidation without influencing glucose oxidation, a suggestion which is supported by the findings of a sigmoidal dose response curve in experiments using isolated rat colonic epithelial cells treated with sulphide *in vitro* (Roediger 1993a). However, preliminary experiments using 1.0 and 5.0 mM instillations *in vivo* did not demonstrate any difference in $^{14}CO_2$ production ex [1-$^{14}$C] n-butyrate (data not shown). The degree of reduction of $^{14}CO_2$ production was similar for butyrate and glucose oxidation (although somewhat higher for butyrate in chronic experiments) suggesting the metabolic defect observed in this model is not as selective as that observed in the human colitic mucosa.

The importance of the observed metabolic changes in colonic epithelial cells treated with NaHS *in vivo* remains in doubt given the results of the histologic studies. No inflammation was observed in the colon of any rat treated with 10–30 mM NaHS for 4 days and no inflammatory changes, architectural alteration or reduction in mucosal sulphomucin content was observed following prolonged (90 days) exposure. Impaired sulphation of macromolecules seems to be an important abnormality in the colonic mucosa of ulcerative colitis, having been demonstrated both with respect to xenobiotics (Ramakrishna 1990) and colonic mucus (Habib 1986). The finding of no significant difference in the number of cells in the top half of the colonic epithelial crypt between control animals and sulphide treated animals suggests that any energy deficiency so produced does not influence the process of sulphate group transfer in the rat colonic epithelium.

That paracellular permeability may be altered in inflammatory bowel disease has been suggested by a number of studies examining both mucosal morphology and physiology. Morphologic studies have included the demonstration of abnormalities of intercellular tight junction formation in active ulcerative colitis (Delpre 1989) and both affected and macroscopically normal tissue from patients with Crohn's disease (Marin 1983, Dvorak 1979). *In vivo* physiological studies have used a number of probes, differing in molecular size, charge, lipophillicity and proposed pathways of absorption. The majority of the published work on mucosal permeability has focussed on Crohn's disease with increased (Hollander 1986, Olaison 1988), normal (Munkholm 1994) and decreased (Jenkins 1986 Olaison 1990) PEG 400 permeability, and elevated lactulose permeability (Andre 1988) all reported. Several studies have examined mucosal permeability in patients with ulcerative colitis using oral probe administration (Bjarnason 1983, Jenkins 1986, Munkholm 1994), none of which have suggested any mucosal permeability abnormality in this condition. The findings of Olaison (1989), however would suggest that oral probe loading may not provide accurate measurements of permeability changes in the colon. That mucosal permeability may be elevated in active ulcerative colitis is suggested by the observation that up to half a rectally administered steroid dose may be absorbed (Powell-Tuck 1976). It has been suggested that the "energy deficiency" observed in the colonic mucosa of patients with ulcerative colitis may allow lumenally derived, proinflammatory agents to breach the "mucosal barrier" (Roediger 1986, Gibson 1992). The model of *in vivo*, sulphide induced diminished n-butyrate oxidation described could be a useful one to test such a hypothesis.

# Chapter 4

# Short Chain Acyl-CoA Metabolism In Rat Colonocytes: Effect Of Sulphides

## 4.1 Introduction

Evidence for diminished n-butyrate oxidation in ulcerative colitis has been presented by several authors (Roediger 1980, Chapman 1994) but such evidence reflects global outcomes in the beta oxidation pathway. While detailed assessments of the intermediates of short chain fatty acid oxidation, the short chain acyl CoA esters, have been undertaken in myocardial, peripheral muscle and hepatic tissues (Corkey 1981, 1988b, Veerkamp 1986, Eaton 1994), such studies have not been performed on the colonic epithelium, despite the importance of n-butyrate oxidation in this tissue. Measurements of CoA esters in the epithelium of patients with ulcerative colitis may provide important clues to the mechanism underlying the observed reduction in n-butyrate oxidation observed in this condition. Evidence has been presented that sulphides may inhibit flavoprotein dependent short chain acyl CoA dehydrogenases through CoA persulphide formation (see 2.2.2). The hypothesis that colonic lumenal sulphides are aetiologically important in ulcerative colitis would be strengthened if an abnormal pattern of acyl CoA ester concentrations in colonic epithelial cells was to be shared between human ulcerative colitis and an experimental model using mucosal tissues exposed to sulphide. Therefore, as a preliminary to future studies of acyl CoA ester concentrations in ulcerative colitis, animal experiments assessing the acyl CoA ester concentrations in rat colonic epithelial cell suspensions exposed to NaHS were performed.

## 4.2 Materials And Methods

### 4.2.1 Reagents

All reagents used for cell incubation studies were as previously described in Chapter 3. Sulphosalicylic acid was purchased from BDH (Poole, England). Magnesium ATP, CoA and acyl CoA standards (acetyl-, butyryl- and crotonyl-CoA) were obtained from Boehringer Mannheim (West Germany).

### 4.2.2 Source Of Cell Suspension

Although the studies described earlier (Chapter 3) showed that reduction of n-butyrate oxidation in isolated colonic epithelial cells could be achieved using the *in vivo* antegrade Roux-en-Y colostomy model, the absence of mucosal inflammation suggested that this model had little to offer over the simpler option of sulphide exposure *in vitro*. Accordingly, these experiments were carried out using isolated cell suspensions created from animals that had not undergone the operative creation of a Roux-en-Y antegrade colostomy nor exposure to NaHS *in vivo*. The preparation of such cell suspensions was otherwise performed as described previously including the omission of mucolytics.

### 4.2.3 Incubation With ATP And Coenzyme A

The activity of intermediates in beta oxidation of short chain fatty acid oxidation has not been studied in colonocytes. The studies of beta oxidation intermediates that do exist have been performed exclusively in hepatic and myocardial tissues and have focussed predominantly on longer chain fatty acid oxidation. It has been shown that the appearance of intermediates during *in vitro* peroxisomal β oxidation of long chain fatty acid is dependent upon added ATP and CoA (at least up to added CoA concentrations of 200 μM (Osmundsen 1987, Bartlett 1990) and that inclusion of ATP (10 mM) in peroxisomal incubations inhibits generation of acetoacetyl-CoA from acetyl-CoA (Hovik 1991). It has also been suggested that ATP activates acyl CoA hydrolases in rat liver peroxisomes (Hovik 1991) but inhibits them in rat brown adipose tissue (Alexson 1989). Accordingly, incubations were carried out in both the presence and absence of added ATP (4.5 mM final concentration) and CoA (0.2 mM final concentration).

### 4.2.4 Length Of Incubation

In order to express acyl-CoA ester values per unit time, it was necessary to demonstrate that intermediate production was linear over the course of the incubation. Accordingly, the time course of acyl-CoA product appearance was assessed. Duplicate incubations of isolated rat colonic epithelial cell suspensions

were stopped at 1, 3, 5 and 10 minutes in the presence of ATP and CoA (concentrations above) and acyl-CoA ester concentrations measured.

### 4.2.4 Choice Of Quenching Agent

The choice of acid to quench the cell suspension incubation requires comment. The most usual practice has been to stop the incubation using 0.5 mL of 10% perchloric acid (see 2.2.3). It was chosen, however to follow the method of Demoz (1995) which suggests the use of sulfosalicylic acid (SSA, final concentration approx 0.5% weight for volume) with 50 μM dithioerythritol (DTE). The differences between perchloric acid and SSA have not been assessed.

### 4.2.5 Sulphide Concentration Used

The findings that sulphide inhibits n-butyrate oxidation *in vitro* in a non linear, dose dependent fashion between 0.5–2.0 mM (Roediger 1993a) and that these levels are of the order that may be seen in the human colonic lumen (Florin 1990, McFarlane 1992) prompted me to use a final concentration of 1.5 mM NaHS in all incubations. This was added as the last substance prior to gassing with 19:1 $O_2$:$CO_2$ (no preincubation with sulphide was carried out).

### 4.2.6 Identification Of Acyl CoA Peaks

While the use of online radioactivity and photodiode detection as described by Eaton (1994) allows identification of acyl CoA peaks with considerable accuracy, such methodology is expensive for multiple assays. Under conditions available in our laboratory (HPLC with UV detection), coelution of a particular peak with its standard species (although clearly a prerequisite) was not considered adequate to identify a particular compound. A peak was accepted as representing a given acyl CoA ester only when standard coelution *and* predicted recovery of a standard "spike" was demonstrated and when alkaline hydrolysis confirmed the observed peak to disappear as would be expected of a CoA ester.

### 4.2.7 Experimental Protocol

All experiments were performed in the morning, beginning at approximately 0900. Six animals were used for each experiment to ensure sufficient cell harvest (greater than 10 mg/mL dry weight cell suspension). Animals were sacrificed by stunning / cervical dislocation and their colons rapidly harvested, rinsed clear of faecal material with normal saline and isolated colonocyte suspensions prepared as described (Chapter 3). One mL of cell suspension was incubated in the presence of 5 mM [1-$^{14}$C] labelled n-butyrate in a final incubation volume of 2.0 mL with or without NaHS (1.5 mM). Incubations additionally were carried out with Mg ATP (4.5 mM) and free CoA (0.2 mM). As previously, incubations were carried out at 37°C in stoppered 25 mL flasks in a shaking water bath, but for the purposes of these experiments incubation was completed at ten minutes by the addition of 0.1 mL 5% sulfosalicylic acid containing dithioerythritol (62.5 μM) (Demoz 1995). $^{14}CO_2$ was collected in 0.5 mL 1.0 M NaOH (after shaking on ice for 90 minutes) and calculated as previously described (Chapter 3). The SSA / DTE quenched incubation mix was homogenised using a glass teflon homogeniser (Braun type 853204) on maximum power setting for 20 seconds. The resulting homogenate was centrifuged (2500g, 10 minutes, 4°C) and 80 μL supernatant used for injecting onto the HPLC. A Waters HPLC system (Millipore Australia, Sydney) was used for HPLC analysis, comprising a 510 HPLC pump, 715 Ultra Wisp sampler and Baseline 810 software. The analytic column was a LiChroCART 250×4 mm column (Merck # 50839, Kilsyth, Victoria, Australia) equipped with a LiChroCART 4-4 (4 mm) guard column (Merck # 1.50963, Darmstadt FRG), and the packing material was LiChrospher 60 RP-select B (5 micron particle size) (Merck Australia). Mobile phase comprised variable amounts of 0.1 M $KH_2PO_4$ (pH = 5.0) (Reagent A) and 60% A : 40% acetonitrile (Reagent B). Acyl CoA ester elution was achieved using a variable flow rate and mobile phase concentrations as shown in Table 4.1 and modified from Corkey (1990). Detection was by UV absorbance at 260 nm using a Waters 486 UV / visible detector (Waters, Sydney Australia). Standard solutions of CoA esters (acetyl-, butyrl- and crotonyl-CoA) and free CoA were prepared as described by DeBuysere (1983) as 5 mM stock

solutions in 1.0 M $KH_2PO_4$ (pH 4.0) and stored at –80°C. A 47.6 µM standard was prepared for each run by diluting stock standard in SSA/DTE (20µL) and Krebs Henseleit buffer containing 2.5% BSA (380 µL). Samples were run in duplicate and the mean value used for calculations. Acyl CoA ester concentration in the reaction mix was calculated according to the formula

$$C = (A_{sample}/A_{std}) \times 47.6 \times V_{inc} \times 1/W$$

Where C = concentration in µmol / L / mg cells dry weight

$A_{sample}$ = Injected sample area

$A_{std}$ = Injected standard area

Vinc = Final incubation volume (= 2.1 mL)

W = dry weight cells in isolated cell suspension.

Sample hydrolysis (to help confirm peak identity) was performed on 100 µL sample supernatant to which was added 7.5 µL 5 M KOH. This mixture was incubated (63°C, 30 minutes) and then acidified with an equivalent amount of SSA/ DTE to ensure pH < 3.0 and assayed as above.

### 4.2.8 Statistical Analysis

Linearity of CoA ester concentrations over time was assessed using the Spearman correlation coefficient. Differences in variance between groups was assessed using Bartlett's test. Comparisons of means of continuous variables ($^{14}CO_2$, CoA ester production) between control and sulphide treated groups was by paired t test. Significance was set at the conventional 5% level. All values are given as mean (SEM).

**Table 4.1** Mobile Phase Concentration And Flow Rates

| Time (min) | Flow (mL/min) | % A | % B |
|---|---|---|---|
| 0 | 0.4 | 90 | 10 |
| 19 | 0.4 | 90 | 10 |
| 20 | 0.8 | 90 | 10 |
| 21 | 0.8 | 90 | 10 |
| 22 | 0.8 | 87.5 | 12.5 |
| 52 | 0.8 | 87.5 | 12.5 |
| 59 | 0.8 | 73 | 27 |
| 79 | 0.8 | 73 | 27 |
| 80 | 0.4 | 90 | 10 |
| 95 | 0.4 | 90 | 10 |

## 4.3 Results

Mean cell harvest for these studies was 13.6 mg, with viability as assessed by trypan blue exclusion greater than 90% in all experiments. The delay between the sacrifice of the first rat and when the six colonic sacs were all harvested and incubating in Ca free KH / EDTA was approximately 8 minutes, meaning that incubations were carried out well within the time frame that $^{14}CO_2$ production is known to be linear (Roediger 1979).

### 4.3.1 Time Course Experiments

The appearance of crotonyl CoA and butyryl CoA was found to be linear over the ten minute time course studied (Figure 4.1, butyryl CoA, $r = 0.99$. $p < 0.0001$; crotonyl CoA $r = 0.99$, $p < 0.0001$). The appearance of acetyl CoA followed a much more rapid and non linear time course over the ten minute incubation (Figure 4.1). Indeed, the observed concentrations of acetyl CoA suggested that an incubation time of less than one minute would have been required to achieve a linear time plot, a time course that would not allow accurate assessment of $^{14}CO_2$ values. Accordingly, values for butyryl and crotonyl CoA are expressed per unit time (as µmol/gm dry wt cells/minute), whereas values for acetyl CoA are expressed as concentration in the reaction mix at ten minutes (in µmol/gm dry wt cells).

### 4.3.2 Incubations Carried Out In The Absence Of Exogenous ATP / CoA

A metabolic effect of sulphide treatment on isolated rat colonic epithelial cells was demonstrated by the reduction of mean (SEM) control $^{14}CO_2$ production from 0.84 (0.07) µmol/gm dry wt cells/minute to 0.18 (0.05) µmol/ gm dry wt cells/minute following addition of 1.5 mM NaHs to the incubation ($t = 12.85$, $p = 0.0002$). Acyl CoA ester values for experiments carried out in the absence of exogenous CoA and ATP are summarised in Table 4.2. In the absence of exogenous ATP and CoA, the rate of appearance of butyryl CoA was not significantly different between control and treated groups (control = 23.1 (3.5) µmol/gm dry wt cells/minute; treated = 21.3 (5.0); t 0.76, $p = 0.49$, paired t test). Under the assay conditions described, the mean (SEM) rate of appearance of crotonyl CoA was not affected by 1.5 mM

sulphide treatment (control = 16.2 (5.0) μmol/gm dry wt cells/minute, treated =11.0 (5.5); t = 1.33, p = 0.26 paired t test). Similarly, acetyl CoA concentrations in the final reaction mix did not differ between the groups (Table 4.2) but free CoA levels were slightly but significantly elevated in the treated group compared to controls (control = 211.2 (46.0) μmol/gm dry wt cells/minute, treated = 245.5 (43.5); t =3.22, p = 0.03, paired t test).

### 4.3.3 Incubations Carried Out In The Presence Of Exogenous ATP / CoA

Results are summarised in Table 4.3. A similar and highly significant reduction in $^{14}CO_2$ production was observed following the addition of 1.5 mM NaHS to isolated cell suspensions incubated in the presence of exogenous ATP and CoA (control = 0.97 (0.06)μmol/ gm dry wt cells / minute, 1.5 mM NaHS treated = 0.26 (0.09) μmol/ gm dry wt cells / minute; t = 7.30, p = 0.0019, paired Students t test). This reduction was associated with a significant increase in the mean rate of appearance of butyryl CoA in the reaction mix (control incubations = 2552.5 (276.1) μmol/ gm dry wt cells/minute, 1.5 mM NaHS treated incubations = 3322.4 (315.0) μmol/gm dry wt cells/minute; t = 7.17, p = 0.002). Concurrently, the mean rate of appearance of crotonyl CoA in sulphide treated incubations was significantly reduced compared to control incubations (control = 274.4 (22.8) μmol/gm dry wt cells/minute, 1.5 mM NaHs treated = 118.4 (40.8) μmol/gm dry wt cells/minute; t = 4.90, p = 0.008). The mean concentration of acetyl CoA in the reaction mix at ten minutes was not significantly different between control and sulphide treated incubations (control = 6941 (1310) μmol/gm dry wt cells, 1.5 mM NaHS treated = 6523 (1057) μmol/gm dry wt cells; t = 1.42, p = 0.22).

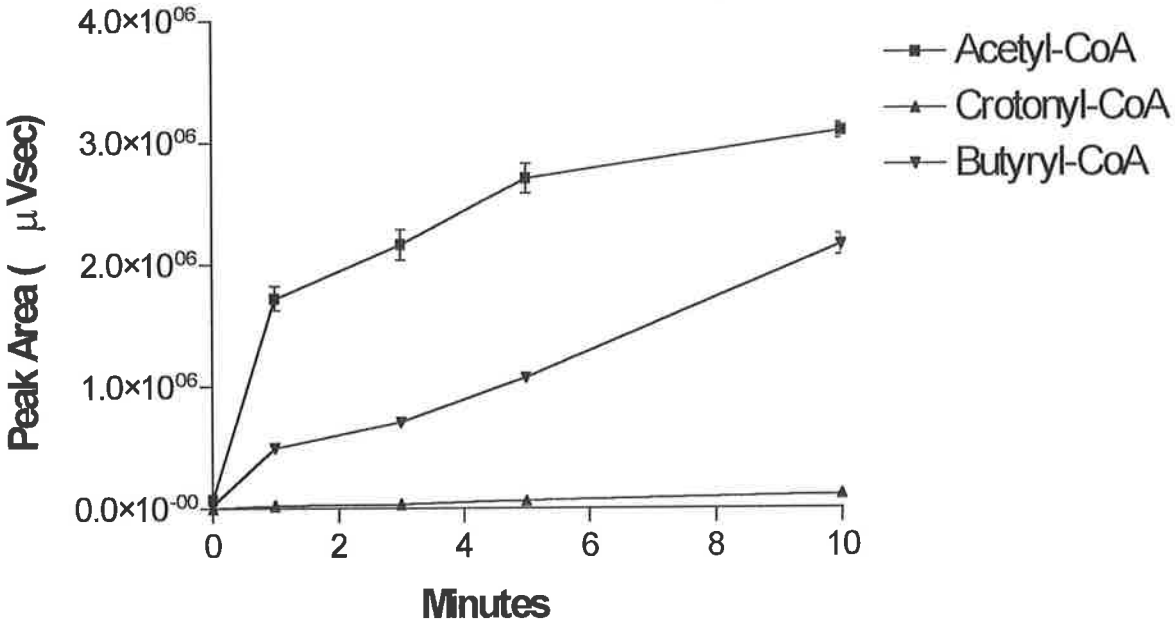



**Figure 4.1** Time course of acyl-CoA ester concentrations over 10 minute incubations. Each point represents the mean (SEM) of triplicate observations from a single colonic epithelial cell suspension.

**Table 4.2** $^{14}CO_2$ and Acyl CoA Ester Values for Incubations Carried Out In The Absence Of Exogenous ATP and CoA. Values are mean (SEM) expressed as * μmol / minute / gm dry weight cells, # μmol / gm dry weight cells in final reaction mix at ten minutes incubation. [1] $p < 0.001$, [2] $p < 0.05$, [3] p = n.s, treated versus control value, paired t test.

| | **Control** | **Treated** |
|---|---|---|
| **$^{14}CO_2$*** | 0.84 (0.07) | 0.19 (0.02)[1] |
| **Free CoA*** | 211 (46) | 245 (43)[2] |
| **Butyryl CoA*** | 25 (3.5) | 21 (6.3)[3] |
| **Crotonyl CoA*** | 16 (5.0) | 11 (5.5)[3] |
| **Acetyl CoA#** | 134 (15.6) | 137 (19.5)[3] |

**Table 4.3** $^{14}CO_2$ and Acyl CoA Ester Values for Incubations Carried Out In The Presence Of Exogenous ATP and CoA. Values are mean (SEM) expressed as * µmol/minute/gm dry weight cells, # µmol/gm dry weight cells in final reaction mix at ten minutes incubation. [1] $p < 0.005$, [2] $p < 0.01$, [3] p = n.s, treated versus control value, paired t test.

| | **Control** | **Treated** |
|---|---|---|
| **$^{14}CO_2$*** | 0.97 (0.06) | 0.26 (0.05)[1] |
| **Butyryl CoA*** | 2552 (276) | 3322 (314)[1] |
| **Crotonyl CoA*** | 274 (23) | 118 (41)[2] |
| **Acetyl CoA#** | 6941 (1310) | 6522 (1057)[3] |

## 4.4 Discussion

That FAD dependent dehydrogenation by colonic epithelial cells is inhibited by sulphides has been suggested indirectly by the observation that in sulphide treated (1.0 mM) isolated colonic epithelial cell suspensions, ketogenesis derived from crotonate, (the enoyl derivative of n-butyrate) was significantly greater than that observed from n-butyrate (Roediger 1993a). The studies here reported provide direct evidence that such FAD dependent acyl-CoA dehydrogenation is one mechanism by which the observed effect of sulphide on colonic epithelial metabolism may be produced. Alterations in acyl CoA profiles could only be demonstrated in incubations carried out in the presence of exogenous ATP and CoA using the methodology described. Similar findings have been reported previously in which detection of beta oxidation intermediates was dependent upon coincubation with CoA and ATP in rat liver and brown fat peroxisomes (Alexson 1989, Bartlett 1990). The finding in control incubations that the rate of crotonyl CoA production was significantly lower than that of butyryl CoA (274 vs 2552 µmol/minute/gm dry weight cells) supports the suggestion that short chain acyl CoA dehydrogenation is an important rate limiting step in short chain fatty acid oxidation (Bremer 1984).

While acetyl CoA concentrations in the final reaction mix in incubations carried out in the presence of exogenous CoA and ATP were reduced in sulphide treated incubations, this was not significantly so. The importance of this observation is hard to assess given the findings of the time course studies which suggest that these values reflect a steady state situation and given the multiplicity of alternative sources and metabolic pathways utilising acetyl CoA within colonic epithelial cells. The reduction observed, while not statistically significant, amounts to approximately 7.5% of control values. Whether such a reduction is physiologically relevant cannot be stated although the reduction in $^{14}CO_2$ values indirectly argues that it may be important. The effect of sulphide on electron transport, which undoubtedly contributes to this, has not been directly addressed however.

It has been suggested that CoA persulphides may play an important role in the physiologic regulation of acyl CoA dehydrogenation (Williamson 1982, Engel 1984, 1992, Shaw 1987), thought to be an important regulatory step in beta oxidation pathway (Bremer 1984). The absorption spectra of long and medium chain fatty acid dehydrogenases has a pattern that corresponds to that of the FAD prosthetic group (Engel 1987). Native short chain acyl CoA dehydrogenases (SCAD) exhibit a similar pattern but also a band at approximately 700 nm responsible for the characteristic green colour of these enzymes (Hoskins 1966, Engel 1971). It has been demonstrated that this is due to a charge transfer reaction between the FAD prosthetic group of SCAD and a CoA persulphide (Williamson 1982). Such a persulphide could result from the combination of free sulphide and the thiol group of free CoA. It has been demonstrated that ox mitochondrial butyryl-CoA dehydrogenase can be "degreened" (losing its CoA persulphide / FAD absorption peak) by incubation in the presence of substrate (butryl CoA) excess and that CoA persulphide saturated enzyme (assessed by absorption spectral changes) exhibits only 10% of the activity of unliganded enzyme (Shaw 1987). The same study suggested that enzyme extracted from healthy ox liver mitochondria was 60% liganded as a SCAD-CoA persulphide complex and proposed that CoA persulphides may be an important regulator of mitochondrial beta oxidation. The strong substrate affinity of SCAD (Km approximately 1–2 μM, (Bremer 1984)) suggests that substrate (butyryl CoA) levels in mitochondria are unlikely to reach the excess concentrations required for the CoA persulphide displacement described by Shaw (1987) to occur and that such a displacement mechanism in the physiologic control of beta oxidation is unlikely. This does not, however, rule out a role for pathophysiologic inhibition of SCAD activity in the presence of elevated concentrations of agents capable of forming CoA persulphides. Sulphur transfer reactions mediated by rhodanese do not generate CoA persulphides (Shaw 1987). The presence of significant concentrations of reduced forms of sulphur, including sulphide, in the colonic lumen may provide an appropriate pathway for the generation of pathologic levels of colonic epithelial mitochondrial SCAD-CoA persulphide complex, capable of inhibiting fatty acid oxidation. The results of

incubations carried out in the presence of exogenous ATP and CoA would be consistent with such a mechanism with higher butyryl CoA and lower crotonyl CoA values observed following sulphide treatment of cell suspensions. The effects of persulphide formation may be expected to be reflected in antioxidant defences within the colonic mucosa such as the ratio of reduced to oxidised glutathione. Recent reports (Khalil 1995) have demonstrated a significant reduction in reduced glutathione in colonic mucosal extracts from carrageenan fed rats, an animal model known to be associated with elevated colonic lumenal sulphide levels (Florin 1995).

A pattern of acyl CoA ester abnormality has been established in rat colonic epithelial cells treated with physiologically relevant concentrations of sulphide. An important step in determining if this is of relevance to the epithelial cell metabolic abnormality observed in ulcerative colitis will be to demonstrate a similar abnormality in colonic epithelial cells isolated from colitic tissue. Such studies are currently being undertaken to explore this possibility.

# Chapter 5

# Studies Of Sulphide And Sulphate Metabolism In The Human Colon

## 5.1 Introduction

Whilst much *in vitro* work supports the hypothesis that reducing sulphur compounds, noteably sulphide, are able to inhibit fatty acid oxidation in colonic epithelial cells (Roediger 1993a, 1993b), a role for lumenal sulphide in the diminished butyrate oxidation of the colitic mucosa is yet to be established. Whilst the demonstration of elevated stool sulphide would be important evidence in support of such a role, contradictory evidence has been presented regarding the level of sulphide in colitic stool compared to controls (Florin 1990, Pitcher 1995b). *In vitro* and *in vivo* studies have also provided contradictory data regarding the importance of sulphate reduction (and hence sulphidogenesis) versus methanogenesis as a means of hydrogen disposal in the colonic lumen (Gibson 1988a, Christl 1992a,b, Strocchi 1991, 1994). If sulphate reducing bacteria outcompete methanogens for available hydrogen (in the presence of available sulphate) stool sulphide might be expected to be higher in nonmethanogenic individuals, as reported by Gibson (1990b).

The aim of this study was to test the hypothesis that stool sulphide is elevated in ulcerative colitis by assessing rectal faecal sulphide levels in patients with ulcerative colitis and comparing this to a control population. Secondary aims were:

1. To assess the correlation, if any between disease activity or extent and stool sulphide levels.
2. Compare direct spectrophotometric and reverse phase high performance liquid chromatography (RPHPLC) methodologies for sulphide determination of faecal sulphide and to assess faecal dialysis as a method for sampling faeces in vivo for the measurement of free sulphide.
3. Compare free and total stool sulphate (a precursor of sulphide derived from sulphate reduction in the colonic lumen) between control and colitic patients.

4. To compare methanogenic status (determined by breath methane analysis) with stool sulphide.

## 5.2 Materials and Methods

### 5.2.1 Reagents

Cellulose (Visking) tubing for in vivo dialysis was obtained from H. B. Selby (Sydney, Australia). Sodium n-butyrate, ferric chloride hexahydrate and maleic acid were obtained from BDH (Poole, England) and n,n-dimethyl-p-phenylenediamine (DPD, as the free base) from Sigma (St Louis, Mo.). All other chemicals were obtained from Ajax Chemical Co. (Sydney, Australia). All chemicals were of analytical grade and ultra pure water (> 18 MΩcm) was used throughout. The working DPD solution comprised 20 mL 9 M sulphuric acid (Ajax Chemical Co., Sydney) plus 0.5 mL stock DPD solution (15 gm/100mL water) and was made fresh daily. Working $FeCl_3$ solution comprised 83 gm $FeCl_3$ hexahydrate in 100 mL water. Maleic acid buffer comprised 10 gm in 100 mL water, titrated to pH 0.6 with concentrated NaOH. 5 amino-sulphasalicylic acid (5-ASA, > 97% pure) and sulphasalazine were obtained from Merck (Schuchardt, Germany) and sulfapyridine from Pharmacia (Uppsala, Sweden).

### 5.2.2 Patient Selection

Patients with ulcerative colitis were identified by three means. Inpatients with active disease requiring admission to hospital were recruited by direct approach in hospital. Outpatients with active disease were recruited with the generous help of the Gastroenterology Unit at The Queen Elizabeth Hospital when seen in the Outpatient Department. Patients with quiescent disease were identified by review of hospital medical records. Those with a discharge diagnosis categorised by ICD-9 as 556.0 (idiopathic proctocolitis) (International Classification of Diseases, Ninth Revision, 1980) were approached by telephone interview and enrolled. All patients gave informed consent and the experimental protocol had the approval of The Queen Elizabeth Hospital and The Royal Adelaide Hospital Ethics of Human Research Committees. Control patients were awaiting haemorrhoidectomy on The

Queen Elizabeth Hospital surgical waiting list. These were used for the following reasons; (1) all these patients were to be admitted to hospital in the near future and be readily available for study with little extra inconvenience to them, (2) haemorrhoids are normal anatomic structures, only requiring surgical management because of symptoms, (3) all had undergone assessment to exclude significant gastrointestinal disease prior to being placed on the waiting list. Patients with colitis were stratified by disease extent (as determined at their last colonoscopy on macroscopic grounds into either limited (disease limited to the colon distal to the splenic flexure only) or pancolitis. Disease activity was assessed by the sigmoidoscopic criteria of contact bleeding (McIntyre 1985) and categorised as either inactive or active disease. Disease duration (defined as time since first symptoms to the time of study was recorded. Past medical history, smoking habit and current medications were reviewed in all patients. Prior to obtaining a measurement of stool sulphide by *in vivo* dialysis and directly on voided stool, 20 mL venous blood was drawn from a peripheral vein and used for measurement of red cell TMT activity as described in Chapter 6.

### 5.2.3 Technique of Sulphide Measurement

Faecal sulphide was measured using two sample types; (1) stool water obtained using faecal dialysis as described by Wrong (1965) and (2) concurrently collected fresh sodium hydroxide and zinc acetate faecal slurries as previously described (Florin 1991a).

#### 5.2.3.1 Stool Dialysis

Seamless Visking tubing (8/32) was used for dialysis bags. These were gently distended with 0.9% NaCl and each end double ligated with 3/0 black silk thread to create a dialysis bag 3.0 cm in length and containing approximately 500 μL saline. The thread at one end was left 10–12 cm long to allow easy retrieval following dialysis.. All bags were made up fresh on the morning of use and all studies were performed in the fasted state (early a.m.). No bowel preparation was used. Patients were asked to defer using their bowels on the morning of study until the study was

complete. With the patient in the left lateral position, and using no anaesthesia, a tubular proctoscope was inserted into the rectum (using the minimum of lubricant). A single dialysis bag was placed in the rectum at 8 cm from the anal verge under direct vision and the proctoscope withdrawn, leaving the long thread attached to the dialysis bag protruding through the anus. After 100 minutes, the bag was removed by gentle traction on the suture and the contents withdrawn immediately using a 27 gauge needle attached to a 2 mL syringe. The faecal dialysate was immediately derivatised using the methylene blue method modified by Savage (Savage, 1990) from that originally described by Cline (Cline, 1969). Briefly, 150 μL of this dialysate was immediately placed in an Eppendorf reaction tube containing 100 μL working DPD solution and 4 μL ferric chloride solution and inverted once only. This derivatised sample was transported to the laboratory on ice in a lightproof container and analysed immediately after 20 minutes were allowed for complete colour development. Zero standards were prepared by adding 150 μL $H_2O$ to 100 μL working DPD solution and 4 μL $FeCl_3$.

#### 5.2.3.2 Faecal Slurries

Following the collection and derivatisation of the faecal dialysate, the subject was asked to void urine into the toilet (to avoid urinary contamination of stool sample), and then to defaecate into a commode and pan lined with waxed laboratory film (Parafilm "M", American National Can, Greenwich, Conn.). A freshly passed stool sample (approx. 1–2 gm) was then collected into (1) a 50 mL polypropylene centrifuge tube (Corning, New York) containing 10 mL of 0.1 M zinc acetate solution (for total acid volatile sulphide estimation) preweighed empty, and following addition of zinc acetate solution (to allow calculation of a weight : weight dilution for faecal slurry) and (2) an identical container containing 10 mL 1 M NaOH for direct spectrophotometric measuement of free sulphide, preweighed in an identical fashion. After reweighing (container, solution plus sample) and homogenisation (Vortex mixer on highest setting until all particulate matter dispersed) one mL of each stool slurry was weighed and then dried in duplicate in an oven overnight (90°C) for estimation of wet and dry weight.

### 5.2.3.3 Assay Procedures

#### 5.2.3.3.1 Free Sulphide

Free sulphide was estimated using three different methods:

(1) RPHPLC of stool dialysate using spectrophotometric detection at 670 nm, using the method of Savage and Gould (1990). Derivatised dialysate was centrifuged (5 min, 10,000 rpm) and 5 µL of supernatant injected onto an SGE polymer coated C18 silica column (SGE-250GL4, C18-P8/5, Adelab Scientific, Norwood, S.A.). Mobile phase comprised dibutylamine (3mL/L) and 40% acetonitrile in MilliQ distilled water titrated to pH 3.0 with 20% phosphoric acid, degassed and filtered in a sonication bath, run at a flow rate of 1 mL per minute and a pump pressure of 2400 psi. Detection was by reading absorbance at 670 nm using a Waters 486 U.V./visible detector (Waters, Sydney Australia). Under the conditions described, standard sulphide solutions produced a peak at 5.6 ± 1 minute.

(2) Direct spectrophotometric analysis of stool dialysate. Derivatised dialysate was centrifuged (5 min, 10,000 rpm) as above. 100 µL of dialysate was added to 2 mL cuvettes containing 1.0 mL maleic acid buffer and 0.9 mL $H_2O$ and mixed by inverting twice. Absorbance was read at 670 nm on a Varian DMS 200 spectrophotometer (Varian, Mulgrave, Victoria).

(3) Direct spectrophotometric assay of NaOH faecal slurry. 0.5 mL of well mixed NaOH slurry was centrifuged at 10000 rpm for 20 min. 150 µL of supernatant was derivatised as per dialysates. Twenty minutes was allowed for colour development (Savage 1990). Derivatised supernatants were then centrifuged (5 min, 10,000 rpm), 100 µL supernatant diluted in maleic acid buffer and $H_2O$ and absorbance read at 670 nm as above.

#### 5.2.3.3.2 Total Sulphide

To ensure insoluble sulphide complexes, present in the particulate fraction of the faecal slurry, were adequately sampled, zinc acetate slurries were mixed using a vortex mixer (1 min, highest setting) immediately prior to 0.3 mL of zinc acetate faecal slurry being added to 0.2 mL of DPD and 8 µL $FeCl_3$ in an Eppendorf

reaction tube. This was quickly sealed and allowed 20 minutes for full colour development. Following centrifugation (5 min, 10,000 rpm), 100 µL of supernatant was diluted in maleic acid buffer and $H_2O$, mixed and absorbance read at 670 nm as above.

To correct for any residual particulate matter in derivatised slurry supernatants and to correct for possible absorbance by faecal pigments, absorbance of derivatised faecal slurries or dialysates was corrected using faecal or dialysate blanks, treated similarly except for the replacement of reagent solutions (DPD and $FeCl_3$) by an equal volume of the carrier solvent (9 M sulphuric acid). This was necessary due to the reported change in absorbance when water only is used (Strocchi 1992). Sulphide standards and blanks (1.0 mM) were freshly prepared using the same derivatisation protocol. Measurement of within run recovery of a sulphide spiked stool sample to correct for variable recoveries as suggested by Strocchi et al (1992) was not used .Stool slurry or dialysate sulphide concentration was calculated according to the formula

$$C = D.F.[A_1-A_0-B]$$

where C = concentration of stool slurry / dialysate sulphide in mmol/L

D = 1/slurry dilution calculated from container weights

F = Factor derived from corrected absorbance of 1 mM sulphide standard (1/absorbance)

$A_1$ = Absorbance of derivatised faecal slurry

$A_0$ = Zero standard absorbance

B = faecal blank absorbance.

Stool sulphide concentration was then calculated by reference to the weight : weight dilution factor obtained for each slurry and expressed as µmol/gm wet weight stool sampled. Dialysate concentrations were expressed as determined directly on the dialysate sample and represent free stool sulphide in mmol per litre stool water.

### 5.2.3.4 Validation Experiments

Linearity of the assay described was determined in the following manner. A 4.0 mM NaHS standard solution made up in distilled water was serially diluted in $H_2O$, normal saline or Dextran 40 to produce sulphide concentrations between 0 and 2.0 mM in 0.25 mM increments. These were derivatised in triplicate and absorbances read at 670 nm using RPHPLC method as above. Linearity for direct spectrophotometric assay for sulphide was similarly assessed using the same range of NaHS concentrations. Sensitivity of the assays was assessed by serial decimal dilutions of a 10 mM NaHS standard solution to 1.0 nM. Derivatised samples were analysed by both RPHPLC and direct spectrophotometric methods.

Within run and between run variability was measured on sulphide standards and samples. Within run variability of sulphide standard was performed by measuring corrected absorbance (direct spectrophotometric method) and area under curve (RPHPLC method) on 6 derivatised 1.0 mM samples, freshly prepared. Between run variability for standard solutions was assessed by comparing the standards obtained on each occasion a patient sample was assayed. Within run variability using the direct spectrophotometric method for stool samples was measured using three freshly passed stool samples. NaOH and zinc acetate slurries were created for each sample and six replicates of each sample derivatised as appropriate for each slurry type. Corrected absorbances were used to calculate the coefficient of variation. Within run variability of RPHPLC measurement was performed on three derivatised dialysate samples. Recovery of sulphide from spiked samples was performed on NaOH and zinc acetate slurries. Fresh faeces was diluted approximately 1:4 with $H_2O$. Three mL of diluted sample was spiked with 300 µL 10 mM NaHS in duplicate. 0.5 mL of the spiked sample was diluted in 4.5 mL 0.1 mL zinc acetate and acid labile (total) sulphide assayed by the direct spectrophotometric method as above. Absorbances of faecal sample (without spike) and the "spike" were measured by similar treatment replacing the spike and sample volumes respectively, with water. As the recovery of free sulphide is known to be low when the spike is added directly to the faecal sample due to avid binding to

faecal cationic species (Florin 1991a), recovery of free sulphide was assessed by adding the 300 μL spike to the NaOH slurry rather than the faecal sample. In view of the findings of Florin (1991a) suggesting that optimum temperature for the methylene blue reaction in faeces was 35°C, recovery experiments were conducted at 21°C and 37°C.

To determine that sulphide was dialysable across Visking tubing and to determine the time required to ensure that recovery was optimised for *in vivo* dialysis, *in vitro* experiments were performed. One hundred mL 2mM NaHS made up in 0.9% NaCl was placed in a stoppered 125 mL conical flask containing twelve 3.0 cm dialysis bags containing 0.9% NaCl. These were incubated at 37°C in a shaking water bath for 180 minutes. Every 15 minutes, a 300 μL sample of the dialysis solution and one dialysate bag were removed, derivatised as above in duplicate and absorbance read at 670 nm using the direct spectrophotometric method. Corrected absorbances were compared with that of a 1.0 mM standard solution freshly prepared and the concentration achieved in the dialysate bag (as a percentage of dialysis solution concentration at that time point) was calculated. In separate experiments to assess the effect of potentially interfering substances which are present in stool *in vivo*, the dialysis solution was made up using an approximation of stool water containing per litre, sodium butyrate 30mmol, sodium acetate 30 mmol, ammonium acetate 15 mmol, potassium bicarbonate 30 mmol, magnesium chloride 40 mmol, potassium hydroxide 40 mmol (osmolarity 370 mM, pH titrated to 8.0 with 1N HCl) (Wrong 1965, Cummings 1981). In a further experiment, the saline/sulphide dialysis solution was replaced by a saline faecal slurry (approximately 5% weight : volume) and the time course to 120 minutes estimated in the same manner as described above.

Experiments were performed to assess interferences which may result from medication commonly used in inflammatory bowel disease. A standard 200 μM NaHS solution was prepared in water and diluted with appropriate volumes of either 20 mM 5-aminosalicylic acid (made up in 0.05 N HCl), 2.0 mM

sulphasalazine, 2.0 mM sulphapyridine (both made up in 0.02 N NaOH) or water , the final concentration of each drug reflecting those reported to occur in the colon with therapeutic doses in patients with inflammatory bowel disease (5-ASA 10 mM, sulphapyridine 1.0 mM, salazapyrine 1.0 mM, (Peppercorn 1973, Hawkey 1985)). Because the methylene blue reaction is pH dependent for optimal absorbances to be achieved, the pH of the derivatised solutions was corrected to 0.8–0.9 with 1.0 N HCl in those solutions made up in 0.02 N NaOH. Corrected absorbances of each derivatised drug containing sulphide solution were compared to that of the solution diluted with water only.

### 5.2.4 Measurement of Breath Methane

Duplicate samples of end expiratory breath were collected from each subject. This was collected by steady full expiration into a plastic mouthpiece (internal diameter 6 mm) connected by way of a three way tap to a 60 mL plastic syringe. Expiration began with the three way tap open to atmosphere and the sample was collected by closing the tap to atmosphere and syringe aspiration simultaneous with end expiration. That a satisfactory end expiratory specimen had been collected was confirmed on all samples by demonstrating sample $O_2$ concentrations below ambient room air collected simultaneously using a Gow-Mack gas chromatograph (Bridgewater N.J.). Breath methane was measured using a Quintron DP Microlyzer (Milwaukee, Wisconsin) gas chromatograph calibrated using room air as a zero standard and a commercially provided 21 ppm methane in $N_2$ standard. The sensitivity of this apparatus is $\pm$ 2 ppm and so a reading of 2 ppm (above room air) was taken as evidence of the presence of methane in breath.

### 5.2.5 Stool Sulphate

#### 5.2.5.1 Assay Procedure

Faecal sulphate was measured using the method described by Florin (1991b)with modification. The same zinc acetate slurry as was used for the measurement of total sulphide in stool was use for sulphate assays as it was shown that stool sulphate (as measured below) was not influenced by the presence of zinc acetate. NaOH slurries

had higher free sulphate levels consistent with a degree of alkaline hydrolysis of sulphate ester linkages (Turvey 1965). For estimation of free faecal sulphate, an aliquot of stool slurry was centrifuged (12000g, 5 min) and 500 µL supernatant was deproteinated with an equal volume of acetonitrile. After 20 minutes on ice, the sample was centrifuged (12000 g, 5 minutes) and 500 µL supernatant diluted in an equal volume of mobile phase diluent (1.2/1.2 mM $Na_2CO_3$/$NaHCO_3$) ready for injection (100 µL) onto the column. For measurement of total sulphate, it was necessary to free bound sulphate in stool. This was achieved by acid hydrolysis (O-, P-, N- but not C- esters, Turvey 1965) as described by Florin (1991b) using a 1:1 dilution of zinc acetate slurry with 0.8M HCl in duplicate. These dilutions were heated overnight in 6 mL pyrex tubes with a gas tight screw top at 80°C. Preliminary experiments had shown that recovery of sulphate was not different between 4 and 24 hours on the heating block. The ensuing solution was centrifuged (12000 g, 5 minutes), neutralised with 1.0M NaOH (in a 2 : 1) dilution, and recentrifuged. The supernatant was then diluted 1 : 1 with mobile phase diluent as above and 50 µL injected onto the column. Sulphate standards (100 µM for free sulphate and 500 µM for total sulphate) were prepared freshly each day and diluted in the manner described. The assay was carried out using a Waters HPLC system incorporating the previously described pump and auto sampling hardware and analytic software. The analytic column was an IC-Pak™ anion HC column (150 × 4.6 mm, 10 µm polymethacrylate particle size, Waters Australia, Sydney) with the column heater set at 35°C.A pre column IC-Pak Anion Guard-Pak™ (Millipore Corp. Milford, MA.) guard column was used. An in line, post column, solid phase chemical suppression cartridge (Alltech Associates Inc, Deerfield, Illinois) was used to increase signal to noise ratio. This was changed following every 8 samples injected. Detection was by a Waters 430 conductivity detector with temperature control. The mobile phase used was 1.4 mM $Na_2CO_3$ / 1.4 mM $NaHCO_3$, run at 2 mL/minute. Background conductivity for the conditions described was 16–20 µS. Retention time for a freshly prepared sulphate standard was 20 minutes.

#### 5.2.5.1 Validation experiments

Linearity of the assay as described was assessed by serial dilution of a 0.5 mM and 2.0 mM $NaSO_4$ standard and plotting observed peak area against expected concentration. Recovery of free sulphate was assessed by the addition of two $Na_2SO_4$ spikes to duplicates of three zinc acetate slurries in an amount to produce a final spike concentration of 150 and 800 μM. Total sulphate recovery was assessed by the addition of a dextran sulphate spike to each of three slurries to a final spike concentration of 500 μM. Within run variability was measured by assaying a 100 μM $Na_2SO_4$ standard and three faecal samples in sextuplicate. Between run variability was measured on standards only, comparing the standards for free and total sulphate for each run performed (n = 5).

### 5.2.6 Statistical Analysis

Statistical analysis was performed using Instat 2 software (Graphpad, San Diego, California). Parametric data were analysed using the unpaired Student's t test or two way ANOVA. Homoscedacicity was assessed using Bartlett's test and data found not to have comparable variance was transformed (logY or 1/Y). If heteroscedacity persisted, a non parametric test was used (Mann-Whitney U test or Kruskal-Wallis test, as appropriate). Data is expressed as mean (SEM). Significance was set at the conventional 0.05 level.

## 5.3 Results

### 5.3.1 Patients Studied

Fifteen control and nineteen patients with ulcerative colitis were enrolled for study. The mean age of controls was 47.1 years and that of colitics 56.5 years ($p = 0.15$, unpaired t test). Patient details are given in Tables 5.1 and 5.2. The prevalence of smokers in the control group was not significantly greater than in patients with colitis (control 5/15, colitis group 3/19; $p = 0.42$, Fishers exact test). The median duration of symptoms for patients with colitis was 48 months. Details of disease extent and activity are given in Table 5.3. Six patients were not taking salicylate therapy for their colitis. Only one patient had disease severe enough to warrant

surgical intervention within six months of being studied. In one of the fifteen control patients, no zinc acetate slurry was made. Of the nineteen colitic patients, one refused rectal dialysis, one was unable to pass stool following rectal dialysis and would not allow collection of a sample by other means and in two other patients, no zinc acetate slurry was created. This left 33 dialysates (15 control, 18 colitic), 33 NaOH slurries (15 control, 18 colitic) and 30 zinc acetate slurries (14 control, 16 colitic) for study. Duplicate breath methane samples were collected in all 34 subjects studied.

### 5.3.2 Validation Experiments

#### 5.3.2.1 Sulphide

Both the direct spectrophotometric assay and RPHPLC assays were shown to be linear over the range 0 - 2.0 mM (direct method r = 0.99, RPHPLC method r = 0.99, Figures 5.1, 5.2) although there was a suggestion of non linearity at the 2.0 mM end of the direct method curve. Sensitivity of the direct spectrophotometric method was shown down to 10 μM level for the direct spectrophotometric method and to 1 μM level with the RPHPLC method using a 50 μL sample injection rather than the usual 20 μL sample. Within run variability was minimal. The within run coefficient of variation (c.v.) using the direct spectrophotometric method for 1 mM sulphide standard was 3% and 1.5% for the RPHPLC method.. The mean within run c.v. of three NaOH faecal slurries analysed in sextuplicate by direct spectrophotometric means was 8.7% (range 6–12%) and for Zn acetate slurries 12% (range 8–15%). The within run c.v for dialysates measured by RPHPLC averaged 3.9 % (range 2.3–5.9). Between run c.v for 1.0 mM sulphide standard was 7.7% (n = 34 observations) and 10.6 % for stool slurries (n = 6). Mean recovery of a sulphide spike added to three stool samples subsequently made into zinc acetate slurries and assayed by direct spectrophotometric method was 89.9% (75.5, 90.8, 103.5%) and that of a spike added to three NaOH stool slurries 83% (87.2, 78, 83%). The mean recovery achieved from three separate NaOH slurries derivatised at 21°C was similar (86.2%) but at 37°C this fell to 73.4%. All assays were subsequently carried out at

room temperature. None of 5-ASA, salazapyrine or sulfapyridine were found to have significant absorbances in the range 600–730 nm. Standard 0.5 mM (final concentration ) sulphide standards made up in the presence of salazapyrine, 5-ASA or sulfapyridine as described, yielded significantly different absorbances, but when pH was corrected to similar levels (pH = 1.0) no significant difference in absorbance was demonstrable.

Assessment of the dialysis of sulphide across Visking tubing showed little difference in the time course observed irrespective of whether the dialysis solution was sulphide in water, "artificial stool water" or a dilute aqueous faecal slurry (Figures 5.3-5). Dialysis could be shown to have reached equilibrium at approximately 90 minutes at a value approximately 90% of the bathing solution. A dialysis time of 100 minutes was therefore chosen for *in vivo* dialysate experiments.

#### 5.3.2.2 Sulphate

The assay as described was found to be linear over the range 0–500 μM and 0–2.0 mM for free and total methodologies respectively (free assay, r =0.998, total assay r = 0.997). Recovery of a 150 μM spike was 99%, 94% and 103% to three zinc acetate stool slurries and that of a 800 μM spike was 78%, 107% and 103%. Recovery of a spike added to three faecal slurries as dextran sulphate (for estimation of total sulphate)was 118%, 104% and 104%. The between run coefficient of variation for 100 μM $Na_2SO_4$ standards treated as for free sulphide estimation was 4.8% and that for 500 for μM standards treated as for total sulphate estimation 3.6%. The within run coefficient of variation for standards averaged 1.4%, for free sulphate on stool slurries 13.4 % and for total sulphate 5.1%.

### 5.3.3 Stool sulphide

Free stool sulphide was estimated by both direct spectrophotometric and RPHPLC methodologies using stool dialysates. There was a highly significant correlation between values obtained by these two methods ($r^2 = 0.89$, $p < 0.0001$). There was a similar although less strong correlation between values obtained comparing stool

slurry and dialysate when measured by the direct spectrophotometric assay ($r^2 = 0.68$, $p < 0.001$). All values subsequently reported are those obtained by the direct spectrophotometric assay, unless otherwise indicated.

Mean free stool sulphide, measured on stool dialysis and directly on stool slurries, and total sulphide did not differ significantly between control and colitic subjects (Table 5.4). Disease extent did not influence observed free stool sulphide levels as measured on either dialysate or stool slurry (Table 5.5). Although there was a trend to higher total stool sulphide levels in control patients, this was not statistically significant ($F = 2.35$, $p = 0.11$, one way ANOVA). There was no discernible influence of disease activity on free stool sulphide measured on stool slurries (control = 0.52 µmol/gm wet weight stool (SEM 0.16), inactive colitis = 0.41 (0.10), active colitis = 0.54 (0.22); $F = 0.18$, $p = 0.83$, one way ANOVA, Table 5.6). Again, although there was a trend to higher total stool sulphide in control patients this was not significant (control = 1.33 µmol/gm wet weight stool (0.21), inactive colitis = 1.03 (0.17), active colitis 0.80 (0.33) ; $F = 1.19$, $p = 0.32$). The level of free stool sulphide (as measured on NaOH stool slurries) in colitic patients who were taking salicylate medication was not significantly different to either control patients or colitic patients not on salicylates (Figure 5.6). Although the number of zinc acetate slurries from patients not on salicylates was too small ($n = 5$) to allow appropriate statistical analysis of differences in total sulphide concentrations, none was apparent. The ratio of free : total sulphide did not differ between control and colitic stool slurries (control 0.32 (0.07), colitic 0.43 (0.08), $t = 1.03$, $p = 0.31$).

### 5.3.4 Stool Sulphate

Zinc acetate stool slurries from 12 control and 15 colitic subjects were able to be studied. The mean (SEM) free sulphate observed in colitic patients was 1.58 (0.39) µmol/gm wet weight stool, a value which did not differ significantly from that observed in control subjects (= 1.8 µmol/gm wet weight stool (0.44); $t = 0.43$, $p = 0.67$, Figure 5.8). The level of free stool sulphate was not influenced by disease

activity (F = 0.39, p = 0.68, one way ANOVA, Table 5.7), or by disease extent (F = 1.27, p = 0.30) (Table 5.8). Bound sulphate did not differ between groups (mean (SEM) control = 2.91 µmol/gm wet weight (0.38), colitis = 2.55 (0.40); t = 0.63, p = 0.53, Figure 5.9) and again disease extent and activity had no influence on observed stool sulphate levels (Tables 5.7, 5.8). The mean ratio of free to bound sulphate did not differ between control and colitic subjects (control = 0.81 (0.21), colitis = 0.77 (0.21) ; U = 41, p = 0.84, Mann Whitney U test). There was no correlation between free stool sulphate and the level of free stool sulphide as determined by direct measurement from stool slurries (Spearman correlation coefficient r = 0.171, 95% CI -0.506 - 0.209, p = 0.38).

### 5.3.5 Breath Methane and Stool Sulphide

Overall, methane was detectable above room air in 32 % of subjects studied (5/15 controls, 6/13 colitics; p = 1.0, Fisher's exact test). The mean free stool sulphide in methanogenic individuals was 0.40 (0.13) µmol/gm wet weight. It was higher in nonmethanogenic individuals (0.52 (0.12)) but this was not significantly different (t = 0.59. p = 0.56, unpaired t test, Figure 5.10). This observation was also true for total sulphide (methanogenic individuals = 1.06 µmol/gm wet weight (0.17), nonmethanogenic individuals = 1.17 (0.18) ; t = 0.37, p = 0.72).

**Table 5.1** Patient details – Controls.

| Patient No. | Past History | Medication | Smoking* |
|---|---|---|---|
| **1** | nil | nil | 20 |
| **2** | nil | nil | 0 |
| **3** | Hypertension, ischaemic heart disease | Verapamil, Isordil | 0 |
| **4** | nil | nil | 20 |
| **5** | Hypertension | Adalat, Indepamide, Capoten | 0 |
| **6** | nil | nil | 0 |
| **7** | nil | nil | 20 |
| **8** | Cholecystectomy | nil | 0 |
| **9** | nil | nil | 0 |
| **10** | gastro-oesophageal reflux | nil | 0 |
| **11** | Ca pancreas (Whipples procedure 1982) | Zantac, Pancrease | 0 |
| **12** | nil | nil | 25 |
| **13** | Hypertension, ischaemic heart disease | Atenolol, Isordil | 0 |
| **14** | nil | nil | 0 |
| **15** | nil | nil | 15 |

* no. cigarettes per day.

**Table 5.2** Patient details – Ulcerative colitis patients.

| Patient No. | Past History | Medication | Smoking* | Disease duration (months) |
|---|---|---|---|---|
| 1 | nil | Stemetil | 10 | 6 |
| 2 | nil | SZP | 0 | 24 |
| 3 | nil | SZP, pred. | 0 | 24 |
| 4 | nil | nil | 0 | 120 |
| 5 | cardiac failure, gastro oesophageal reflux | SZP, spironolactone, frusemide, felodepine, doxepin | 0 | 148 |
| 6 | gastro-oesophageal reflux, ischaemic heart disease | SZP, Isoptin, Zantac, Cartia, Isordil | 0 | 60 |
| 7 | nil | Pred. | 0 | 2 |
| 8 | nil | SZP | 0 | 15 |
| 9 | Epilepsy, depression | Carbemazepin, Melleril, Ethosuximide | 0 | 6 |
| 10 | NIDDM | 5ASA, pred., glicazide, ketoprofen | 0 | 1 |
| 11 | nil | hydrocortisone (IV) | 0 | 24 |
| 12 | Ca prostate | SZP, Zoloft | 10 | 86 |
| 13 | Cardiac failure | SZP, frusemide, spironolactone, voltaren | 0 | 180 |
| 14 | nil | pred., SZP, AZT | 0 | 96 |
| 15 | NIDDM | SZP, Diamicron, Amphomax | 0 | 48 |
| 16 | IDDM | insulin, SZP | 10 | 108 |
| 17 | Gout | Allopurinol, SZP | 0 | 180 |
| 18 | Ca stomach (gastrectomy 1980), | SZP, pred., Renitec | 0 | 72 |
| 19 | Hypertension | pred., Visken, Calctriol | 0 | 6 |

* no. cigarettes per day, pred. = oral prednisolone, AZT = azathioprine, SZP = oral salazopyrine

**Table 5.3.** Disease activity and extent (ulcerative colitis patients).

| | **Active disease** | **Inactive disease** | **Total** |
|---|---|---|---|
| **Limited Disease** | 3 | 8 | 11 |
| **Pancolitis** | 3 | 5 | 8 |
| **Total** | 6 | 13 | 19 |

**Figure 5.1**. Linearity of direct spectrophotometric sulphide assay using NaHS standard solutions in the range 0 - 2.0 mM. $r = 0.99$, $p < 0.001$, Spearman correlation)

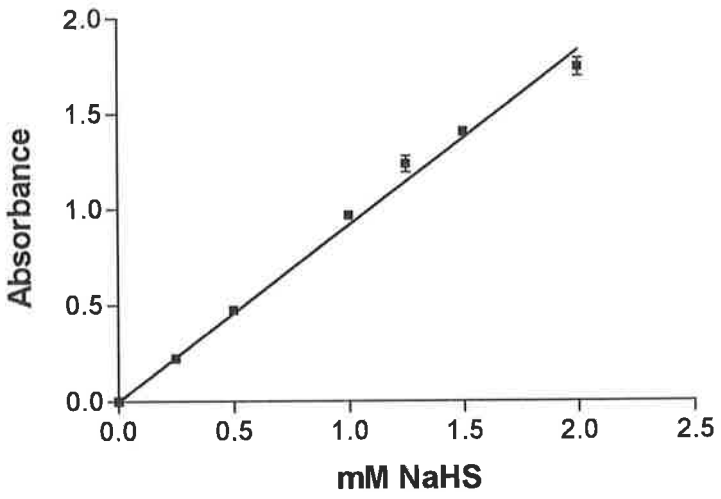


**Figure 5.2.** Linearity of RPHPLC sulphide assay using NaHS standard solutions in the range 0 - 2.0 mM. ($r = 0.99$, $p < 0.001$, Spearman correlation)

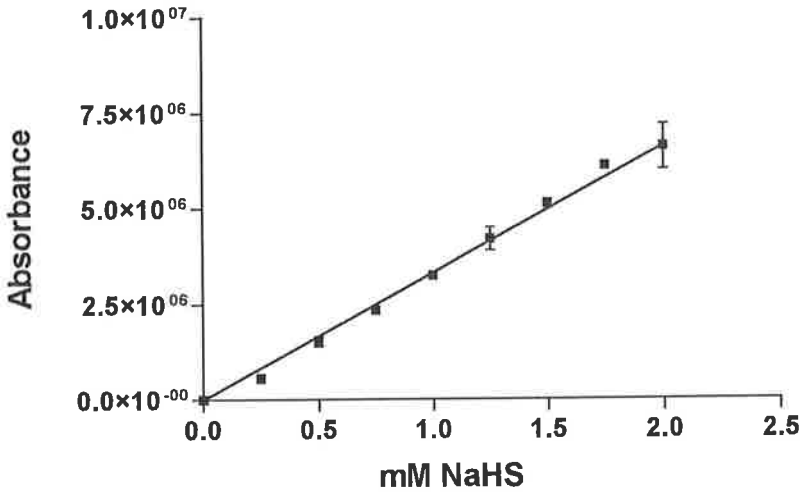



**Figure 5.3.** Time course of sulphide dialysis across Visking tubing. Dialysate was 150 mM NaCl containing (at $t = 0$) 2.0 mM NaHS. Each point represents the mean (S.E.M.) of three experiments. $Y_{max} = 86.7\%$ (one phase exponential association).

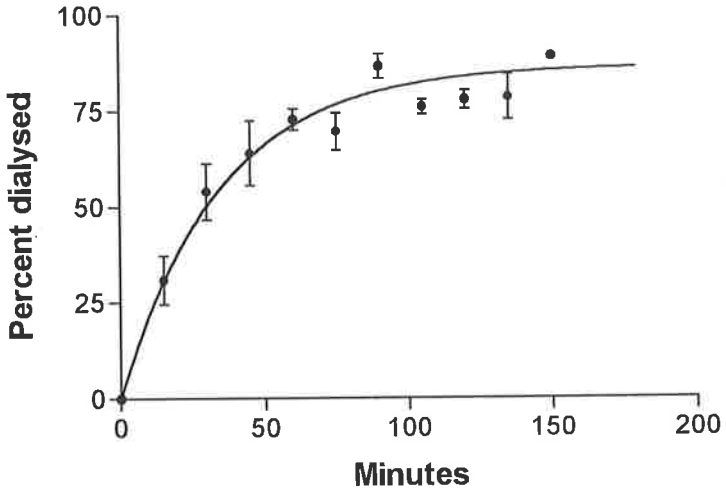


**Figure 5.4** Time course of sulphide dialysis across Visking tubing. Dialysate contained NaHS 2.0 mM (at t = 0), sodium butyrate 30 mM, sodium acetate 30 mM, ammonium acetate 15 mM, potassium bicarbonate 30 mM, magnesium chloride 40 mM, potassium hydroxide 40 mM (osmolarity 370 mM, pH titrated to 8.0 with 1N HCl). Each point represents the mean (S.E.M.) of three experiments. $Y_{max}$ = 92.1% (one phase exponential association).

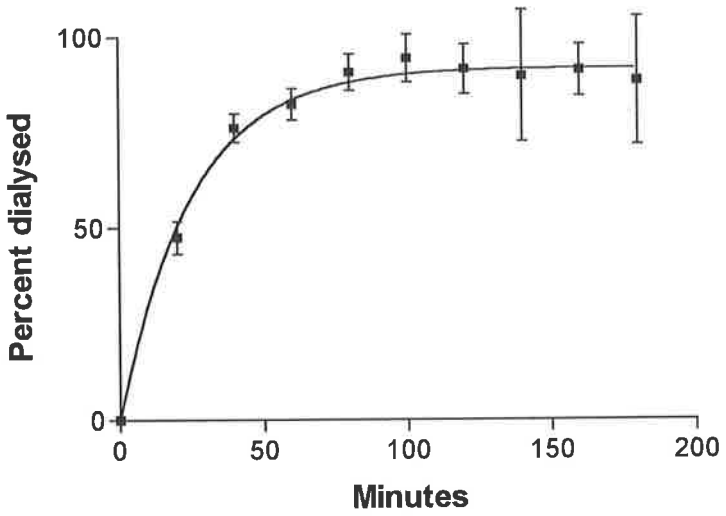


**Figure 5.5** Time course of sulphide dialysis across Visking tubing. Dialysis solution comprised freshly passed faeces made to a 1:20 slurry with 150 mM NaCl. Each point represents the mean (S.E.M.) of three experiments. $Y_{max} = 0.87$ (one phase exponential association).

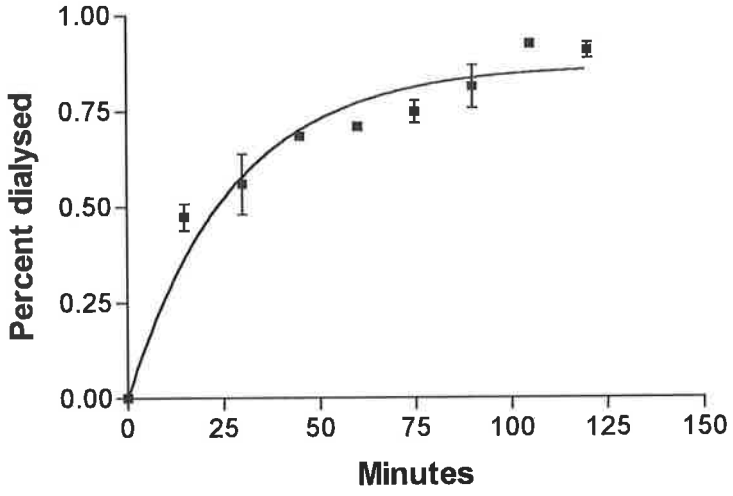


**Table 5.4**. Stool sulphide levels in control subjects vs all colitic subjects. Values are mean (SEM) for numbers given in each group. There is no significant difference between control and colitic subjects for dialysate, free stool sulphide or total stool sulphide (Students t test).

| | **Control** | **Ulcerative Colitis** |
|---|---|---|
| Free sulphide - Dialysate (mmol/L dialysable stool $H_2O$) | 0.049 (0.011) n = 15 | 0.049 (0.009) n = 18 |
| Free sulphide - NaOH stool slurry (μmol/gm wet wt stool) | 0.52 (0.17) n = 15 | 0.45 (0.10) n = 18 |
| Total sulphide (μmol/gm wet wt stool) | 1.33 (0.21) n = 14 | 0.96 (0.15) n = 16 |

**Table 5.5** Stool sulphide by Disease Extent. Values are mean (SEM) for number of observations indicated for each group.

| | **Control** | **Limited Colitis** | **Pancolitis** |
|---|---|---|---|
| Free sulphide - Dialysate (mmol/L dialysable stool $H_2O$) | 0.049 (0.011) n = 15 | 0.054 (0.045) n = 11 | 0.043 (0.012) n=7 |
| Free sulphide - NaOH stool slurry (µmol/gm wet wt stool) | 0.52 (0.17) n = 15 | 0.64 (0.14) n = 10 | 0.22 (0.07) n = 8 |
| Total sulphide (µmol/gm wet wt stool | 1.33 (0.21) n = 14 | 1.23 (0.19) n = 8 | 0.69 (0.21) n = 8 |

**Table 5.6** Stool sulphide by disease activity. Values are mean (SEM) for number of observations indicated for each group.

| | **Control** | **Inactive Colitis** | **Active Colitis** |
|---|---|---|---|
| Free sulphide - Dialysate (mmol/L dialysable stool $H_2O$) | 0.049 (0.011) n = 15 | 0.036 (0.005) n = 12 | 0.077 (0.02) n = 6 |
| Free sulphide - NaOH stool slurry (µmol/gm wet wt stool) | 0.52 (0.17) n = 15 | 0.40 (0.10) n = 12 | 0.54 (0.23) n = 6 |
| Total sulphide (µmol/gm wet wt stool | 1.33 (0.21) n = 14 | 1.03 (0.17) n = 11 | 0.80 (0.33) n = 5 |

**Figure 5.6.** Effect of salicylate therapy on free stool sulphide (measured by direct spectrophotometric method). ASA+ = patients on salicylates, ASA- = patients not on salicylates.

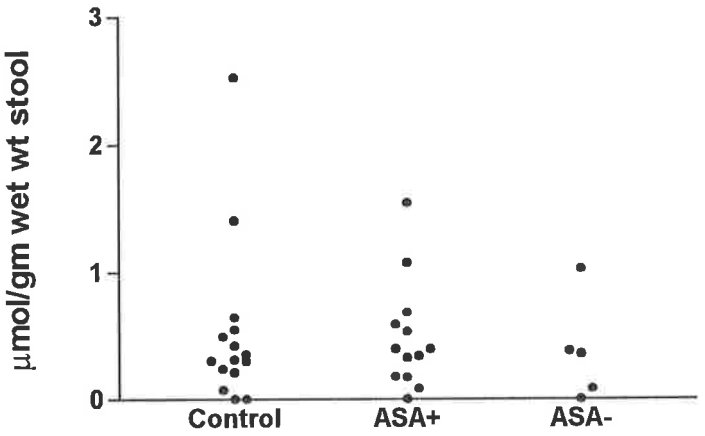



**Figure 5.7.** Effect of salicylate therapy on total stool sulphide (measured by direct spectrophotometric method). ASA+ = patients on salicylates, ASA- = patients not on salicylates.

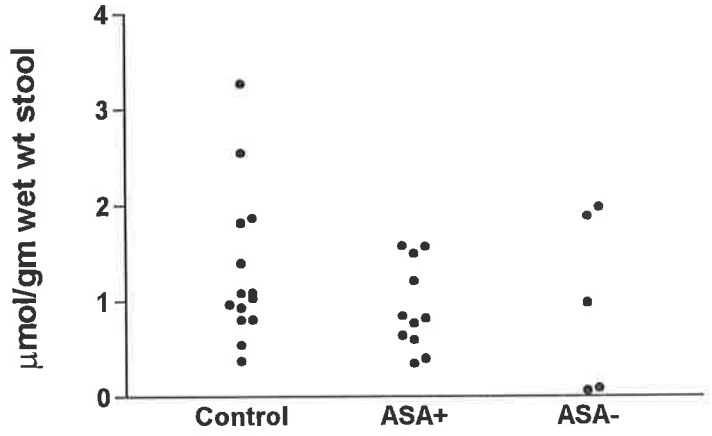


**Figure 5.8** Free stool sulphate.

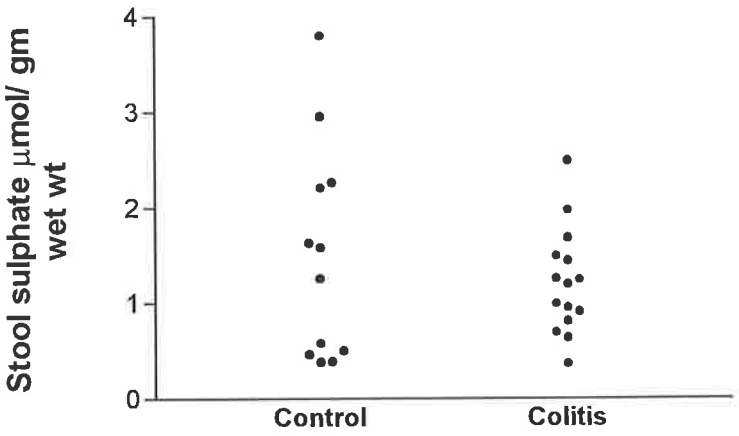


**Table 5.7** Stool Sulphate by Disease Activity. Values are mean (SEM) in µmol/gm wet weight stool for the number of observations indicated.

| | Control (n = 12) | Inactive Colitis (n =10) | Active Colitis (n = 5) |
|---|---|---|---|
| Free Sulphate | 1.50 (0.32) | 1.24 (0.17) | 1.16 (0.29) |
| Bound Sulphate | 2.91 (0.38) | 2.82 (0.56) | 2.01 (0.41) |

**Table 5.8** Stool Sulphate by Disease Extent. Values are mean (SEM) in µmol/gm wet weight stool for the number of observations indicated.

| | Control (n = 12) | Limited Colitis (n = 8) | Pancolitis (n = 7) |
|---|---|---|---|
| Free Sulphate | 1.50 (0.32) | 0.99 (0.13) | 1.46 (0.24) |
| Bound Sulphate | 2.91 (0.38) | 1.94 (0.53) | 3.25 (0.54) |

**Figure 5.9.** Bound stool sulphate.

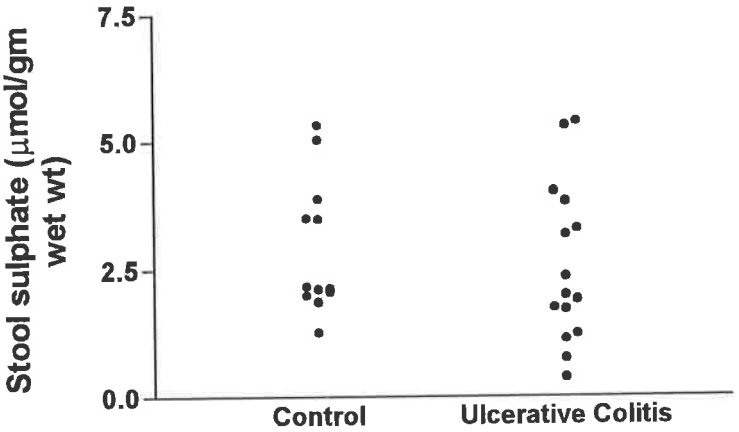
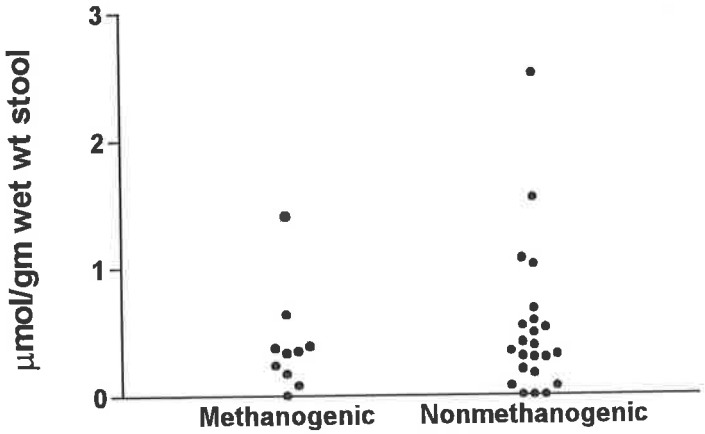



**Figure 5.10**. Free stool. sulphide (direct spectrophotometric method, NaOH slurries) in methanogenic and non methanogenic individuals. (Unpaired Students t test, t = 0.59, p = 0.56.)



## 5.4 Discussion

That hydrogen sulphide may be aetiologically important in ulcerative colitis is an attractive hypothesis providing as it may do, possible reasons for several features of colitis hitherto unexplained. These include the distal origin of the disease, the associated reduction in butyrate oxidation in both active and quiescent disease, its association with non smoking and its tendency to remission and relapse. Central to the validity of such a hypothesis however is the demonstration of a significant difference between control and colitic patients with respect to either lumenal levels of sulphide or a difference in the mucosal detoxification of sulphide. Such a difference has been suggested in the past and indeed an association between disease activity but not disease extent has been reported (Florin 1990). Similarly, Gibson (1991) reported higher rates of sulphidogenesis in colitic stool compared to control stool in vitro. The results reported now are at variance with this suggestion however, with no significant difference found in lumenal sulphide levels found between healthy control patients and patients with ulcerative colitis.

It has been reported that salicylates reduce sulphidogenesis *in vitro* (Pitcher 1995c) and this has been suggested as a possible mode of therapeutic action for salicylates. As these drugs are the only agents which favourably influence the incidence of disease relapse, the vast majority of patients with ulcerative colitis use them. This may have the confounding influence of reducing sulphide levels in colitic patients and masking any difference between the groups. Such a bias might be expected particularly in a group of quiescent colitics who are more likely to be prescribed salicylates than patients with very active disease because of oral intolerance in the latter group. Indeed it has recently been reported that stool acid labile (total) sulphide levels are highest in patients with active colitis who were not receiving salicylates (Pitcher 1995c). In that study, stool sulphide was not elevated in colitics that were taking salicylates compared to control patients. Despite the *in vitro* work noted above, no differences in lumenal sulphide, either free or total, were found between patients on salicylates and those not taking these compounds in this study. The numbers of patients not taking salicylate was small (n = 6) and of these only

two patients had active disease, reflecting the difficulty in recruiting patients not taking these medications. It was not considered appropriate or ethical to ask patients to withhold salicylates prior to study.

Several methodological factors require consideration. The methodology used in this study for sulphide assay is similar to that used by Florin (1991a) and Strocchi (1992) using both reagent and faecal blanks to overcome turbidity associate with faecal samples. RPHPLC methodology should overcome this problem but we have found that total sulphide measurements taken from the same stool sample consistently underestimated the value obtained for free sulphide (data not shown). A further refinement to potentially overcome the problem of turbidity has been the use of stool dialysis. The absorbance of stool dialysate faecal blanks was always significantly lower than the corresponding NaOH faecal blanks and usually not much greater than the reagent blank. Given the significant correlation between dialysate and NaOH stool slurry sulphide values, it would seem that dialysis is an effective method of obtaining, *in vivo*, a sample which accurately reflects stool water in terms of sulphide concentration. Absolute values are not the same however as dialysis obtains a value related to dialysable stool water rather than weight of stool assayed. Dialysis has the virtue of providing a sample which can be immediately derivatised, minimising the problem of sulphide oxidation and volatility. The finding of a highly significant correlation between RPHPLC and direct spectrophotometric values for dialysate derived stool sulphide supports the validity of stool dialysis as a method for sampling free stool sulphide. However, dialysis would seem to introduce a further reduction in free stool sulphide measured given the incomplete (approximately 90%) recovery at 100 minutes. This may relate to sulphide adsorbance to the cellulose tubing used, an issue not presently assessed.

Recovery from NaOH faecal slurries was not as variable as that observed with zinc acetate slurries. Strocchi et al (1992) have reported the incorporation of a within run correction for variable recovery. For the purposes of this study, it was chosen

not to include this modification; rather it was preferred to report the values obtained without correction for recovery and provide data regarding recovery variability from which the reader may draw their own conclusions.

Based on the evidence obtained in this study, it would appear that the null hypothesis is upheld, ie that there is no significant difference in the level of lumenal sulphide between control patients and patients with ulcerative colitis. Care should, however, be taken before accepting these findings. Gibson et al (1991) suggested that stool sulphide was influenced by stool dilution, i.e. that the diarrhoea of ulcerative colitis selected out SRB and so elevated stool sulphide. This study contains a preponderance of patients with inactive colitis without diarrhoea and includes only six patients with active disease, all of whom had stool frequencies of greater than 6 per day. It may be that the population studied has influenced these results in favour of quiescent disease which according to Florin (1990) would have lower sulphide levels. It might further be argued that if the absolute lumenal sulphide level has a pathogenic role in colitis, a difference might be expected to exist between control and quiescent colitics, given the observation that the metabolic abnormality is seen in quiescent colitics (Roediger 1980, Chapman 1994). The observation of no difference in lumenal sulphide levels between control and quiescent colitic subjects provides further support for the null hypothesis. Alternatively, elevated lumenal sulphide in ulcerative colitis may be an epiphenomenon related to sulphur amino acid fermentation consequent upon active disease and increased lumenal protein content as suggested by Florin (1995). Duncan et al (1990) have found no differences in mercapto fatty acid production in anaerobic batch culture of control and colitic stool using cysteine as a sulphur source, although protein content of the stool slurries was not directly assessed.

The mean levels of free sulphide in stool slurries reported for control patients in this study (0.52 µmol/gm wet weight stool) is approximately thrice the mean levels previously reported (Florin 1991a) (0.17 µmol/gm wet weight stool) using similar stool collection and assay methodologies. Strocchi (1992) did not measure free

sulphide but reported a mean total sulphide concentration for eight healthy volunteers (4 methanogenic an 4 nonmethanogenic) of 1.6 μmol/gm wet weight stool, a value that closely compares to that found in this study. In contrast, the value for total (acid labile) sulphide reported by Florin was only 0.66 μmol/gm wet weight stool, again in a group with equal numbers of methanogenic an non methanogenic subjects.

On the basis of in vitro incubation of stool homogenates with an exogenous sulphur source (5 mM cysteine), Tangerman (1994) has suggested that the amount of sulphide that may be found in bound form in stool is limited to approximately 2 μmol/gm wet weight stool. Above this level free sulphide might be expected to increase due to saturation of available sulphide "chelators" such as $Zn^{++}$ and $Cu^{++}$. It could be hypothesised that colitic patients have a primary reduction in this capacity to "buffer" free sulphide. If this was so, episodic increases in lumenal sulphide (perhaps induced by increased lumenal sulphate availability, changes in stool pH or increased stool dilution for whatever reason) may be manifested by a significantly greater increase in the free (and presumedly physiologically active) sulphide fraction than in control subjects. The finding of this study of no difference in the ratio of free to total sulphide between control and colitic patients would not support such a hypothesis.

Several in vitro studies have suggested that methanogenesis and sulphate reduction / sulphidogenesis are to some extent mutually exclusive events in the colonic lumen (Gibson 1988a,b, MacFarlane1992, Strocchi1991, 1994) although it is disputed as to which mechanism is the predominant hydrogen sink. If such a dichotomy were to exist in vivo, it might be expected that lumenal sulphide levels in non methanogenic individuals would be significantly higher than in methanogenic subjects. The finding of no significant difference in either free or total stool sulphide between methanogenic and nonmethanogenic subjects in this study provides evidence that such a dichotomy may not exist in vivo. Similar findings have been reported by Florin (1991a) and Strocchi (1992). Importantly, however, it should be noted that

in neither this study nor those quoted above were the presence of viable SRB in nonmethanogenic subjects measured. Furthermore, the variability of methanogenic and SRB counts within different segments of the colon (MacFarlane 1992) means that observations made on rectal faecal specimens and breath methane may not accurately reflect "local" events within the colonic lumen.

# Chapter 6

# Thiol-Methyltransferase Activity In The Human Colonic Mucosa

## 6.1 Introduction

While there is increasing interest in the levels of stool sulphide both in human ulcerative colitis (Florin 1990, Pitcher 1995a, 1995b) and in animal models of ulcerative colitis (Florin 1995), little attention has been paid to local detoxification mechanisms active against sulphide in the colon. As previously outlined, proposed mechanisms include cationic binding, rhodanese mediated cyano group transfer yielding thiocyanate, oxidation to sulphate and methylation (see 2.6.2–5). The exact importance of each in protecting the colonic mucosa from the adverse effects of sulphide is unknown but thiol-methyltransferase (TMT) mediated methyl group transfer is likely to be an important form of mucosal sulphide detoxification. Such a role was first proposed by Weisiger (1980) and although the prenatal development and distribution of this enzyme in the colon has been documented (Pacifici 1991, 1993), little work has been undertaken to assess its potential role in human colonic disease. Although TMT activity is higher in the colon than the small bowel (Pacifici 1993) and the methylated products methanthiol and dimethylsulphide are significantly less toxic to the colonic mucosa (Roediger 1993b), the ability of TMT to protect the colonic epithelial cell from the toxic effects of mercaptides has not been addressed. It is hypothesised that methyl group transfer mediated by TMT may be important in protecting the colonic mucosa from the adverse effects of lumenal sulphide and that regional differences in TMT activity may exist that may explain the distribution of disease observed in clinical practice. To test this hypothesis, the studies to be described aim to:

1. Assess the effect of sulphide on metabolic performance of human isolated colonic epithelial cells and the effect of the methyl group donor, S-adenosyl methionine (SAM), in ameliorating any observed sulphide effect.
2. To assess regional differences in the above effects and in mucosal TMT activity.

3. To correlate the degree of sulphide inhibition of butyrate oxidation and the degree to which this may be influenced by SAM with mucosal TMT activity.
4. To assess whole mucosal TMT activity as a measure of isolated colonic epithelial cell TMT activity.
5. To assess RBC membrane TMT activity as a marker of mucosal TMT actvity.
6. To correlate stool sulphide and RBC membrane TMT activity.

A paucity of colonic resections for colitis (of any form) during the time that these studies were proposed has meant that a direct examination of the mechanisms of sulphide detoxification in colitic mucosa has not been possible.

## 6.2 Materials and Methods

### 6.2.1 Reagents

Chemicals and reagents for cell incubation studies were identical to those previously outlined (Chapter 3). Unlabelled S-adenosyl-methionine 1,4 butane disulphonate (SAMe) used for incubation studies was a gift from Bioresearch S.P.A. Gruppo BASF, Milan. The radiolabelled methyl donor S-adenosyl-1-[methyl-$^{14}$C]-methionine (S.A. 53 mCi /mmol) was purchased from Amersham International, (Amersham, U.K.), dithiothreitol (DTT) and the model TMT substrate 2-mercaptoethanol were from Sigma (St Louis, Mo.) All solvents (acetonitrile, methanol, chloroform) were of HPLC grade (B.D.H., Poole, U.K.). Tris HCl (Sigma, St Louis, Mo.), pH 7.4, was made to 5.0 mM with ultrapure water. Standard working solutions were prepared in the following concentrations using 5 mM Tris HCl : methyl mercaptoethanol 1.0 mM, mercaptoethanol 60 mM (both from Sigma, St Louis, Mo.). Unlabelled S-adenosyl methionine (SAM, 324 μM for TMT assay was made fresh for each run. The working hot SAM solution comprised 4 volumes unlabelled 324 μM SAM and one volume S-adenosyl-1-[methyl-$^{14}$C]-methionine.

### 6.2.2 Experimental Protocol

#### 6.2.2.1 Incubation Studies

All studies were performed on tissues obtained from patients undergoing colectomy for carcinoma of the colon or diverticular disease at The Queen Elizabeth Hospital. All patients had undergone a standard bowel preparation (4 L polyethelene glycol the day prior). Peripheral venous blood (20 mL) for estimation of erythrocyte TMT activity was drawn from an intravenous cannula prior to induction of anaesthesia and collected in two 10 mL containers containing 125 IU lithium heparin. At surgery, the colon was mobilised in routine fashion but operative technique was altered such that the bowel was divided both proximally and distally prior to division of the vascular pedicle. This allowed the collection of tissue with as little warm ischaemia time as possible. Specimens were classified as proximal if the tissue harvested originated proximal to the middle colic artery and as distal if from beyond the splenic flexure The colon was opened along the antemesenteric border, pinned flat and strips of mucosa taken with flat scissors. Mucosa was harvested from macroscopically normal areas only and never less than 10 cm from a tumour. Harvested mucosal strips were collected into calcium free Krebs-Henseleit solution (Ca free K.H.) containing 0.25% BSA at 37°C for preparation of isolated cell suspension, and 2 mucosal strips, representing approximately 10 mg tissue (wet weight) were collected into a preweighed 1.5 mL Eppendorf tube for estimation of whole mucosal TMT activity. The specimens were then transported to the laboratory, the usual time between division of the vascular pedicle and reaching the laboratory being 7-9 minutes.

Isolated colonocyte suspensions were prepared using the method of Roediger (1979b) with modification. Harvested mucosal strips were washed in Ca free K.H 0.25% BSA to remove adherent blood then incubated in Ca free K.H. containing 0.25% BSA and 10 mM EDTA without hyaluronidase for 20 minutes. The mucosal strips were divided into two portions, one half were incubated in the same solution containing 20mM DTT for 20 minutes and the other half incubated in the same solution without DTT for the same period of time. This omission of DTT was

found to be necessary in the tissue to be used for TMT assay as this compound is a substrate for TMT (Weinshilboum 1979). At the end of 20 minutes the mucosal strips were washed twice in Ca free KH containing 0.25% BSA to remove EDTA and vigorously stirred to disaggregate colonic crypts. The remaining tissue was removed by sieving through a coarse sieve, the suspension spun down (1250 rpm, 2 minutes) and resuspended in ordinary K.H. containing 2.5% BSA. Cell suspensions prepared without the aid of DTT were used for estimation of isolated cell microsomal TMT activity according to the method of Babidge (1995) (described below). Those prepared in the presence of DTT were used for metabolic studies. Whole mucosal specimens were used for estimation of whole mucosal microsomal TMT activity.

Isolated cell incubations to assess the individual degree of sulphide inhibition of butyrate oxidation were carried out as follows. One mL of cell suspension was incubated in duplicate in the presence of 5 mM [1-$^{14}$C] n-butyrate (specific activity approx 19,500 cpm/μmol), with or without the addition of 1.5 mM NaHS and 5.0 mM SAMe. The final incubation volume was 2.0 mL Incubations were carried out at 37°C in an atmosphere of 19:1 $O_2$:$CO_2$ for 40 minutes in rubber stoppered 25 mL conical glass flasks equipped with a glass centre well as previously described (Chapter 3). Incubations were terminated by the addition of 0.5 mL 10% perchloric acid to the incubation mix, and $CO_2$ produced was collected in 0.5 mL 2M NaOH placed in the flask centre well. After shaking on ice for 90 minutes, 100 μL aliquots of the centre well NaOH solution were mixed with 5 mL scintillation cocktail and counted using a liquid scintillation counter (model Delta 300, Searle Analytic, U.K.). $^{14}CO_2$ production (as μmol/min/gm dry weight of cells) was calculated as described previously. Sulphide effect was measured as the percentage drop in $^{14}CO_2$ produced compared to control (butyrate alone) and SAMe effect as the percentage of observed reduction with sulphide restored by SAMe.

Red cell membranes were prepared according to the method of Pazmino (1978) with modification. Briefly, 20 mL peripheral blood was centrifuged (800 g,

10 minutes). The plasma fraction was discarded, replaced by an equivalent volume of normal saline. This was repeated twice, discarding the buffy coat. Six mL of reconstituted blood was then added to 24 mL ice cold ultra pure water to lyse the cells and centrifuged (13,000 g, 10 minutes) in a Beckmann J2MC centrifuge. The supernatant was discarded, the pellet washed thrice with three mL 5.0 mM Tris HCl (pH 7.4), resuspended in 6.0 mL Tris HCl and recentrifuged (13000 g, 6.5 min). The resulting pellet was resuspended in 120 µL Tris and stored at –80°C until assay.

The microsomal fraction of whole mucosal strips were prepared in the following manner. Harvested tissue (approximately 1gm) was added to 5.0 mL ice cold 0.25 M sucrose and minced using a Ystral (Dottingen, West Germany) morcellator bathed in iced water (power setting 10). The minced tissue was filtered through a gauze swab, then homogenised in a glass teflon homogeniser and divided into duplicate aliquots. The homogenate was centrifuged (12000 g 15 minutes, 4°C), the supernatant discarded and recentrifuged (105000 g, 60 minutes, 4°C). This pellet containing the microsomal fraction was resuspended in 200 µL 5 mM Tris HCl buffer and stored at –80°C until assay. Isolated colonic epithelial cell microsomal fractions were prepared in a similar fashion, with the omission of mincing and gauze filtration.

Determination of TMT activity was performed using the modification of the method of Weinshilboum (1979) described by Babidge (1995). This assay relies on the conversion of a model substrate, 2 mercaptoethanol to [$^{14}$C]methyl-mercaptoethanol by TMT using the methyl donor, S-adenosyl-L-[methyl-$^{14}$C]-methionine. All samples were thawed on ice and well mixed prior to assay. Five microlitres of 60 mM 2-mercaptoethanol and 5 µL hot working SAM solution were added to 20 µL of sample and incubated in Eppendorf tubes on a heating block (37°C) for 50 minutes. The reaction was stopped by the addition of 30 µL acetonitrile. After standing on ice for 15 minutes the sample was centrifuged (12000g, 10 minutes) and 20 µL supernatant injected onto the HPLC column. The

analysis was performed using a Waters HPLC system (Millipore Australia, Sydney), comprising a 510 HPLC pump, 715 Ultra Wisp sampler and Baseline 810 software. The analytic column was an Alltech Spherisorb ODS-2 (250 ×4.6 mm, 5 micron particle size) (Alltech Australia, Sydney). Detection was by a Berthold HPLC radioactivity detector (#LB507A) (Bartlett Instruments, Melbourne) with a 1.0 mL admixture cell (Z-1000-4) and T mixer. Simultaneous UV detection of methyl mercaptoethanol using an inline Waters 486 UV detector (Millipore Australia) set at 214 nm was also used for validation purposes. An isocratic mobile phase comprising 20% methanol, 80% ultrapure water was used at a flow rate of 1 mL/minute and the scintillation fluid used was Ultima Flo AP (Canberra Packard) with a scintillation fluid to mobile phase ratio of 2:1. The retention time of $^{14}C$-methylmercaptoethanol detected radioactively was 6.9 minutes and by UV detection 5.9 minutes (due to the 1.0 mL admixture cell). Sample protein concentrations were measured using the modification of the method of Lowry (1951) described by Leggett-Bailey (1967). Under the conditions described, linearity of the assay for both red cell membrane and whole mucosal TMT was assessed by serial 1:1 dilutions of the sample with the highest protein concentration using 5mM Tris HCl. Both RBC and whole mucosal assays showed linearity over a protein concentration 0–5 mg/mL with a small departure from linearity above this level. Samples were accordingly diluted to obtain protein concentrations in this range. It was found that baseline began to rise to twice that seen at the beginning of each run after approximately 20 samples and so each run was limited to 18 injections. Between runs the column was washed sequentially with methanol, chloroform, methanol, and mobile phase for thirty minutes each. The detector cell was washed with hydrogen peroxide (30%) and then flushed with 30 mL ultrapure water. The next run was not started until baseline was less than 20 dpm.

#### 6.2.2.2 Calculations

Peak area was converted to dpm using a conversion factor derived from the peak area (in μVsec) observed following the injection of an aliquot of labelled SAM of known activity. The specific activity of labelled SAM was then used to calculate

pmol [$^{14}$C] methylmercaptoethanol formed and TMT activity according to the formula;

$$Q = A \times F \times 1/SA_f \times 60/50 \times 1/W$$

Where Q = TMT activity in pmol [$^{14}$C] methylmercaptoethanol formed/hour/mg protein

A = peak area in μVsec

F = conversion factor (dpm / unit peak area in μVsec)

$SA_f$ = dpm per pmol SAM working solution, derived from specific activity [$^{14}$C] SAM

W = mg protein / 20 μL sample.

### 6.2.3 Red Cell Membrane TMT Activity and Stool Sulphide

In a separate study to assess the relationship between stool sulphide and red blood cell membrane TMT activity, 10 control and 15 colitic subjects (5 limited, 10 pancolitis, 5 active, 10 inactive) were studied. These patients form a subgroup of those studied in Chapter 5. Not all patients were included as the assays required were not available at the time these studies were conducted. Free stool sulphide was measured on NaOH stool slurries as described in Chapter 5 and values correlated with RCM TMT measured as above on blood drawn immediately prior to stool collection.

### 6.2.4 Statistical Analyses

All results are expressed as mean (SEM). Data were stored using Excel 5.0 software (Microsoft Corporation) and analysed using Graphpad Prism V 1.03 and Instat 2 (Graphpad Software, San Diego). As observations in cell incubation studies were paired, comparison of means between groups was performed using repeated measures ANOVA reporting a two sided p value. Comparison of means between patient groups were by unpaired Student's t test. The relationship between values measured simultaneously was tested using the Spearman correlation coefficient. A p value of less than 0.05 was considered significant.

## 6.3 Results

### 6.3.1 Patients Studied

Twenty one colonic resection specimens were studied (12 distal, 9 proximal). The indications for operation are given in Table 6.1. All but one (distal) isolated cell suspensions were considered viable by virtue of $^{14}CO_2$ production comparable to that previously reported (Roediger 1980). This non viable cell suspension was excluded from analysis entirely. In one distal experiment a failure to include the SAMe meant that whilst sulphide effect could be assessed, SAMe effect could not. TMT values for this patient and cell suspension are included in the analysis. This meant that observations of metabolic performance were available for 9 proximal and 10 distal cases. Whole mucosal TMT values were measured in all 21 patients studied but isolated cell values were only measured in 11 cases. The study group comprised 12 males and 9 females (distal group 6:6, proximal group 6:3) and the mean age of patients was 67.0 (2.2) years (proximal cases 71.2 (3.0), distal cases 63.5 (2.7); $p = 0.07$, unpaired Students t test). No patient was taking any drug known to be metabolised by thiomethyl- or thiopurine methyltransferases. There was only one current smoker in both groups although there were two reformed smokers in both the distal and proximal groups. None of these subjects had smoked for 5 years. The mean (SEM) cell harvest was 10.4 (1.9) gm for proximal cases and 8.8 (1.0) gm for distal cases ($t = 0.78$, $p = 0.44$ unpaired t test).

### 6.3.2 Effect of Sulphide on n-Butyrate Oxidation

A significant reduction in n-butyrate oxidation, as measured by $^{14}CO_2$ production, was observed in incubations carried out in the presence of 1.5 mM NaHS compared to control values (Table 6.2, Figure 6.1). In distal cases, mean control values of 0.77 (0.04) µmol/min/gm dry weight cells were observed, falling to 0.49 (0.07) µmol/min/gm dry weight cells with sulphide treatment. A similar reduction was observed in the proximal colon (control = 0.67 (0.03), sulphide treated = 0.39 (0.06) µmol/min/gm dry weight cells). The reduction in $^{14}CO_2$ production consequent upon sulphide treatment was not significantly different between

proximal or distal cell suspensions when expressed as a percentage reduction of control values (proximal = 43.3% (6.7), distal 40.4% (6.7); t = 0.30, p = 0.77).

### 6.3.3 Effect of SAMe on Sulphide Induced Reduction of $^{14}CO_2$ Production

An improvement in metabolic performance, again measured as $^{14}CO_2$ production, was observed in incubations performed containing SAMe (5 mM) and NaHS (1.5 mM) as compared to NaHS alone (Table 6.2, Figures 6.1, 6.2). The mean (SEM) $^{14}CO_2$ production in SAMe treated incubations in distal cases was 0.63 (0.07) µmol/min/gm dry weight cells and 0.62 (0.07) µmol/min/gm dry weight cells in proximal cases.

In incubations performed using distal colonic epithelial cells, $^{14}CO_2$ production was statistically significant between groups ($F = 22.87$, $p < 0.0001$, repeated measures analysis of variance). The reduction consequent upon sulphide treatment was highly significant ($q = 9.56$, $p < 0.001$, Tukey Kramer multiple comparison test). The reversal of sulphide effect by SAMe was incomplete however as a significant difference existed between control and NaHS / SAMe treated incubations ($q = 4.93$, $p < 0.01$ Tukey Kramer test). Whilst there was also a statistically significant difference in $^{14}CO_2$ production in proximal cases ($F = 29.42$, $p < 0.0001$, repeated measures analysis of variance), the pattern of SAMe effect was not the same. Whilst the reduction in $^{14}CO_2$ observed with sulphide treatment compared to controls was similar to that observed in distal cases ($q = 10.06$, $p < 0.001$ Tukey Kramer test), the effect of SAMe was more complete with no significant difference observed between control and NaHS / SAMe treated groups ($q = 1.53$, $p > 0.05$, Tukey Kramer test).

### 6.3.4 Red Cell Membrane TMT Activity

Red blood cell membrane TMT activity was estimated in 20 patients. The mean RCM TMT activity was 157.0 pmol/hour/mg protein and did not differ significantly between patients undergoing proximal or distal colonic resection (proximal = 146.4 (10.7), distal = 163.9 (24.9); U = 48.0, p = 0.7, Mann Whitney U test). RCM

TMT activity was observed to correlate with whole mucosal TMT activity (Figure 6.3) although the correlation was not strong (Spearman correlation coefficient $r = 0.53$, $p = 0.017$, $n = 20$).

As part of the separate study described in Section 6.2.3, red blood cell membrane TMT activity was estimated in a separate group of patients comprising ten control and 15 colitic subjects. Control values again varied widely over an approximately fivefold range (mean (SEM) = 201.0 (36.2) pmol/hour/mg protein). Values obtained from blood drawn from colitic subjects (mean (SEM) = 249.2 (24.8) pmol/hour/mg protein) did not differ significantly from control values ($t = 1.13$, $p = 0.27$, unpaired t test). No correlation was observed between red cell TMT activity and free stool sulphide (Spearman correlation coefficient $r = 0.07$ (95% CI –0.36 to 0.48,), $n = 22$, $p = 0.75$), or total stool sulphide ($r = –0.13$, (95% CI –0.53 to 0.31,), $n = 22$, $p = 0.55$) (Figure 6.4).

### 6.3.5 Mucosal TMT Activity

Both whole mucosal and isolated cell TMT activity was estimated in 11 specimens (6 distal, 5 proximal). Whole mucosal microsomal TMT activity was found to correlate significantly with that measured from the microsomal fraction of isolated cell suspensions (Spearman correlation coefficient $r = 0.80$, $p = 0.004$ , Figure 6.5). Accordingly, whole mucosal microsomal TMT activities were used for all following comparisons. The mean (SEM) whole mucosal TMT activity for proximal colon (641.2 (55.1) pmol/hour/mg protein) was not significantly different to that for the distal colon (= 891.3 (117.7) pmol / hour /mg protein ($U = 34.0$, $p = 0.26$, Mann Whitney U test)). The distribution of values was dissimilar, however, with a bimodal distribution apparent in distal values (Figure 6.6). Whole mucosal TMT activity was not observed to correlate with either the degree of sulphide inhibition $^{14}CO_2$ production or the degree to which SAMe may reverse this inhibition either in the proximal colon, the distal colon or when considering the pooled data (Figures 6.7, 6.8).

**Table 6.1 Indication for operation**

| | Proximal Group | Distal Group |
|---|---|---|
| Benign polyp | 1 | 1 |
| Carcinoma | 8 | 9 |
| Diverticular | 0 | 2 |

**Table 6.2** Metabolic performance of isolated human colonocyte suspensions derived from proximal and distal colon following incubation for 40 minutes in the presence of substrates shown. N-butyrate = 5 mM, NaHS = 1.5 mM, SAMe = 5.0 mM. Values are mean (SEM) µmol $^{14}CO_2$ produced/min/gm dry weight cells. [a] $p < 0.001$, [b] $p < 0.01$, [c] p = n.s., Tukey Kramer post test versus control values.

| | Proximal (n = 9) | Distal (n = 11) |
|---|---|---|
| n-butyrate | 0.66 (0.03) | 0.77 (0.04) |
| n-butyrate+ NaHS | 0.39 (0.06)[a] | 0.49 (0.07)[a] |
| n-butyrate + NaHS + SAMe | 0.62 (0.07)[c] | 0.62 (0.07)[b] |

**Figure 6.1** Effect of NaHS (1.5mM) and SAMe (5 mM) on $^{14}CO_2$ production by isolated colonic epithelial cell suspensions . Distal colonic epithelial cell experiments. See Table 6.2 for statistical analysis.

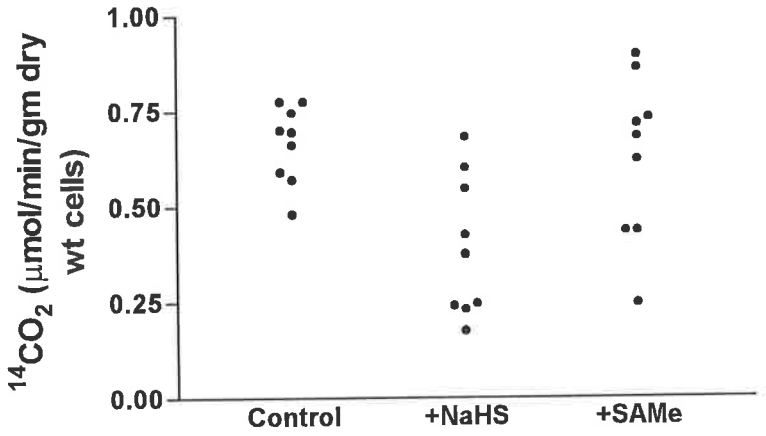



**Figure 6.2.** Effect of NaHS (1.5mM) and SAMe (5 mM) on $^{14}CO_2$ production by isolated colonic epithelial cell suspensions. Proximal colonic epithelial cell experiments. See Table 6.2 for statistical analysis.

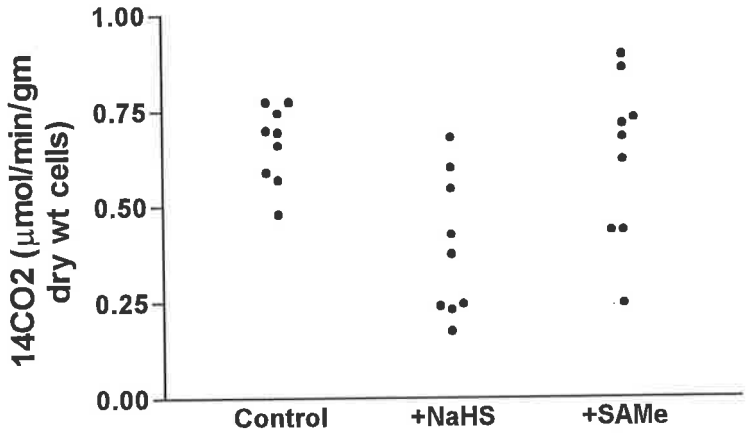


**Figure 6.3.** Correlation between whole mucosal and red blood cell membrane TMT activities. Spearman correlation coefficient r = 0.53, p = 0.017, n = 20)

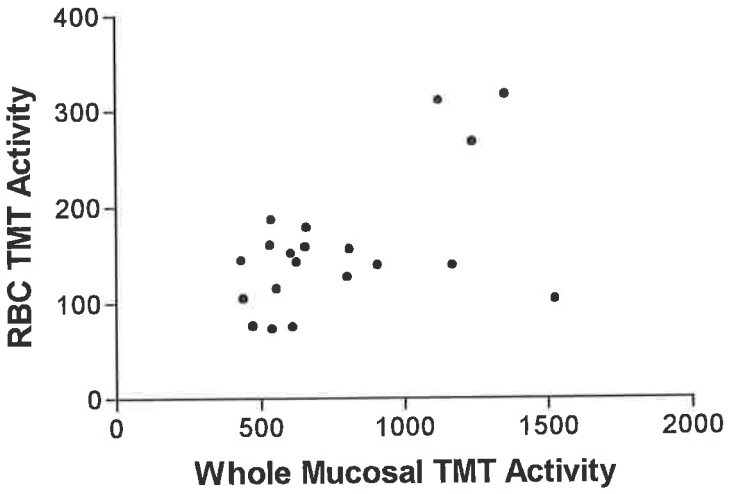



**Figure 6.4.** Relationship between erythrocyte TMT activity and free stool sulphide. r = 0.07, p = 0.75

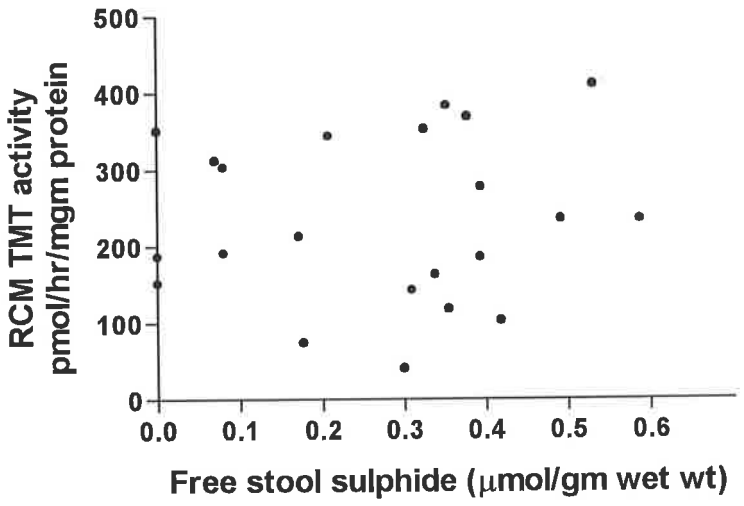


**Figure 6.5.** Correlation between whole mucosal and isolated cell microsomal fraction TMT activity. Spearman correlation coefficient r = 0.80, p = 0.004, n = 11.

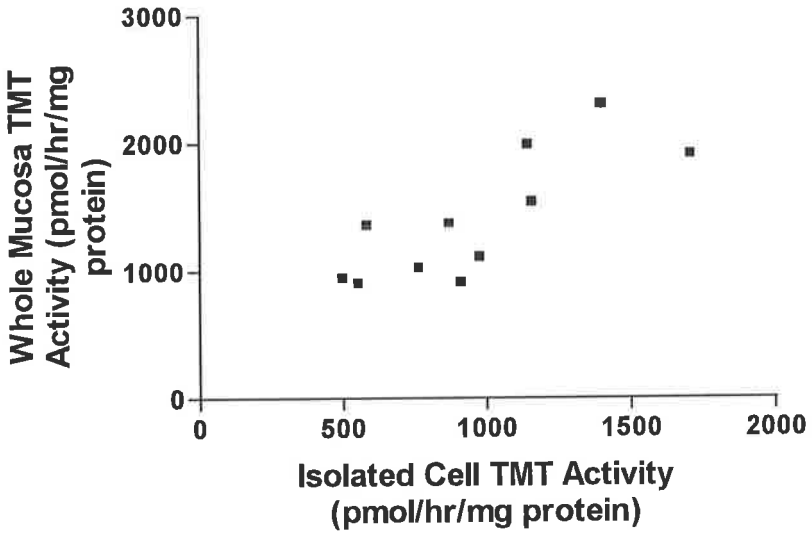



**Figure 6.6.** Whole mucosal TMT values.

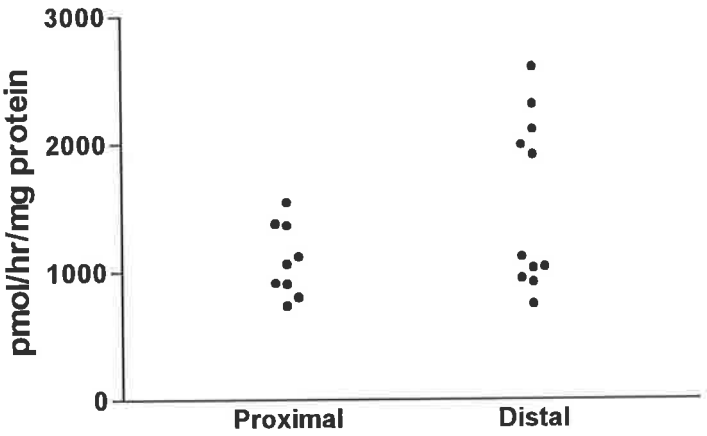


**Figure 6.7.** Relationship between whole mucosal TMT activity and the reduction (% control values) in $^{14}CO_2$ production observed in all (proximal and distal) cell suspensions studied. Spearman correlation coefficient r = –0.25 (95% CI –0.64 to 0.25), p = 0.31.

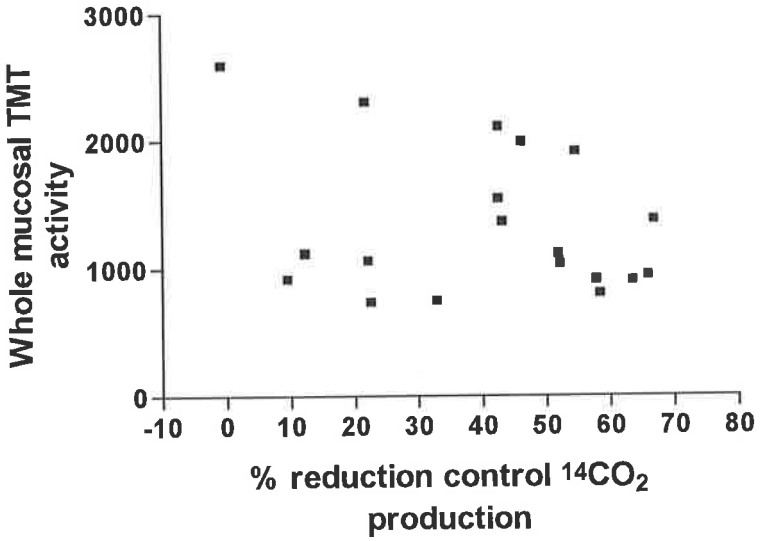
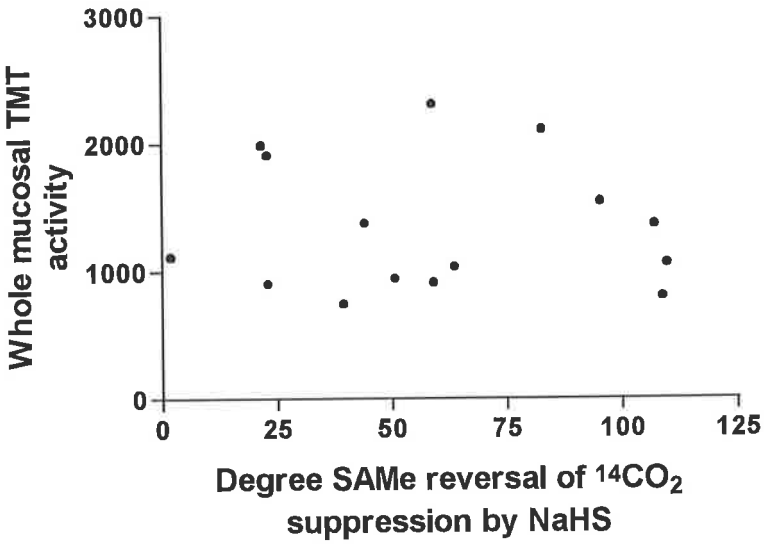


**Figure 6.8**. Relationship between whole mucosal TMT activity and the percentage of observed sulphide effect reversed by SAMe. Spearman r = –0.17 (95% CI –0.59 to 0.32), p = 0.49.



## 6.4 Discussion

The diminished n-butyrate oxidation observed in colonic epithelial cell suspensions treated with 1.5 mM NaHS differs from that previously reported . Roediger (1993) reported that 2 mM NaHS reduced n-butyrate oxidation (also measured as $^{14}CO_2$ production) significantly more in the rectosigmoid region (–74.7%) compared to the right colon (–42.3%). This difference is unlikely to be explained methods of bowel preparation or sample collection as both studies were performed in the same laboratory but may be explained by the fact that the earlier study used preincubation of cells with sulphide. No difference was observed in sulphide effect between right colonic and splenic flexure / descending colon incubations in the study reported by Roediger. In the study presently reported, 8 of 11 distal operative specimens were resections that included the splenic flexure. Because of the surgical anatomy, the majority of mucosa harvested came from the proximal side of the specimen, ie the descending colon. In only three specimens was mucosa harvested from the rectosigmoid region, and although the mean values for sulphide effect are similar in these two subgroups, it is possible that greater numbers of distal specimens may reveal a difference.

The finding that there is a significant difference in SAMe effect between proximal and distal incubations is important. The reversal of sulphide effect was less marked in the distal colon, suggesting that the distal colon may be more susceptible to sulphide effect by virtue of less effective mucosal sulphide detoxification. This was not due to differences in mucosal TMT activity as mean values were similar in proximal and distal cases. The observation of a bimodal distribution of TMT activity in the distal colon, however, may suggest that a subgroup of individuals may be more susceptible to sulphide effect in the distal colon. To confirm that such a subgroup does exist will require significantly greater numbers than have been presented in this study, but such a finding may provide an explanation for the distal origin of colitis. RBC TMT activity is known to be genetically controlled (Weinshilboum 1992) and this might be expected to be true for mucosal TMT. This may provide an explanation for the observed genetic influences in the incidence of

ulcerative colitis. Sulphide concentrations are greatest in the distal colon (MacFarlane 1992) and it is possible that the bimodal distribution of distal TMT activity is a reflection of higher lumenal sulphide concentrations. Although stool sulphides were not correlated with mucosal TMT in this study, this explanation is considered unlikely given the finding that stool sulphide does not correlate with RBC TMT activity and the significant (albeit not strong) correlation between RBC TMT and whole mucosal TMT here reported.

The wide variability of RBCM TMT activity seen in healthy individuals in this study is comparable to that reported by Babidge (1995). Similarly, Weinshilboum (1992) and Keith (1983) demonstrated a five fold variability in RBCM TMT activity in large groups of randomly selected individuals. The RBCM TMT activities obtained by Babidge (1995) were derived from patients undergoing colectomy and blood was drawn at induction of anaesthesia and following bowel preparation, a factor not prevalent in this study of ambulant patients. There was no significant difference between control and colitic RCM TMT activity in this study. Recent work from the U.K. supports this finding (Pitcher 1995a). In the study quoted it was hypothesised that TMT activity may be induced by higher stool sulphide levels. In this study, however, no correlation was found between RCM TMT activity and either free or total stool sulphide levels. This is perhaps not surprising as the colonic mucosa would seem to act as an efficient barrier to lumenal sulphide. Mucosal levels of TMT activity would perhaps be a more useful value to correlate with stool sulphide. Such a study would be difficult to perform as currently, measurement of mucosal TMT activity requires significant amounts (approximately 10 mgm) of tissue which can only be provided from resection specimens. As such specimens are obtained following a cathartic bowel preparation, which is likely to influence stool sulphide, the concurrent measurement of stool sulphide and mucosal TMT activity will rely on the development of TMT assays able to be performed on biopsy material.

The observation that neither the degree of sulphide reduction of $^{14}CO_2$ production nor the degree of SAMe reversal of such an effect correlated (either positively or negatively) with TMT activity suggests that TMT activity is not a rate limiting factor in mucosal sulphide detoxification.). It has recently been suggested that levels of SAM show an aboral gradient in the colonic mucosa and that such levels are reduced in areas of colonic mucosa that are actively inflamed compared to control mucosa (Thiede 1995). Such an abnormality may result in impaired methylation of sulphides and render lumenal levels of sulphide normally seen in the colon toxic to colonocytes. SAM is recycled within the colonocyte by a folate dependent process via S-adenosyl-homocysteine, homocysteine and thence by methylation (terahydrofolate dependent) to methionine. Perhaps surprisingly for a tissue with such a high metabolic rate and cell turnover, the gastrointesinal tract is a relatively poor source of folic acid. Studies of the tissue distribution of folate in rats (Clifford 1990) would suggest that less than 3% of total body folate can be attributed to the gastrointesinal tract as a whole. Systemic folic acid deficiency is a well recognised feature of ulcerative colitis (Elsborg 1979) and depletion of intermediate term folate stores (red cell folate levels) has been associated with an increased risk of the development of dysplasia in pancolitic patients on surveillance programmes (Laschner 1993) and in dimethylhydrazine treated rats (Cravo 1992). Whilst multiple factors may have a causal role for folate deficiency in ulcerative colitis including reduced intake, reduced absorption and inhibition of folate enzymes by drug therapy, the possibility that susceptibility of the colonic epithelium to sulphide toxicity may be related to a failure of folate dependent methionine (and consequently SAM) regeneration is worthy of further study.

# Chapter 7

## General Discussion and Future Research Directions

The aetiology of both the major phenotypes of inflammatory bowel disease remains unclear despite considerable advances in our understanding of the pathways responsible for the generation of intestinal inflammation. It is increasingly accepted that a complex interaction between the lumenal contents , the epithelial cell, and mucosal defence mechanisms is likely responsible and research efforts have in recent times been directed predominantly in one of these three broad directions. The studies undertaken for this thesis have focussed on the role of one aspect of colonic lumenal physiology which may be of relevance to ulcerative colitis, that of colonic sulphur metabolism and the role reduced forms of sulphur such as sulphide may play in colitis. Several lines of evidence support such a role. Animal models which rely on the delivery of oxidised forms of sulphur, noteably those utilising degraded carrageenan and dextran sodium sulphate, are associated with mucosal inflammation resembling that observed in ulcerative colitis. Such models require the presence of both an intact microflora (Onderdonk 1977, 1978, 1981, Ohkusa 1987) and the delivery of sulphate to the colon (Ishioka 1986) and at least in the case of the carrageenan model, are associated with elevated lumenal sulphide levels (Florin 1995). Reduced forms of sulphur, particularly sulphide, are found in the colonic lumen in health as a consequence of both bacterial dissimilatory sulphate reduction (Gibson 1991) and fermentation of sulphur amino acids (Kadota 1972, Florin 1991). Colonic lumenal carriage of bacteria capable of sulphidogenesis via dissimilatory sulphate reduction has been uniformly reported to be elevated in patients with ulcerative colitis ( Florin 1990, Pitcher 1995b) but evidence regarding lumenal levels of sulphide has been conflicting, having been reported as normal (Pitcher 1995b), elevated (Florin 1990, Gibson 1991) or selectively elevated in patients not receiving salicylates (Pitcher 1995c) compared to controls. Finally, the inhibition of n-butyrate oxidation observed in colonic epithelial cells harvested from both quiescent and active ulcerative colitis can be reproduced *in vitro* by incubation with what is regarded as physiologically relevant concentrations of sulphide. On the

basis of this evidence, the general hypothesis that lumenal mercaptides, in particular hydrogen sulphide, contribute to the aetiology of ulcerative colitis by the inhibition of short chain fatty acid oxidation has been proposed. To address this hypothesis, animal studies examining the effect of *in vivo* administration of sulphide to the "in flow" rat colonic mucosa (Chapter 3) and the effect of sulphide treatment of isolated rat colonic epithelial cells on the intermediates of fatty acid oxidation (Chapter 4) have been performed. Additionally, the concentrations of stool sulphide and sulphate in healthy human control subjects has been compared with that observed in ulcerative colitis (Chapter 5) and the role of colonic mucosal sulphide detoxification in healthy human tissue has been explored (Chapter 6).

The studies of rat colonic epithelial exposure to sulphide have used both *in vivo* (Chapter 3) and *in vitro* (Chapter 4) exposures. It may be argued that in vitro exposure of the entire disaggregated colonic epithelial cell to an agent such as sulphide, which is normally only encountered by the lumenal aspect of the cell, is unphysiologic. $^{14}CO_2$ production values from isolated rat colonic epithelial cell suspensions were more markedly reduced in *in vitro* experiments (1.5 mM NaHS) than in *in vivo* studies in which stool sulphide concentrations of at least 2 mM were achieved. Nevertheless, the finding that n-butyrate oxidation can be inhibited by *in vivo* exposure of the rat colonic epithelium to levels of sulphide that have been reported to exist in human ulcerative colitis is the first time, as far as the author is aware, that diminished colonic mucosal n-butyrate oxidation has been reproduced in an animal model using a physiologically relevant agent. The mechanism for this inhibition would seem to involve, at least in part, the inhibition of flavoprotein dependent short chain acyl dehydrogenase activity although the role of electron transport chain toxicity, well known to be associated with sulphide exposure has not been addressed.

The development of mucosal inflammation in association with the metabolic abnormality observed in sulphide treated colonic epithelial cells would provide strong evidence for the importance of reduced fatty acid oxidation in the

development of colitis. Significant inflammatory changes were not observed in rats treated for either four or ninety days suggesting that diminished fatty acid oxidation is not a causal factor for colitis in the rat colon. This suggestion needs to be considered, however, in light of the finding that n-butyrate oxidation was reduced by 42% in mucosal biopsy specimens in patients with quiescent ulcerative colitis in the study reported by Chapman (1994). Similar findings have been reported by Roediger (1980) with respect to quiescent colitis (most of which were specimens obtained following colectomy for dysplasia (W. Roediger, personal communication.) but active colitis was associated with a much greater inhibition of n-butyrate oxidation (of the order 90%). The degree of inhibition of n-butyrate oxidation observed in rat colonic epithelial cells following *in vivo* sulphide exposure (10mM) was of the order 40% of control values. Thus it may be that a greater degree of inhibition of fatty acid oxidation may result in mucosal inflammation and such inhibition as produced by the use of 2-bromo-octanoate has been associated with mucosal inflammation (Roediger 1986a). Attempts to achieve such a further reduction of n-butyrate oxidation through the use of higher instilled concentrations of sulphide produced no evidence of a dose response effect at the 20 and 30 mM levels and a very limited number of experiments using higher concentrations (70–100 mM) were associated with the development of signs of systemic sulphide toxicity (tachypnoea, seizures and death) shortly after a single dose administration. It would seem likely that sulphide exposure alone is an insufficient explanation for the pathogenesis of mucosal inflammation, at least in this experimental model.

Although the results of studies of lumenal sulphide concentrations in patients with ulcerative colitis did not reveal a significant difference when compared to control values (Chapter 5), it is likely that this is in part related to the population studied (see 5.4 for discussion). The elevated ratio of free to total sulphate within the colonic lumen reported by Pitcher (1995b) could not be reproduced. While it is recognised that measurement of stool sulphate and sulphide are only very basic means of assessing the colonic lumenal sulphur metabolic "milieu" and that more dynamic measures of colonic sulphur metabolism such as sulphate reduction rates

may provide contrary insights, it is tentatively concluded that no difference exists between control and colitic subjects with respect to stool sulphide levels. The strength of this conclusion must be tempered by the limitations imposed by the population studied and the potentially confounding factor of drug therapy.

Perhaps one of the most interesting findings of the studies described is that relating to the detoxification of sulphide by isolated colonic epithelial cells *in vitro* (Chapter 6). The hypothesis that methyl group transfer mediated by TMT may be important in protecting the colonic mucosa from the adverse effects of lumenal sulphide would seem to be supported by the results obtained. In the proximal colon, SAMe fully restored $^{14}CO_2$ production to control values indicating that methylation is an important mechanism of sulphide detoxification. The observation that colonic epithelial cell suspensions derived from the distal colon exhibit a differential ability to reverse sulphide induced inhibition of n-butyrate oxidation when compared to cell suspensions derived from the proximal colon may explain the previously observed susceptibility of distal suspensions to sulphide (Roediger 1993b). The distal colon is thought to be more reliant on n-butyrate oxidation than the distal colon in health (Roediger 1980a), thus if the distal colon is less able to detoxify sulphide, sulphide effects might be expected to be proportionately greater in this part of the colon. Such a susceptibility to sulphide may help explain the distal predilection for ulcerative colitis.

It has been shown previously that red cell membrane and hepatic TMT activities correlate and appear to be under genetic control (Keith 1983, Weinshilboum 1992). The finding of a significant correlation between red cell membrane and whole mucosal TMT activities was not therefore unsurprising. The demonstration of a bimodal distribution of distal colonic epithelial cell TMT activity was unexpected however. It raises the possibility of a population subgroup with a (perhaps genetically determined) susceptibility to stool sulphide. This is made less likely, however by the poor correlation between mucosal TMT activity and sulphide or SAMe effect. Larger studies of whole mucosal TMT activity in both health (with

respect to *in vitro* sulphide effect) and disease (comparing control, disease control and colitic patients) will be required to establish the importance of this form of mucosal defence against sulphide in mucosal disease affecting the colon. The role of folate dependent methionine regeneration in the colonic epithelial cell and the possible importance of this in ulcerative colitis (see 6.4) is a further related area worthy of study.

The finding that salicylates inhibit sulphate reduction *in vitro* and that stool sulphide concentrations were elevated in colitic patients not receiving such medication (Pitcher 1995c) highlights the need to distinguish between abnormalities which arise as a consequence of treatment and those of potentially aetiologic importance. If elevated stool sulphides are associated with untreated ulcerative colitis, the question must be asked as to the responsible mechanism, ie is it a consequence of increased sulphate reduction or is it a consequence of greater levels of protein fermentation as suggested by Florin (1995). As far as the author is aware, this question has not been satisfactorily addressed as yet. In particular, studies utilising models of mucosal inflammation known to be associated with elevated stool sulphide such as the carrageenan model, and comparative studies of the relative contribution of sulphate reduction and protein fermentation to stool sulphide in patients with ulcerative colitis versus controls may indicate whether elevated stool sulphide should occupy a central role in our understanding of the pathogenesis of ulcerative colitis or whether it represents yet another epiphenomenon.

## References

Abraham R., Fabian R.J., Goldberg L., Coulston F.
Role of lysosomes in carrageenan induced caecal ulceration.
Gastroenterology 1974 67;1169-1181

Ahlman B., Leijonmarck C-E., Lind C., Vinnars E., Wernerman J.
Free amino acids in biopsy specimens from the human colonic mucosa.
J. Surg. Res. 1993 55; 647-653

Alexson S.E., Osmundsen H., Berge R.K.
The presence of acyl CoA hydrolase in rat brown adipose tissue peroxisomes.
Biochem. J. 1989 262; 41-46

Allen D.C., Connolly N.S., Biggart J.D.
Mucin profiles in ulcerative colitis with dysplasia and carcinoma.
Histopathology 1988 13; 413-2

Allison A.C., Harington J.S., Birbeck M.
An examination of the cytotoxic effects of silica on macrophages.
Journal Exp. Med. 1966 124; 141

Allison C., MacFarlane G.T.
Effect of nitrate upon methane production by slurries of human faecal bacteria.
J. Gen. Microbiol. 1988 134; 1397-1405

Allison M.C., Cornwall S., Poulter L.W. Dhillon AP Pounder RE
Macrophage heterogeneity in normal colonic mucosa and in inflammatory bowel disease.
Gut 1988 29 1531-1538

Aminlari M., Gilanpour H.
Comparative studies on the distribution of rhodanese in different tissues of domestic animals.
Comp. Biochem. Physiol. 1991 99B; 673-77

Andre F., Andre C., Emery Y., Forichon J., Descos L., Minaire Y.
Assessment of the lactulose mannitol test in Crohn's disease.
Gut 1988 29; 511-15

Annison E.F., Hill K.J., Lewis D.
Studies on the portal blood of sheep II. Absorption of volatile fatty acids from the rumen of the sheep.
Biochem J. 1957 66; 592-99

Anonymous
Intestinal permeability.
Lancet 1985 1; 256-58

Ardawi M.S.M., Newsholme E.A.
Fuel utilisation in colonocytes of the rat.
Biochem. J. 1985 231; 713-19

Aslam M., Batten J.J., Florin T.H.J., Sidebotham R.L., Baron J.H.
Hydrogen sulphide induced damage to the colonic mucosal barrier in the rat.
Gut 1992 33 (suppl2); S69 (abstract)

Axelsson L.G., Landstrom E., Bylund-Fellenius A.C., Midtvedt T.
Dextran sulphate sodium induced colitis and sulphasalazine treatment in bacteria free mice.
Gastroenterol. 1995 108; A775

Babidge W.J., Millard S.H., Roediger W.E.W.
Thiol methyltransferase activity in colonocytes and erythrocyte membranes.
J. Clin. Pathol. 1995 48; 641-44

Bartholemew T.C., Powell G.M., DoDgson K.S., Curtis C.G.
Oxidation of sodium sulphide by rat liver, lungs and kidney.
Biochem. Pharmacol. 1980 29; 2431-2437

Bartlett K., Hovik R. Eaton S., Watmough N.J., Osmundsen H.
Intermediates of peroxisomal beta oxidation; a study of the fatty acyl CoA esters which accumulate during peroxisomal beta oxidation of [U-$^{14}$C] hexadecanoate.
Biochem. J. 1990 270; 175-80

Bauche F., Sabourault D., Guidicelli Y., Nordmann J., Nordmann R.
Inhibition in vitro of acyl CoA dehydrogenase by 2-mercaptoacetate in rat heart mitochondria.
Biochem. J. 1983 215; 457-64

Baxter C.F., van Reen R., Pearson R.B., Rosenberg C.
Sulfide oxidation in rat tissues.
Biochim. Biopys. Acta. 1958a 27; 584-89

Baxter C.F., van Reen R.
The oxidation of sulfide to thiosulfate by metalloprotein complexes and by ferritin.
Biochim. Biophys. Acta 1958b 28; 573-76

Beauchamp R.O., Bus J.S., Popp J.A., Boreiko C.J., Andjelkovich D.A.
A critical review of the literature on hydrogen sulphide toxicity.
C.R.C. Critical Reviews in Toxicology 1984 13; 25-97

Beck J.F., Donini J.C., Maneckjee A.
The influence of sulfide and cyanide on axonal function
Toxicology 1983 26;37-45

Beck-Speier I., Hinze H., Holzer H.
Effect of sulfite on the energy metabolism of mammalian tissues in coorelation to sulfite oxidase activity.
Biochim. Biophys. Acta. 1985 841; 81-89

Beerens H., Romond C.
Sulphate reducing anaerobic bacteria in human faeces.
Am. J. Clin. Nutr. 1977 30; 1770-76

Beijerinck W.M.
Uber Spirillum desulfuricans als ursache von sulfatreduktion.
Centralblatt fur Bakteriologie und Parisitenkunde 1895 1; 1-114 (quoted by Gibson 1990)

Berger R.L., Lium R.
Abdominal postganglionic sympathectomy; A method for the production of an ulcerative colitis like state in dogs.
Ann. Surg. 1960 152; 266-73

Berglin E., Edlund B., Nyberg G.K., Carlsson J.
Potentiation by L-cysteine of the bacteriocidal effect of hydrogen peroxide in Escherichia coli.
J.Bacteriol. 1982 152; 81-88

Berglin E., Carlsson J.
Potentiation by sulphide of hydrogen peroxide killing of Escherichia coli.
Infect. Immun. 1985 49; 538-43

Berry R.K., Hansard S.L., Ismail R.J., Wysocki A.A.
Absorption, deposition and placental transfer of sulphate sulphur by gilts.
J. Nutr. 1969 97; 399-408

Bhaskar L., Ramakrishna B., Balasubramanian K.A.
Colonic mucosal antioxidant enzymes and lipid peroxide levels in normal subjects and patients with ulcerative colitis.
J. Gastroenterol. Hepatol. 1995 10; 140-43

Biester H.E.. Schwarte H.
Intestinal adenoma in swine.
Am. J. Pathol. 1931 7; 175-86

Biester H.E.
Studies on infectious enteritis in swine II. The pathogenesis of infectious enteritis.
J. Am. Vet. M.A. 1928 72; 1003-1022

Bjarnason I., Peters T.J.
A persistent defect in intestinal permeability in coeliac disease demonstrated by a $Cr^{51}$ labelled EDTA absorption test.
Lancet 1983 1; 323-25

Bjarnason I., OMorain C., Levi A.J., Peters T.J.
Absorption of Cr51 labelled Ethylenediaminetetraacetate in inflammatory bowel disease.
Gastroenterology 1983 85; 318-22

Bohe M., Borgstrom S., Ohlsson G.K.
Determination of immunoreactive trypsin, pancreatic elastase and chymotrypsin in extracts of human faeces and ileostomy drainage.
Digestion 1983 27; 8-15

Brad field G., Somerfield P., Meyn T., Holby M., Babcock D., Bradley D., Segel I.H.
Regulation of sulphate transport in filamentous fungi.
Plant Physiol. 1970 46; 720-

Bremer J., Osmundsen H.
Fatty acid oxidation and its regulation.
Fatty acid Metabolism and its Regulation ( Numa S. editor) 1984 Elsevier Science Publishers.
Chapter 5.

Brennan P.C., Fritz T.E., Flynn R.J., Poole C.M.
Citrobacter fruendii associated with colitis in laboratory mice
Lab. Anim. Care 1965 15; 266-75

Breuer R.I., Buto S.K., Christ M.L., Bean J., Vernia N., Pauluzi P., Di Paolo M.C., Caprilli R.
Rectal irrigation with short chain fatty acids for distal ulcerative colitis.
Dig. Dis. Sci. 1991 36; 185

Bruner F., Ciccioli P., Di Nardo F.
Further developments in the determination of sulphur compounds in air by gas chromatography.
Anal. Chem. 1975 47; 141-144

Brunold C., Schiff J.A.
Studies of sulfate utilisation by algae. 15. Enzymes of assimilatory sulfate reduction in Euglena and their cellular localisation.
Plant Physiol. 1976 57; 430-36

Bures J., Horak V., Buresova E. Fixa B., Komarkova O Hartmann M
Colicinogeny in chronic inflammatory bowel disease.
Scand J Gastroenterol 1986 21; 819-823

Burke D.A., Axon A.T.R.
Ulcerative colitis and Escherichia coli with adhesive properties
J Clin Pathol 1987 40; 782-786

Burke D.A., Axon A.T.R., Clayden S.A., Dixon M.F., Johnson D., Lacey R.W.
The efficacy of oral tobramycin in the treatment of ulcerative colitis.
Ailment. Pharmacol. Therap. 1990 2;123-29

Buytenhuys F.A.
Ion chromatography of inorganic and organic ionic species using refractive index detection.
J. Chromatogr. 1981 218; 57-64

Cabantchick Z.I., Wolosin J.M., Ginsberg H., Ziemel O.
Structural and functional properties of the anion transport system isolated from human erythrocyte membranes. In "FEBS Symposium on the Biochemistry of Membrane Transport" Semeneza G., Carafoli E. (editors) Springer Verlag, Berlin. 1977, pp 328

Cabre F., Marin C., Cascante M., Canela E.I.
Occurrence and comparison of sulfite oxidase activity in mammalian tissues.
Biochem. Med. Metab. Biol. 1990 43; 159-62

Calkins B.M.
A meta analysis of the role of smoking in inflammatory bowel disease.
Dig. Dis Sci. 1989 34; 1841-54

Cantanzaro P.J., Schwartz H.G., Graham R.C.
Spectrum and possible mechanism of carrageenan cytotoxicity.
Am. J. Pathol 1971 64; 387-404

Cappiello M., Franchi M., Guiliani L., Pacifici G.M.
Distribution of 2-napthol sulphotransferase and its endogenous substrate adenosine 3'-phosphate-5'-phosphosulphate in human tissues.
Eur. J. Clin. Pharmacol. 1989 37; 317-20

Chacko A., Cummings J.H.
Nitrogen losses from the human small bowel: obligatory losses and the effect of physical form of food.
Gut 1988 29; 809-15

Chadwik V.S., Phillips S.F., Hofman A.F.
Measurements of intestinal permeability using low molecular weight polyethylene glycol (PEG 400).
Gastroenterology 1977 73; 247-51

Chalifoux L.V., Bronson R.T.,
Colonic adenocarcinoma associated with chronic colitis in cotton top tamarins (Sanguinus oedipus)
Gastroenterology 1981 80; 942-46

Chapman M.A.S., Grahn M.F., Giamundo P., O'connell P.R., Onwu D., Hutton M., Maudsley J., Norton B., Rogers J., Williams N.J.
New mucosal technique to measure mucosal metabolism and its use to map substrate utilisation in the healthy human large bowel
Br J Surg 1993a 80 445-449

Chapman M.A.S., Grahn M.F., Hutton M., Williams N.S.
Metabolic adaptation of terminal ileal mucosa after ileoanal pouch construction
Br. J. Surg. 1993b 80; 1478 (abstract)

Chapman M.A.S., Grahn M.F., Boyle M.A., Hutton M., Rogers J., Williaims N.S.
Butyrate metabolism is impaired in the colonic mucosa of sufferers of quiescent ulcerative colitis.
Gut 1994 35; 73-76

Chapman M.A.S., Grahn M.F., Hutton M., Williams N.S.
Butyrate metabolism in the terminal ileal mucosa of patients with ulcerative colitis.
Br. J. Surg. 1995 82; 36-38

Chester J.H. Ross J.S., Malt R.A., Weitzman S.A.
Acute colitis produced by chemotactic peptides in rats and mice
Am. J. Pathol. 1985 121; 284-290

Chou J.Y., Robinson J.C., Wang S.
Effects of sodium butyrate on synthesis of human chorionic gonadotropin in trophoblastic and non trophoblastic tumours..
Nature 1977 268; 543-44

Christl S.U., Gibson G.R., Cummings J.H.
Role of dietary sulphate in the regulation of methanogenesis in the human large intestine.
Gut 1992a 33; 1234-38

Christl S.U., Murgatroyd P.R., Gibson G.R., Cummings J.H.
Production, metabolism and excretion of hydrogen in the large intestine.
Gastroenterology 1992b 102; 1269-1277

Clark R.A., Szot S.Venkatasubramaniam K, Schiffman E.
Chemotactic factor inactivation by myeloperoxidase mediated oxidation of methionine.
J.Immunol. 1980 124; 2020-2026

Clark R.A., Szot S.
Chemotactic factor inactivation by stimulated human neutrophils mediated by myeloperoxidase catalysed methionine oxidation.
J. Immunol 1982 128; 1507-13

Clemedson C.J., Sorbo B. Ullberg S.
Autoradiographic observations on ingested S35 thiocyanate and C14 cyanide in mice.
Acta Physiol Scand. 1960 48; 382-89

Clifford A.J., Heid M.K., Muller H.J., Bills N.D.
Tissue distribution and prediction of total body folate in rats.
J. Nutr. 1990 120; 1633-39

Cline J.D.
Spectrophotometric determination of hydrogen sulphide in natural waters
Limnol. Oceanogr. 1969 14; 454-58

Cocchetto D.M., Levy G.
Absorption of orally administered sodium sulphate in humans.
J. Pharm. Sci. 1981 70; 331-33

Cole D.E.C., Mohyuddin F., Scriver C.R.
A microassay for analysis of serum sulphate.
Anal. Biochem. 1979 100; 339-42

Cole D.E.C., Scriver C.R.
Microassay of inorganic sulphate in biological fluids by controlled flow anion chromatography.
J. Chromatogr. 1981 225; 359-67

Cope G.F., Heatley R.V., Kelleher J.
Colonic mucus and ulcerative colitis.
In "Ulcerative Colitis" (Morain C.D. editor) CRC Press, 1991

Corazza G.R., Frazzoni M., Dixon M.F.
Quantitative assessment of the mucosal architecture of jejunal biopsy specimens; a comparison between linear measurement, stereology and computer aided microscopy
J Clin Pathol 1985 38; 765-770

Corkey B.E., Brandt M., Williams R., Williamson J.R.
Assay of short chain acyl Coenzyme A intermediates in tissue extracts by high pressure liquid chromatography.
Anal. Biochem. 1981 118; 30-41

Corkey B.E.
Analysis of acyl co-enzyme A esters in biological samples.
In "Methods in Enzymology" vol 166 (Harris R.A., Sokatch J.R. editors) pp55-70 Academic Press, New York 1988a.

Corkey B.E., Hale D.E., Glennon M.C., Kelley R.I., Coates P.M., Kilpatrick L., Stanley C.A.
Relationship between unusual hepatic acyl coenzyme A profiles and the pathogenesis of Reye syndrome.
J. Clin. Invest. 1988b 82; 782-88

Corkey B.E., Deeney J.T.
Acyl CoA regulation of metabolism and signal transduction.
In "Fatty acid oxidation: clinical biochemical and molecular aspects". 1990 Alan R. Liss, New York. pp217-232

Cowan A.E., Korman M.G., Hoffmann A.F.
Metabolism of lithocholate in healthy man. 1. Biotransformation and biliary excretion of intravenously administered lithocholate, lithocholylglycine and their sulphates.
Gastroenterology 1975 69; 59-66

Cravo M.L., Mason J.B., Dayal Y., Hutchinson M., Smith D., Selhub J., Rosenberg I.H.
Folate deficiency enhances the development of colonic neoplasia in dimethylhydrazine treated rats.
Cancer Res. 1992 52; 5002-6

Cummings J.H.
Short chain fatty acids in the colon.
Gut 1981 22; 763-779

Cummings J.H.
Dietary fibre
Brit Med Bull 1981 1; 65-70

Cummings J.H., Mac Farlane G.T.
The control and consequences of bacterial fermentation in the human colon.
J.Appl. Bacteriol. 1991 70; 443-59

Curtis C.G., Bartholemew T.C., Rose F.A., Dodgeson K.S.
Detoxication of sodium $^{35}S$ sulphide in the rat.
Biochem. Pharmacol. 1972 21; 2313-21

Cutter G.A., Oatts T.J.
Determination of dissolved sulphide and sedimentary sulphur speciation using gas chromatography-photoionisation detection.
Anal. Chem 1984 59; 717-721

Danehy J.P., Zubritsky C.
Iodometric method for the determination of diothionite, bisulfite and tiosulphate in the presence of each other and its use in following the decompositon of aqueous solutions of sodium dithionate.
Anal. Chem. 1974 46; 391-95

DeAmelio R., Rossi P., LeMoli S., Ricci R., Montano S., Pallone F.
In vitro studies on cellular and humoral chemotaxis in Crohn's disease using the under agarose gel technique.
Gut 1981 22; 566-70

DeBuysere M.S., Olson M.S.
The analysis of acyl-coenzyme A derivatives by reversed phase high performance liquid chromatography.
Anal. Biochem. 1983 133; 373-79

Delpre G., Avidor I., Steinherz R., Kadish U Ben-Bassat M
Ultrastructural abnormalities in endoscopically and histologically normal and involved colon in ulcerative colitis
Amer J Gastroenterol 1989 84;1038-1046

Demoz A., Garras A., Asiedu D.K., Netteland B., Berg R.K.
Rapid method for the separation and detection of tissue short chain coenzyme A esters by reversed phase high performance liquid chromatography.
J. Chromatog. B. 1995 667; 148-52

Der Garabedian M.
The sulphide oxidase of higher vertebrates.
Compt. Rend. 1945 220; 373 (quoted by Beauchamp 1984)

de Silva H.J., Gatter K.C., Millard P.R., Kettlewell M Mortensen N.J., Jewell D.P.
Crypt cell proliferation and HLA-DR expression in pelvic ileal pouches
J Clin Pathol 1990 43 824-828

de Silva H.J., Jones M., Prince C., Kettlewell M., Mortensen N.J., Jewell D.P.
Lymphocyte and macrophage subpopulations in pelvic ileal pouches
Gut 1991b 32; 1160-1165

de Silva H.J., Millard P.R., Soper N., Kettlewell M., Mortensen N.J., Jewell D.P
Effects of the faecal stream and stasis on the ileal pouch mucosa
Gut 1991a 32; 1166-1169

de Yong P., Burggraaf M.
An ion chromatographic method for the simultaneous determination of inorganic phosphate bromide nitrate and sulphate in human serum.
Clin. Chim. Acta 1983 132;63-71

Dickens F., Weil-Malherbe H.
Metabolism of normal and tumour tissue; The metabolism of intestinal mucous membrane.
Biochem. J. 1941 35; 7-15

Dodgson K.S., White G.F., Fitzgerald J.W.
Sulfatase activity in the gastrintestinal tract. In "Sulfatases of Microbial Origin". Dodgson K.S., White G.F., Fitzgerald J.W. (editors) CRC Press, Boca Raton, 1984. Volume 2, pp 156- 59.

Donowitz M.
Arachidonic acid metabolites and their role in inflammatory bowel disease; an update requiring addition of a pathway.
Gastroenterology 1985 88; 580-587

Dreyfuss J.
Characterisation of a sulphate and thiosulphate transporting system in Salmonella typhimurium.
J. Biol. Chem. 1964 239; 2292-96

Dudeck M., Frendo J., Koj A.
Subcellular compartmentation of rhodanese and 3-mercaptopyruvate sulphurtransferase in the liver of some vertebrate species.
Comp. Biochem. Physiol. 1980 65B; 383-86

Duncan A., Kapaniris O., Roediger W.E.W.
Measurement of mercaptoacetate levels in anaerobic batch culture of colonic bacteria.
F.E.M.S. Microbiol Ecol. 1990 74; 303-308

Dvorak A.M., Connel A.B., Dickerson G.R.
Crohn's disease; a scanning electron microscope study.
Human Pathol. 1979 10; 165-77

Dziewiatkowski D.D.
Fate of ingested sulfide labelled with radioactive sulfur in the rat.
J.Biol. Chem. 1945 161; 723 (quoted in Beauchamp 1984)

Eaton S., Turnbull D.M., Bartlett K.
Redox control of beta oxidation in rat liver mitochondria.
Eur. J. Biochem. 1994 220; 671-681

Ellestad-Sayed J.J., Nelson R.A., Adson M.A., Palmer W.M., Soule E.H.
Pantothenic acid, coenzyme A and human chronic ulcerative colitis and granulomatous colitis.
Am. J. Clin. Nutr. 1976 29; 1333-1338

Elsborg L., Larson L.
Folate deficiency in niflammatory bowel disease.
Scand. J. Gastroenterol. 1979 14; 1019-24

Elson C.O., Sartor R.B., Tennyson G.S., Riddell R.H.
Experimental models of inflammatory bowel disease.
Gastroenterology 1995 109; 1344-67

Engel P.C., Massey V.
Green butyryl Coenzyme A dehydrogenase. An enzyme-coenzyme A complex.
Biochem. J. 1971 125; 889-902

Engel P.C., Williamson G., Shaw L.
Butyrl CoA dehydrogenase; Aspects of acceptor and substrate specificity. In: Bray R.C., Engel P.C., Mahew S.G. eds. "Flavins and Flavoproteins" Berlin, Walter de Gruyher 1984; 403-12

Engel P.C.
Acyl Coenzyme A dehydrogenases. In: Muller F. ed. "Chemistry and Biochemistry of Flavoenzymes" CRC Press, Boca Raton, 1992 vol 3; 598-655

Engster M., Abraham R.
Caecal response to different molecular weights and type of carrageenan in the guinea pig.
Toxicol. Appl. Pharmacol. 1976 38; 265-82

Fabia R., Willen R., Ar'Rajab A., Andersson R., Ahren B., Bengmark S.
Acetic acid induced colitis in the rat; A reproducible experimental model for acute ulcerative colitis
Eur Surg Res 1992 24; 211-225

Ferry D.M., Butt T.J., Broom M., Hunter J., Chadwick V.S.
Bacterial chemotactic peptides and the intestinal mucosal barrier.
Gastroenterology 1989 97; 61-7

Fields J.Z., Keshavarzian A., Eiznhamer D., Frommel T., Winship D., Holmes E.
Low levels of blood and colonic glutathione in ulcerative colitis.
Gastroenterology 1994 106; A680

Filipe M.I.
Mucins in the human gastrointestinal mucosa; A review.
Invest. Cell. Pathol. 1979 2; 195-216

Finnie I.A., Taylor B.A., Rhodes J.M.
Mucosal metabolism in ulcerative colitis; a reappraisal of the butyrate hypothesis
Clin Sci 1992 83; 17p-18p

Finnie I.A., Taylor B.A., Rhodes J.M.
Ileal and colonic epithelial metabolism in quiescent ulcerative colitis; increased glutamine metabolism in distal colon but no defect in butyrate metabolism.
Gut 1993 34 1552-1558

Fiocchi C.
Immune events associated with inflammatory bowel disease.
Scand J Gastroenterol 1990 (Supp172)4-12

Fischer E.
Bildung von Methylenblau als Reaction auf Schwefelwasserstoff.
Chem. Ber. 1883 26; 2234-36 (quoted in Cline 1969)

Flatt R.E., Weisbroth S.H., Kraus A.L.
Metabolic, traumatic, mycotic and miscellaneous diseases of rabbits. In "The Biology of the Laboratory Rabbit" Weisbroth S.H., Flatt R.E., Kraus A.L. (editors). Academic Press, New York, 1974. pp 35-51

Fleming S.E., Fitch M.D., DeVries S., Liu M.L. Kight C.
Nutrient utilisation by cells isolated from rat jejunum, caecum and colon.
J. Nutr. 1991 121;869-78

Florin T.H.J., Gibson G.R., Neale G., Cummings J.H.
A role for sulphate reducing bacteria in ulcerative colitis?
Gastroenterology 1990 98; A170

Florin T.H.J.
Hydrogen sulphide and total acid volatile sulphide in faeces, determined with a direct spectrophotometric method.
Clin. Chim. Acta. 1991a 196; 127-134

Florin T., Neale G., Gibson G.R., Christl S.U., Cummings J.H.
Metabolism of dietary sulphate; absorption and excretion in humans.
Gut 1991b 32; 766-73

Florin T.H.J., Neale G., Goretski S., Cummings J.H.
The sulphate content of food and beverages.
Journal of Food Composition and Analysis 1993 6; 140-51

Florin T.H.J., Khalil D., Lenarczyk A., Cowley D.M.
Sulphur metaboloism in colons of carrageenan fed rats.
J. Gastroenterol. Hepatol. 1995 10; A56

Fonkalsrud E.W., Phillips J.D.
Reconstruction of malfunctioning ileoanal pouch procedures as an alternative to permanent ileostomy.
Am. J. Surg. 1990 160; 245-48

Fozard B.J., Pemberton J.H.
Results of pouch surgery after ileo anal anastomosis; the implications of pouchitis
World J Surg 1992 16;880-884

Franklin W.A., McDonald G.B., Stein H.O., Gatter K.C., Jewell D.P., Clarke L.C., Mason D.Y.
Immunohistologic demonstration of abnormal colonic crypt cell kinetics in ulcerative colitis
Hum Pathol 1985 16; 1129-1132

Frankenberg L.
Enzyme therapy in cyanide poisoning; effect of rhodanese and sulphur compounds.
Archs. Toxic. 1980 45; 315-23

Frizzell R.A., Markschied-Kaspi L., Schultz S.G.
Oxidative metabolism of rabbit ileal mucosa.
Am. J. Physiol. 1974 226; 1142-48

Garcia R.A., Stipanuk M.H.
The splanchnic organs, liver and kidney have unique roles in the metabolism of sulphur amino acids and their metabolites in the rat.
J. Nutr. 1992 122; 1693-1701

Gardiner K.R., Erwin P.J., Anderson N.H., Barr JG Halliday MI Rowlands BJ
Colonic bacteria and bacterial translocation in experimental colitis
Br J Surg 1993 80; 512-516

Gibson G.R., Cummings J.H., MacFarlane G.T.
Competition for hydrogen between sulphate reducing bacteria and methanogenic bacteria from the human large intestine.
J.Appl. Bacteriol. 1988a 65; 241-247

Gibson G.R., MacFarlane G.T., Cummings J.H.
Occurrence of sulphate reducing bacteria in human faeces and the relationship to dissimilatory sulphate reduction to methanogenesis in the gut.
J. Appl. Bacteriol. 1988b 65; 103-111

Gibson G.R., Cummings J.H., MacFarlane G.T.
Use of a three stage continuos culture system to study the effect of mucin on dissimilatory sulphate reduction and methanogenesis by mixed populations of human gut bacteria.
Applied and Environmental Microbiology 1988c 54; 2750-55

Gibson G.R.
Physiology and ecology of the sulphate reducing bacteria.
J. Appl. Bacteriol. 1990a 69; 769-97

Gibson G.R., Cummings J.H., Allison C., Segal I, Vorster H.H., Walker A.R.P.
Alternative pathways for hydrogen disposal during fermentation in the human colon.
Gut 1990b 31;679-683

Gibson G.R., Cummings J.H., MacFarlane G.T.
Growth and activities of sulphate reducing bacteria in gut contents of healthy subjects and patients with ulcerative colitis.
FEMS Microbiology Ecology 1991 86;103-12

Gibson G.R., MacFarlane G.T., Cummings J.H.
Sulphate reducing bacteria and hydrogen metabolism in the human large intestine.
Gut 1993 34; 437-39

Gibson P.R., Jewell D.P.
Local immune mechanisms in inflammatory bowel disease and colorectal carcinoma; natural killler cells and their activity.
Gastroenterology 1986 90;12-19

Gibson P.R., Vandepol E., Barratt P.J., Doe W.J.
Ulcerative colitis; a disease characterised by the abnormal colonic epithelial cell?
Gut 1988c 29; 516-521

Gibson P.R., Pavli P.
Pathogenic factors in inflammatory bowel disease.
Dig Dis 1992 10;17-28

Gillespie J.H., Timoney J.F.
"Hagen and Bruers Infectious Diseases of Domestic Animals" 7th edition Cornell University Press, Icatha , New York. 1981

Gitnick G.
Etiology of inflammatory bowel diseases; Where have we been? Where are we going?
Scand J Gastroenterol 1990 (supp 175) 93-96

Glick M.E., Falchuk Z.M.
Dintrochlorobenzene induced colitis in the guinea pig; studies of colonic lamina propria lymphocytes.
Gut 1981 22 120-25

Glotzer D.J., Glick M.E., Goldman H.
Proctitis and colitis following diversion of the faecal stream.
Gastroenterology 1981 80; 438-41

Gorozny J., Weyand C.M., Waase I.
T cell subpopulations in inflammatory bowel disease; evidence for defective induction of T8+ suppressor/cytotoxic T lymphocytes.
Clin Exp Immunol 1985 61; 593-600

Granlund-Edstedt M., Johansson E., Claesson R., Carlsson J.
Effect of sulphide ions on complement factor 3.
Infect. Immun. 1991 59; 696-99

Granlund-Edstedt M., Johansson E., Claesson R., Carlsson J.
Effect of anaerobiosis and sulphide on lilling of bacteria by polymorphonuclear leukocytes.
J. Periodont Res.1993 28; 346-53

Grisham M.B., Hernandez L.A., Granger D.N.
Xanthine oxidase and neutrophil infiltration in intestinal ischaemia
Am J Physiol 1986 251; G567-G574

Gunina A.I.
Transformation of sulfur 35 labelled hydrogen sulfide introduced into blood.
Dokl. Akad. Nauk. SSR.1957 112; 902
(quoted in Beauchamp 1984)

GutmannI., Wahlefeld A.W.
L-(+)-lactate. Determination with lactate dehydrogenase and NAD. In "Methods of Enzymatic Analysis" Bergmeyer H.U. (editor) Vol. 3 Academic Press, New York 1974. pp 1463-68

Habib N.A., Dawson P.M., Krause T., Blount M.A., Kersten D., Wood C.B.
A study of histochemical changes in mucus from patients with ulcerative colitis, Crohns disease and diverticular disease of the colon
Dis Colon Rectum 1986 29; 15-17

Haddad P.R., Heckenberg A.L.
Trace determination of sulphide by reversed phase ion interaction chromatography using pre-column derivatisation.
J. Chromatogr. 1988 447; 415-20

Haggard H.W., Henderson Y., Charlton T.J.
The influence of hydrogen sulphide on respiration.
Am. J. Physiol. 1922 61; 289 (quoted by Beauchamp 1984)

Hanauer S.B.
Inflammatory Bowel disease re-visited; Newer drugs
Scand J Gastroenterol 1990 (supp 175) 97-106

Hanson P.J., Parsons D.S.
Factors affecting the utilisation of ketone bodies and other substrates by rat jejunum; effects of fasting and diabetes
J. Physiol. 1978 278; 55-67

Harmer R.C., Maika S.D., Richardson J.A. Tang J.P., Taurog J.D.
Spontaneous inflammatory disease in transgenic rats expressing HLA B27 and human beta-2 microglobulin; An animal model of HLA B27 associated human disorders.
Cell 1990 63; 1099-1112

Harig J.M., Soergel K.H., Komorowski V.R.A., Wood C.M.
Treatment of diversion colitis with short chain fatty acid irrigation.
New Engl. J. Med. 1989 320; 23-28

Hague A., Elder D.J.E., Hicks D.J., Parasekeva C.
Apoptosis in colorectal tumour cells; induction by the short chain fatty acids butyrate, propionate and acetate and by the bile salt deoxycholate.
Int. J. Cancer 1995 60; 400-406

Hawkey C.J., Boughton-Smith N.K., Whittle B.J.R.
Modulation of human colonic arachadonic acid metabolism by sulfasalazine.
Dig. Dis. Sci. 1985 30; 1161-1165

Hawkins C.
Historical Review. In "Inflammatory Bowel Diseases" (Allen R.N., Keightley M.R.B., Alexander-Williams J., Hawkins C. editors) 1983, pp 1-7. Churchill Livingstone, London

Hayden L.J.
Hydrogen sulphide in biochemical systems.
Proceedings of the International Conference on Hydrogen Sulphide Toxicity Banff 1984. (Prior M.G., Roth S.H., Green F., Hulbert W., Reiffenstein R. editors) pp 91-97

Hayman E.G., Envall E., Ruoslahti E.
Butyrate restores fibronectin at cell surface in transformed cells.
Exp. Cell Res. 1980 127; 478-81

Henning S.J., Hird F.J.R.
Ketogenesis from butyrate and acetate in the caecum and colon of rabbits.
Biochem. J. 1972 130; 785-90

Hermanowicz A., Gibson P.R., Jewell D.P.
The role of phagocytes in inflammatory bowel disease.
Clin Sci 1985 69; 241-249

Hill G.L.
Ileostomy Function. . In "Inflammatory Bowel Diseases" (Allen R.N., Keightley M.R.B., Alexander-Williams J., Hawkins C. editors) 1983, pp 73-77. Churchill Livingstone, London

Hodgson H.J.F., Potter B.J., Jewell D.P.
Immune complexes in ulcerative colitis and Crohn's disease
Clin Exp. Immunol. 1977 29;187-196

Hodgson H.J.F., Potter B.J., Skinner J., Jewell D.P.
Immune complex mediated colitis in rabbits.
Gut 1978 19; 225-232

Hollander D., Vadleim C., Bretholz E., Petersen C.M., Delahunty T., Rotter J.I.
Increased intestinal permeability in patients with Crohn's disease and their relatives; a possible aetiologic factor.
Ann. Int. Med. 1986 105; 883-85

Hosakawa Y., Shimomura Y., Harris R.A., Ozawa T.
Determination of short chain acyl-coenzyme A esters by high performance liquid chromatography.
Anal. Biochem. 1986 153; 45-49

Hoskins D.
The electron transporting flavoprotein as a common intermediate in the mitochondrial oxidation of butyryl Coenzyme A and sarcosine.
J.Biol. Chem 1966 241; 4472-4479

Hovik R., Brodal B., Bartlett K., Osmundsen H.
Metabolism of acetyl CoA by isolated peroxisomal fractions; formation of acetate and acetoacetyl CoA.
J. Lipid Res. 1991 32; 993-99

International Classification of Diseases, Ninth Revision, Clinical Modification ; ICD-9 CM.2nd edition, 1980. U.S. Dept. Health and Human Services, Washington.

Ireland A., Jewell D.P.
5-aminosalicylic acid (5ASA) has no effect on butyrate metabolism in human colic epithelial cells.
Gastroenterology 1990 98; A176 (abstract)

Ishioka T., Kuwabara N., Oohashi Y., Wakabayashi K.
Induction of colorectal tumours in rats by sulphated polysacharrides
C.R.C. Critical Reviews in Toxicology 1986 17; 215-244

Iyengar R., Stuehr D.J., Marletta M.J.
Macrophage synthesis of nitrite, nitrate and N-nitrosamines; Precursors and the role of the respiratory burst.
Proc. Natl. Acad. Sci. 1987 84; 6369-73

Jacobsson S., Falk O.
Determinationof hydrogen sulphide by porous layer open tubular gas chromatography-mass spectroscopy.
J. Chromatogr. 1989 479; 194-99

Jakoby W.B., Ziegler D.M.
The enzymes of detoxification
J. Biol. Chem. 1990 265; 20715-20718

Jenkins R.T., Goodacre R.L., Rooney P.J., Bienenstock J., Sivakumaren T., Walker W.H.C.
Studies of intestinal permeability in niflammatory bowel diseases using polyethylene glycol 400.
Clinical Biochemistry 1986 19; 298-302

Jenkins D., Goodall A., Drew K., Scott B.B.
What is colitis? Statistical approach to distinguishing clinically important inflammatory change in rectal biopsy specimens.
J Clin Pathol 1988 41; 72-79

Jenkins D., Goodall A., Scott B.B. Ulcerative colitis; one disease or two? (quantitative histological differences between distal and extensive disease).
Gut 1990 31; 426-430

Jennings M.A., Florey H.W.
Autoradiographic observations on the mucous cells of the stomach and intestine.
Quart. J. Exp. Physiol. 1956 41; 131-52

Jewell D.P., Patel C.
Immunology of inflammatory bowel disease.
Scand J Gastroenterol (supp) 1985 114; 119-126

Johnson P.W., Yaegaki K., Tonzetich J.
Effect of volatile thiol compounds on protein metabolism by human gingival fibroblasts.
J. Periodont. Res. 1992 27; 553-61

Jorgensen B.B.
A comparison of methods for the quantification of bacterial sulphate reduction in coastal marine sediments; 1. Measurement with radiotracer techniques.
Geomicrobiology Journal 1978 1; 11-27

Jubb K.V.F., Kennedy P.C.,
Pathology of domestic amnimals Vol 2, Academic Press Inc., New York. pp 119

Kadota H., Ishida Y.
Production of volatile sulphur compounds by microorganisms
Annu. Rev. Microbiol. 1972 26; 127-38

Kage S., Nagata T., Kimura K., Kudo K.
Extractive alkylation and gas chromatographic analysis of sulphide.
J. Forensic Sci. 1988 33; 217-22

Kameyama J., Narui H., Inui M., Sato T.
Energy levels in large intestinal mucosa in patients with ulcerative colitis.
Tohuku J. Exp. Med. 1984 143; 253-54

Keith R.A., Van Loon J., Wussow L.F., Weinshilboum R.M.
Thiol methyl pharmacogenetics; heritability of human erythrocyte thiol methyltransferase activity.
Clin. Pharmac. Ther. 198334; 521-28

Kennedy P.C., Cello R.M.
Colitis of boxer dogs
Gastroenterology 1966 51(5); 926-29

Khalil D., Florin T.H.J., Lenarczyk A., Moody S.A., Cowley D.M.
Colonic mucosal metabolism of sulphur in carrageenan fed rats.
J. Gastroenterol. Hepatol 1995 10; A29

Khan A.
Biochemical effects of hydrogen sulphide.
Proceedings of the International Conference on Hydrogen Sulphide Toxicity Banff 1984. (Prior M.G., Roth S.H., Green F., Hulbert W., Reiffenstein R. editors) pp 139-56

Kirsner J.B.
Experimental "colitis" with particular reference to hypersensitivity reactions in the colon.
Gastroenterology 1961 40; 307-312

Kirsner J.B.
Inflammatory bowel disease; Nature and pathogenesis.
Disease a Month 1991 37; 609-66

Kmiot W.A., Hesselwood S.R., Smith N., Thompson H., Harding L.K., Keighley M.R.B.
Evaluation of the inflammatory infiltrate in pouchitis with 111In labelled granulocytes
Gastroenterology 1993 104; 981-988

Kmiot W.A., Youngs D., Tudor R., Thompson J., Keighley M.R.B.
Mucosal morphology, cell proliferation and faecal bacteriology in acute pouchitis
Br J Surg 1993 80; 1445-1449

Kolten W.A., Smith R.J., Loehner D., Durdy P., Coller J.A.et al
Alteration in intestinal permeability after ileal anal pouch anastomosis.
Dis Colon Rectum 1993 36; 922-926

Koltun W.A., Schoetz D.J. Jr., Roberts P.L., Murray J.J., Coller J.A., Veidenheimer M.C.
Indeterminate colitis predisposes to perineal complications after ileal pouch-anal anastomosis.
Dis. Colon Rectum 1991 34; 857-60

Kombian S.B., Warenycia M.W., Mele F.G., Reiffenstein R.J.
Effects of acute intoxication with hydrogen sulphide on central amino acid transmitter systems.
Neurotoxicology 1988 9; 587-596

Koo A., Leung F.W.
Microcirculation in the colon; Macromolecular permeability and loss of endothelial viability in experimental colitis in rats.
Prog. Appl. Microcirc. 1990 17; 90-100

Koopman B.J., Jansen G., Wolthers B.G.
Determination of inorganic sulphate in plasma by reversed phase chromatography using ultra violet detection and its application to plama samples of patients receiving different types of haemodialysis.
J. Chromatogr. 1985 337; 259-66

Korelitz B.I., Sommers S.C.
Responses to drug therapy in ulcerative colitis; evaluation by rectal biopsy and mucosal cell counts
J. Clin. Gastroenterol. 1984 6; 123-7

Kraft S.C., Fitch F.W., Kirsner J.B.
Histologic and immunohistochemical features of "Auer" colitis in rabbits.
Am. J. Pathol. 1963 43; 913-23

Kristjansson K.J., Schonheit P., Thauer R.K.
Different Ks values for hydrogen of methanogenic bacteria and sulphate reducing bacteria; an explanation for the apparent inhibition of methanogenesis by sulphate.
Arch. Microbiol. 1982 131; 278-82

Kruh J.
Effects of sodium butyrate, a new pharmacologic agent, on cells in culture.
Mol. Cell Biochem. 1982 42; 65-82

Kuhn R., Lohler J., Rennick D., Rajewsky K., Muller W.
IL-10 deficient mice develop chronic enterocolitis
Cell 1993 75; 263-74

Kulwich R., Struglia L., Pearson P.B.
Metabolic fate of $^{35}S$ labelled sulphate in baby pigs.
P.S.E.B.M. 1958 97; 408-10

Lajoie S.F., Bank S., Miller T.L., Wolin M.J.
Acetate production from hydrogen and [$^{13}C$] carbon dioxide by the microflora of human faeces.
Appl. Environ. Microbiol. 1988 5; 2723-27

Lamers J.M.J., Hulsmann W.C.
Pasteur effect in the in vitro vascularly perfused rat small intestine.
Biochim. Biophys. Acta. 1972 275; 491-95

Langman J.M., Rowland R., Vernon-Roberts B.
Carageenan colitis in the guinea pig; pathological changes and the importance of ascorbic acid deficiency in disease induction.
Aust J Exp Biol Med Sci 1985 63; 545-553

Lapin B.A., Yakovleva L.A.
Colitis in Siamang gibbons. In "Comparative Pathology in Monkeys" C.C. Thomas (pub)
Springfield, Illinois, 1962. pp11-39

Lashner B.A.
Red cell folate is associated with the development of dysplasia and cancer in ullcerative colitis.
J. Cancer Res. Clin. Oncol. 1993 119; 549-54

Lauritsen K., Laursen L., Bukhave K., Rask-Madsen J
In vivo profiles of eicosanoids in ulcerative colitis, Crohns colitis and Clostrdium difficile colitis.
Gastroenterology 1988 95; 11-17

Lauterbach F., Czekay R., Sund R.B.
Compartmentation of intestinal drug sulphoconjugation; Incorporation of luminal and contraluminal [$^{35}$S]sulphate into 1-napthol by the isolated mucosa of guinea pig jejunum and colon.
Biochem. Pharmacol. 1993 46; 1339-1347

Leggett Bailey J.
Estimation of protein. In "Techniques in Protein Chemistry" Leggett Bailey J. (editor) Chapter 11
Elsevier, New York, 1967

Leder A., Leder P.
Butyric acid, a potent inducer of erythroid differentiation in cultured erythroleukaemic cells
Cell 1975 5; 319-22

Lester R.G., Grim E.
Substrate utilisation and oxygen consumption by canine jejunal mucosa in vitro.
Am. J. Physiol. 1975 229; 139-43

Lev R., Spicer S.S.
Specific staining of sulphate groups with Alcian blue at low pH.
J. Histochem. Cytochem. 1964 12; 309

Leyh T.S.
The physical biochemistry and molecular genetics of sulphate activation.
Crit. Rev. Biochem. Mol. Biol. 1993 28; 515-42

Liau Y.H., Horowitz M.I.
The importance of PAPS in determining sulphation in gastrointestinal mucosa.
Digestion 1976 14; 372-75

Lih-Brody L., Geradi F., ILyia E et al
Protein oxidative products are increased in the mucosal lesions of inflammatory bowel disease.
Gastroenterology 1994 106; A720

Lillard D.W., Seib P.A.
Monosulphate esters of ascorbic acid. In "Carbohydrate Sulphates" ACS Symposium 77,
Schweiger R. (editor). American Chemical Co., Washinton. 1978, pp1

Little J.R., Eisen H.N.
Preparation and characterisation of antibodies specific for the 2,4,6, trinitrophenyl group
Biochemistry 1966 5; 3385-95

Loftus E.V., Tremaine W.J., Nelson R.A., Shoemaker J.D., Sandborn W.J., Batts K.P.
Topical pantotheinol does not affect fecal short chain fatty acid levels or colonic Coenzyme A
levels in patients with active ulcerative colitis.
AGA/AASLD meeting San Diego 1995 (abstract)

Lohmuller J.L., Pemberton J.H., Dozois R.R., Ilstrup D., van Heerden J.
Pouchitis and extraintestinal manifestations of inflammatory bowel disease after ileal pouch anal
anastomosis.
Ann. Surg. 1990 211; 622-27

Lowry O.H. Rosebrough N.J., Farr A.L., Randall R.J.
Protein measurement with the Folin phenol agent.
J. Biol. Chem. 1951 193; 265-75

Lopez A.
Nasal lesions exposed for four hours to atmospheres containing 0, 14, 280 or 560 mg / $m^3$ $H_2S$.
Am. J. Vet. Res. 1988a 49; 1107-379

Lopez A., Prior M., Lillie L.E., Gulayets C., Atwal O.S.
Histologic and ultrastructural alterations in the lungs of rats exposed to sublethal concentrations
of hydrogen sulphide.
Vet Pathol. 1988b 25; 376-84

Lucke H., Stange G., Murer H.
Sulphate-sodium cotransport by brush border membrane vesicles isolated from rat ileum.
Gastroenterology 1981 80; 22-30

Lund J., Stjernstrom H., Vinnars E., Jorfeldt L., Bergholm U., Wiklund L.
The influence of abdominal surgery on the splanchnic exchange of amino acids.
Acta Chir. Scand. 1986 152; 191-97

Lutz J., Linkswiler H.M.
Calcium metabolism in postmenopausal women consuming two levels of dietary protein.
Am. J. Clin. Nutr. 1981 34; 2178-86

MacDermott R.F., Stenson W..F.
Alterations of the immune system in ulcerative colitis and Crohn's disease.
Advances in Immunology 1988 42; 285-28

MacPherson A.J.S., Khoo U.Y., Bjarnason I.
Inflammatory bowel disease of mice- and men?
Lancet 1994 343; 130-131

MacFarlane G.T., Cummings J.H., Allison C.
Protein degradation by human intestinal bacteria
J. Gen. Microbiol 1986a 132; 1647-56

MacFarlane G.T., Allison C.
Utilisation of protein by human gut bacteria.
FEMS Microbiol. Ecol. 1986b 38; 19-24

MacFarlane G.T., Allison C., Gibson S.A.W., Cummings J.H.
Contribution of the microflora to proteolysis in the human large intestine.
J. Appl. Bacteriol. 1988 64; 37-46

MacFarlane G.T., Gibson G.R., Cummings J.H.
Comparison of fermentation reactions in different regions of the human colon.
J. Appl. Bacteriol. 1992 72; 57-64

MacPherson B.R., Pfeiffer C.J.
Experimental production of diffuse colitis in rats.
Digestion 1978 17; 135-150

Madara J.L., Podolsky D.K., King N.W., Sehgal P.K., Moore R., Winter H.S.
Characterisation of spontaneous colitis in cotton top tamarins (*Sagiunus oedipus*) and its response to sulfasalazine
Gastroenterology 1985 88; 13-19

Mahida Y.R., Patel S., Gionghetti P., Vaux D., Jewell D.P.
Macrophage subpopulations in lamina propria of normal and inflamed colon and terminal ileum
Gut 1989 30; 826-834

Mangani F., Bruner F.
Interferences in determination of hydrogen sulphide in air by gas chromatography with flame photometric detection.
Anal Chem. 1983 55; 2193-94

Marangella M., Petrarulo M., Cosseddu D., Vitale C., Linari F.
Plasma profiles and removal rates of inorganic sulphate, and their influence on serum ionized calcium, in patients on maintainance haemodialysis
Clin. Sci. 1991 80; 489-495

Marko-Varga G., Csiky I., Jonsson J.A.
Ion chromatographic determination of nitrate and sulphate in natural waters containing humic substances.
Anal. Chem. 1984 56; 2066-69

Marcus R., Watt J.
Seaweeds and ulcerative colitis in laboratory animals.
Lancet 1969 2; 489-90

Marin M.L., Greenstein A.J., Geller S.A., Gordon R.E., Aufses A.H.
A freeze fracture study of Crohn's disease of the terminal ileum; changes in epithelial tight junction organization.
Am. J. Gastroenterol. 1983 78; 537-47

Matthews D.M.
Protein absorption; Development and present state of the subject.
1991 Wiley Liss, New York. pp 218-24

Maxton D.G., Bjarnason I., Reynolds A.P., Catt S.D. Peters T.J., Menzies I.S.
Lactulose Cr51 labelled ethylenediaminetetra-acetate, L-rhamnose and polyethylene glycol 500 as probe markers for assessment in vivo of human intestinal permeability.
Clin. Sci. 1986 71; 71-80

McDermott R.P., Bragdon M.J., Kodner I.J., Bertovich M.J.
Deficient cell-mediated cytotoxicity and hyporesponsiveness to interferon and mitogenic lectin activation by inflammatory bowel disease peripheral blood and intestinal mononuclear cells
Gastroenterology 1986 90; 6-11

McDonald G.B., Jewell D.P.
Class 2 antigen (HLA-DR) expression by intestinal epithelial cells in niflammatory bowel diseasees of the colon
J Clin Pathol 1987 40; 312-317

McIntyre A., Young G.P., Taranto T., Gibson P.R., Ward P.B.
Different fibres have different regional effects on luminal contents of rat colon.
Gastroenterology 1991 105; 1274-81

McIntyre P.B., Macrae F.A., Berghouse L., English J., Lennard-Jones J.E.
Theraputic benefits from a poorly absorbed prednisolone enema in distal colitis.
Gut 1985 26; 822-24

McKay L.F., Eastwood M.A., Brydon W.G.
Methane excretion in man- a study of breath, flatus and faeces.
Gut 1985 26; 69-74

Mayall T., MacPherson A., Bjarnason I., Forgacs I., Peters T.
Mitochondrial function of colonic epithelial cells in inflammatory bowel disease.
Gut 1992 33; S24

Mee A.S., McLauchlin J.E., Hodgson H.J.F., Jewell DP.
Chronic immune colitis in rabbits.
Gut 1979 20; 1-5

Melanby J., Williamson D.H.
Acetoacetate. In "Methods of Enzymatic Analysis" Bergmeyer H.U. (editor) Academic Press, New York 1974. Vol. 4 pp 1840-43

Mian N., Anderson C.E., Kert P.W.
Effect of O-sulphated groups in lactose and n-acetyl neuraminyl-lactose on their enzymic hydrolysis.
Biochem. J. 1979 181; 387-99

Michal G., Bergmeyer H.U.
Co Enzyme A. In "Methods of Enzymatic Analysis" Bergmeyer H.U. (editor) Academic Press, New York 1974 Second edn, Volume 4 pp 1967-87

Miller D.L., Schedl H.P., Bouska J., Phillips S.F.
Food restriction and recovery of nonabsorbed indicators from the small intestine of the rat.
Digestion 1987 38; 83-89

Miller E., Hlad C.J., Levine S., Holmes J.H., Elrick H.
The use of radioisotopes to measure body fluid constituents. 1. Plasma sulphate.
J. Lab. Clin. Med. 1961 58; 656-61

Miller R.S., Hoskins L.C.
Mucin degradation in human colon ecosystems. Fecal population densities of mucin degrading bacteria as measured bt a "most probable number" method.
Gastroenterology 1981 81;759-65

Miller T., Wolin M.J.
Enumeration of Methanobrevibacter smithii in human faeces.
Arch. Microbiol. 1982 131; 14-18

Michalk D., Manz F.
Determination of inorganic sulphate by indirect atomic absorption spectrophotometry.
Clin. Chim. Acta 1980 107; 43-48

Mitchell T.W., Savage J.C., Gould D.H.
High performance liquid chromatography detection of sulphide in tissues from sulphide treated mice
Journal Of Applied Toxicology 1993 13; 389-394

Mombaerts P., Mizoguchi E., Grusby M.J. Glimcher L.H., Bahn A.K., Tonegawa S.
Spontaneous development of inflammatory bowel disease in T cell receptor mutant mice.
Cell 1993 75; 275-82

Mopper K., Delmas D.
Trace determination of organic thiols by liquid chromatography and precolumn flurometric labelling with o-phthalaldehyde.
Anal Chem. 1984 56; 2557-60

Morris G.P., Beck P.L., Herridge M.S.,Depew W.T., Szewczuk M.R., Wallace J.L.
Hapten induced model of chronic inflammation and ulceration in the rat colon
Gastroenterology 1989 96; 795-803

Morris M. Levy G.
Serum concentration and renal excretion by normal adults of inorganic sulphate after acetaminophen, ascorbic acid or sodium sulphate.
Clin. Pharmacol. Ther. 1983 33; 529-36

Mottot N.K.
Carrageenan ulceration as a model for ulcerative colitis.
Lancet 1970 (2); 1361

Murthy S.N., Doble A,.T.A., Silver D., Lauderdale C.
A theraputic comparison of ad libitum and enteric metronidazole in dextran sulphate mediated colitis in mice.
Gastroenterol. 1994 106; A741

Nairn R.C., Savvas R., Hocking G., Kovala M., Rolland J.M.
Immunoreactive inflammation in fetal colon implants in syngeneic rats.
Am. J. Pathol. 1979 96; 647-50

Nasmyth D.G., Godwin P.G.R., Dixon M.F., Williams N.S., Johnson D
Ileal ecology after pouch anal anastomosis or ileostomy; a study of mucosal morphology, faecal bacteriology, faecal volatile fatty acids and their interrelationship
Gastroenterology 1989 96; 817-24

Ng W., Tonzetich J.
Effect of hydrogen sulphide and methyl mercaptan on the permeability of oral mucosa.
J. Dent. Res. 1984 63; 994-97

Nicholls P.
The effect of sulphide on cytochrome aa3. Isosteric and allosteric shifts of the reduced a-peak.
Biochim. Biophys. Acta. 1975 396; 24-35

Nicholls R.J.
Pouchitis Workshop;Clinical Diagnosis
Int. J. Colorect. Dis. 1989 4; 205-29

Nuguid T.P., Bacon H.E., Boutwell J.
An investigation of the volume of output and chemical content of ileal discharges following total colectomy and ileostomy.
Surg. Gynecol. Obstet. 1961 113; 733-42

Ohkusa T., Yamada M., Takenaga T., Kitazuma C., Yamamoto M., Sesabe M.et al
Protective effect of metronidazole in experimental ulcerative colitis induced by dextran sulphate sodium.
Jpn. J. Gastroenterol 1987 84; 2337-46

Okabe S., Pfeiffer C.J., Roth J.L.A.
A method of producing penetrating gastric and duodenal ulcers in rats.
Am. J. Dig. Dis. 1971 16; 277-284

Okayasu I., Hatakeyama S., Yamada M., Ohkusa T Inagaki Y Nakaya R
A novel method in the induction of reliable experimental acute and chronic ulcerative colitis in mice.
Gastroenterology 1990 98; 694-702

Olaison G., Sjodahl R., Tagesson C.
Decreased absorption of peroral polyethylene glycol (PEG 1000)in Crohn's disease.
Acta Chir. Scand. 1987 153; 373-77

Olaison G., Leanderssen P., Sjodahl R., Tagesson C.
Intestinal permeability to polyethylene glycol (PEG 600) in Crohn's disease. Peroperative determination in a defined segment of small intestine.
Gut 1988 29; 196-99

Olaison G., Sjodahl R., Leanderssen P., Tagesson C.
Abnormal intestinal permeability pattern in colonic Crohn's disease.
Scand. J. Gastroenterol. 1989 24; 571-76

Olaison G., Sjodahl R., Tagesson C.
Abnormal intestinal permeability in Crohn's disease.
Scand. J. Gastroenterol. 1990 25; 321-28

O'Morain C., Segal A.W., Walker D., Levi A.J.
Abnormalities of neutrophil function do not cause the migration defect of Crohn's disease.
Gut 1981 22; 817-22

Onderdonk A.B. Hermos J.A., Bartlett J.G.
The role of intestinal microflora in experimental colitis.
Am. J. Clin. Nutr. 1977 30; 1819-25

Onderdonk A.B., Hermos J.A., Dzink J.L., Bartlett J.G.
Protective effect of metronidazole in experimental colitis.
Gastroenterology 1978 74; 521-26

Onderdonk A.B. Franklin M.L., Cisneros R.L.
Production of experimental ulcerative colitis in gnotobiotic guineapigs with simplified microflora.
Infect. Immun. 1981 32; 225-31

Onderdonk A.B., Cisneros R.L., Bronson R.T.
Enhancement of experimental ulcerative colitis by immunisation with bacteroides vulgatus.
Infect. Immun. 1983 42; 783-88

Onderdonk A.B.
Experimental models for ulcerative colitis.
Dig Dis Sci 1985(supp) 30 40S-44S

Ouyang Q., El-Youseff M., Yen-Lieberman B., Sapatnekar.W., Youngman K.R., Kusagami K., Fiocchi C.
Expression of HLA-DR antigens in inflammatory bowel disease mucosa; role of intestinal lamina propria mononuclear cell derived interferon gamma
Dig Dis Sci 1988 33; 1528-1536

Pacheco S., Hillier K., Smith S.
Increased arachadonic acid levels in phospholipids of human colonic mucosa in inflammatory bowel disease.
Clin. Sci. 1987 73; 361-64

Pacifici G.M., Franchi M., Colizzi C., Giuliani L., Rane A.
Sulphotransferase in humans; development and tissue distribution.
Pharmacology 1988 36; 411-419

Pacifici G.M., Santerini S., Guiliani L., Rane A.
Thiol methyltransferase in humans; Development and tissue distribution.
Dev. Pharmacol. Ther. 1991 17; 8-15

Pacifici G.M., Romiti P., Santeri S., Giuliani L.
S methyltransferases in human intestine; differential distribution of the microsomal thiol methyltransferase and cytosolic thiopurine methyltransferase along the human bowel.
Xenobiotica 1993 23; 671-679

Painter R.G., Sklar L.A., Jesaitis A.J., Schmitt M., Cochrane C.G.
Activation of neutrophils by N-formyl chemotactic peptides.
Fed Proc. 1984 43; 2737-2742

Pascoe P.J., Peake M.J., Walmsley R.N.
Determinationof inorganic sulphate in plasma with a centrifugal analyzer.
Clin. Chem. 1984 30; 275-77

Pazmino P.A., Weinshilboum R.M.
Human erythrocyte phenol-O-methyltransferase; radiochemical microassay and biochemical properties.
Clin. Chim. Acta. 1978 89;317-29

Peck H.D.
Some evolutionary aspects of inorganic sulphur metabolism.
University of Maryland lectures on theoretical and applied aspects of modern microbiology. Baltimore, University of Maryland (quoted by Pfennig 1981)

Pena A.S.
Changing patterns of inflammatory bowel diseases.
Scand J Gastroenterol 1990 (supp172) 1-3

Peppercorn M.A., Goldman P.,
Distribution studies of salicylazosulfapyridine and its metabolites.
Gastroenterology 1973 64; 240-45

Persson S.
Hydrogen sulphide and methyl mercaptan in periodontal pockets.
Oral Microbiol. Immunol. 1992 7; 378-79

Peterson L.C.
The effect of inhibitors on the oxygen kinetics of cytochrome c oxidase.
Biochim. Biophys. Acta. 1977 460; 299-307

Pfeiffer C.J.
Animal models of colitis. In "Animal Models of Intestinal Disease" Pfeiffer C.J. (editor). CRC Press, Boca Raton, 1979. pp 147-57

Pfennig N., Widdel F.
Ecology and physiology of some anaerobic bacteria from the microbial sulphur cycle. In "Biology of Inorganic Nitrogen and Sulphur" (Bothe H., Trebst A., editors) 1981 Springer Verlag, Heidelberg. pp170-77

Pfennig N., Widdel F., Truper H.G.
The dissimilatory sulphate reducing bacteria.
In "The sulphate reducing bacteria" Postgate J.R. (editor). 2nd edition. pp 926-940. Cambridge University Press, Cambridge

Phillips R.K.S.
Pelvic pouches
Br J Surg 1991 78; 1025-1026

Piotrowski J., Slomiany A., Murty V.L.N., Fekete Z., Slomiany B.L.
Inhibition of Helicobacter pylorii colonisation by sulphated gastric mucin.
Biochem. Int. 1991 24; 749-56

Pitcher M.C.L., Gibson G.R., Neale G., Cummings J.H.
Gentamycin kills multiple drug resistant sulphate reducing bacteria in patients with ulcerative colitis.
Digestive Diseases Week 1994 A-506 2021 (abstract)

Pitcher M.C.L., Beatty E.R., Waring R.H., Cummings J.H.
Is there a defect in sulphur metabolism in ulcerative colitis?
Gut 1995a 37; A22 (abstract)

Pitcher M.C.L., Beatty E.R., Gibson G.R., Cummings J.H
Incidence and activities of sulphate reducing bacteria in patients with ulcerative colitis.
Gut 1995b 36 (suppl 1) A63

Pitcher M.C.L., Beatty E.R., Cummings J.H
Salicylates inhibit bacterial sulphide production within the colonic lumen in ulcerative colitis.
Gut 1995c 37 (suppl 2) A15

Pitt P., De Bruijn K.M., Beeching M.F., Goldberg E., Blendis L.M.
Studies on breath methane; the effect of ethnic origins and lactulose.
Gut 1980 21; 951-59

Pochart P., Done J., Lemann F., Goderel I., Rambaud J.
Interrelations between populations of methanogenic archaea and sulphate reducing bacteria in the human colon.
FEMS Microbiol. Lett. 1992 98; 225-28

Podolsky D.K., Isselbacher K.J.
Glycoprotein composition in colonic mucosa
Gastroenterology 1984 87;991-998

Podolsky D.K., Madara J.L., King N.Sehgal P., Moore R., Winter H.S.
Colonic mucin composition in primates ; Selective alterations associated with spontaneous colitis in the cotton top tamarin
Gastroenterology 1985 88; 20-25

Pomeroy R.
Auxiliary pretreatment by zinc acetate in sulphide analyses.
Anal. Chem. 1957 26; 571-72

Postgate J.R.
The sulphate reducing bacteria
2nd edition, 1984. Cambridge University Press, Cambridge.

Powell-Tuck J., Lennard Jones J.E., May C.S., et al
Plasma prednisolone levels after administration of prednisolone 21-phosphate as a retention enema in colitis.
Br. Med. J. 1976 1; 193-95

Prasad M.R., Sauter J., Lands W.E.M.
Quantative determination of acyl chain composition of subnanomole amounts of cellular long chain long chain acyl coenzyme A esters.
Anal. Biochem. 1987 162; 202-12

Price R.A., Keith R.A., Spielman R.S., Weinshilboum R.M.
Major gene polymorphism for human erythrocyte (rbc) thiol methyltransferase (TMT).
Genet. Epidemiol. 1989 6; 651-62

Pritchard P.J., Porteous J.W.
Steady state metabolism and transport of D-glucose by rat small intestine in-vivo.
Biochem. J. 1977 164; 1-14

Rabassa A.A., Rogers A.I.
The role of short chain fatty acid metabolism in colonic disorders
Am J Gastroenterol 1992 87 419-423

Rabin B.S., Rogers S.J.
A cell mediated immune model of inflammatory bowel disease in the rabbit.
Gastroenterology 1978 75; 29-33

Rachmilewicz D., Karmeli F., Eliakim R.
Platelet activating factor- A possible mediator in the pathogenesis of ulcerative colitis
Scand J Gastroenterol 1990 (supp 172) 19-21

Ramage J.K., Stanisz A., Scicchitano R., Hurt R.H., Purdue M.H.
Effect of immunologic reactions on rat intestinal epithelium. Correlation of increased permeability to Cr51 EDTA and ovalbumin during acute inflammation and anaphylaxis.
Gastroenterology 1988 9; 1368-75

Ramakrishna B.S., Roberts Thomson I.R., Pannall P.R., Roediger W.
Impaired sulphation of phenol by the colonic mucosa in quiescent and active ulcerative colitis.
Gut 1991 32; 46-49

Ramakrishna B.S., Roediger W.E.W.
Bacterial short chain fatty acids; their role in gastrintestinal disease.
Digestive diseases 1990 8; 337-345

Ramaswamy S.G., Jakoby W.B.
Sulphotransferase Assays. In "Methods in Enzymology" Bergmeyer H. (editor) Academic Press. N.Y. 1987 Vol 143 pp 201-17,

Raouf A.H., Tsai H.H., Parker N., Hoffmann J., Walker R.J., Rhodes J.M.
Sulphation of colonic and rectal mucin in inflammatory bowel disease; reduced sulphation of rectal mucus in ulcerative colitis.
Clin. Sci. 1992 83; 623-26

Rauh S.M., Schoetz D.J. Jr., Roberts P.L., Murray J.J., Coller J.A., Veidenheimer M.C.
Pouchitis; Is it a wastebasket diagnosis?
Dis. Colon Rectum 1991 34; 685-9

Reichert F.L., Mathes M.E.
Experimental lymphoedema of intestinal tract and its relation to regional cicatrising enteritis.
Ann. Surg. 1936 104; 601-16

Reiffenstein R.J.
Neurochemical and neurophysiological effects of high dose hydrogen sulphide.
Proceedings of the International Conference on Hydrogen Sulphide Toxicity Banff 1984. (Prior M.G., Roth S.H., Green F., Hulbert W., Reiffenstein R. editors) pp 117-123

Reiffenstein R.J., Hulbert W.C., Roth S.H.
Toxicology of hydrogen sulphide.
Annu. Rev. Pharmacol. Toxicol. 1992 32; 109-34

Reiter C., Muller S. Muller T.
Improved method for determination of sulphate in human serum using ion chromatography.
J. Chromatogr. 1987 413; 251-256

Rhodes J.M., Jewell D.P.
Motility of neutrophils and monocytes in Crohns disease and ulcerative colitis
Gut 1983 24; 73-77

Rhodes J.M., Gallimore R., Ellias E., Kennedy J.
Faecal sulphatase in health and inflammatory bowel disease.
Gut 1985 26; 466-469

Roediger W.E.W.
The functional activity of the colonic mucosa in health and in ulcerative colitis.
D. Phil. Thesis, Oxford 1979a

Roediger W.E.W., Truelove S. C.
Method of preparing isolated colonic epithelial cells (colonocytes) for metabolic studies
Gut 1979b 20; 484-488

Roediger W.E.W.
Role of anaerobic bacteria in the metabolic welfare of the colonic mucosa in man.
Gut 1980a 21; 793-798

Roediger W.E.W.
Utilisation of nutrients by isolated epithelial cells of the rat colon.
Gastroenterology 1980b 83; 424-29

Roediger W.E.W.
The colonic epithelium in ulcerative colitis; an energy deficient disease?
Lancet 1980c 2 712-15

Roediger W.E.W., Heyworth M., Willoughby P., Piris J., Moore A., Truelove S.C.
Lumenal ions and short chain fatty acids as markers of functional activity of the mucosa in ulcerative colitis.
J. Clin. Pathol. 1982 35; 323-26

Roediger W.E.W., Lawson M.J., Kwok V., Kerr-Grant A., Pannall P.R.
Colonic bicarbonate output as a test of disease activity in ulcerative colitis
J Clin Pathol 1984 37; 704-707

Roediger W.E.W., Nance S.H.
Metabolic induction of experimental colitis by inhibition of fatty acid oxidation.
Br.J. Exp. Pathol. 1986a 67; 773-

Roediger W.E.W., Radcliffe B.C., Deakin E.J., Nance S.H.
Specific metabolic defect of sodium nitrite on fat metabolism by mucosal cells of the colon.
Dig. Dis. Sci. 1986b 31; 535-39

Roediger W.E.W., Lawson M.J., Nance S.H., Radcliffe B.C.
Detectable colonic nitrite levels in inflammatory bowel disease- mucosal or bacterial malfunction.
Digestion 1986c 35; 199-204

Roediger W.E.W.
The role of colonic Mucosal metabolism in the pathogenesis of ulcerative colitis. In
"Inflammatory bowel diseases- basic research and clinical implications"
Falk symposium 46 (Goebell H Peskar BM Malchow H eds) Kluwer Academic Publishers 1987

Roediger W.E.W.
What sequence of pathogenetic events leads to ulcerative colitis
Dis Colon Rectum 1988 31; 482-48

Roediger W.E.W.
Faecal anions and lactate in severe ulcerative colitis
Dig Dis Sci 1989 34; 1801

Roediger W.E.W., Nance S.
Selective reduction of fatty acid oxidation in colonocytes; Correlation with ulcerative colitis
Lipids 1990 25; 646-652

Roediger W.E.W., Duncan A., Kapaniris O., Millard S.
Sulphide impairment of substrate oxidation in rat colonocytes; a biochemical basis for ulcerative colitis.
Clin. Sci. 1993a 85; 623-27

Roediger W.E.W., Duncan A., Kapaniris O., Millard S.
Reducing sulphur compounds of the colon impair colonocyte nutrition; implications for ulcerative colitis.
Gastroenterology 1993b 104; 802-09

Roediger W.E.W.
The place of short chain fatty acids in colonocyte metabolism in health and ulcerative colitis; the impaired colonocyte barrier. In "Physiological and clinical aspects of short chain fatty acids" Cummings J.H., Rombeau J.L., Sakata T. (editors). Cambridge University Press, Cambridge. 1995 pp 337-352

Roediger W.E.W., Duncan A., Babidge W., Millard S.
Methionine derivatives diminish sulphide damage to colonocytes; implications for ulcerative colitis.
Gut 1996 (In press)

Roth S.H., Green F., Hulbert W., Reiffenstein R. editors) pp 79-89
King M.T., Reiss P.D.
Separation and measurement of short chain acyl Coenzyme A compounds in rat liver by reversed phase high performance liquid chromatography.
Anal. Biochem. 1985 146; 173-79

Roth S.H., Hannah R.S.
Proceedings of the International Conference on Hydrogen Sulphide Toxicity Banff 1984. (Prior M.G., Roth S.H., Green F., Hulbert W., Reiffenstein R. editors) pp 139-56

Rowe W.A., Bayless T.M.
Colonic short chain fatty acids; fuel from the lumen?
Gastroenterology 1992 103;336-339

Rowland I.R.
Role of the gut flora in toxicity and cancer. Academic Press, London 1988

Roy A.B., Trudinger P.A.
The biology of inorganic compounds of sulphur .
Cambridge Univ. Press 1970

Rozman P., Kim H.J., Madhu C., Klaassen C.D.
Tissue sulphate determination by ion chromatography.
J. Chromatogr. 1992 574; 146-49

Rozman P., Kim H.J., Madhu C., Gregus Z., Klaassen C.D.
Homeostasis of sulphate and 3'-phosphoadenosine 5'-phosphosulphate in rats with deficient dietary intake of sulphur.
Drug Met. Disp. 1992 20; 374-78

Ryan R.W., Gourlie M.D., Titton R.C.
Release of rhodanese from Pseudomonas aeruginosa by cold shock and its localisation within the cell.
Can. J., Microbiol. 1978 25; 340-45

Ryder S.D., Walker R.J., Jones H., Rhodes J.M.
Rectal bismuth subsalicylate as therapy for ulcerative colitis.
Aliment. Pharmacol. Ther. 1990 4; 333-38

Sabbagh E.D., Cuebas D., Schulz H.
3-mercaptopropionic acid; a potent inhibitor of fatty acid oxidation in rat heart mitochondria.
J. Biol. Chem. 1985 260; 7337-42

Sadlak B., Merz H., Schorle H., Schimpi A., Feller A.C., Horak I.
Ulcerative colitis like disease in mice with an interuptted interleukin 2 gene.
Cell 1993 75; 253-61

Sandbourne W.J., Tremaine W.J., Batts K.P. Pemberton J.H., Phillips S.F.
Faecal short chain fatty acid concentrations are lower in patients with pouchitis and primary sclerosing cholangitis
Gastroenterology 1993 104; A775

Sartor R.B., Cromartie W.J., Powell D.W., Schwab J.H.
Granulomatous enterocolitis induced in rats by purified bacterial cell wall fragments
Gastroenterology 1985 89; 587-95

Satsangi J., Jewell D.P., Rosenberg W.M.C., Bell J.I.
Genetics of inflammatory bowel disease.
Gut 1994 35; 696-700

Sava G., Bertoli G., Giraldi T.
Effects of sodium sulfide on bile flow in the anaethsetised rat.
Riv. Farmacol. Ter. 1980 11; 15 (quoted in Beauchamp 1984)

Savage J.C., Gould D.H.
Determination of sulphide in brain tissue and rumen fluid by ion interaction reversed phase high performance liquid chromatography
Journal of Chromatography 1990 526; 540-545

Schenker M.
"Quo Vadis?" Summary of the International Conference on Hydrogen sulphide toxicity.
Proceedings of International Conference on Hydrogen Sulphide Toxicity. Banff Canada 1989
Prior M.G. et al (editors)

Schiff J.A., Fankhauser H.
Assimilatory sulphate reduction. In "Biology of Inorganic Nitrogen and Sulphur" (Bothe H., Trebst A., editors) 1981 Springer Verlag, Heidelberg

Schiff J.A., Saida T.
Overview; Inorganic sulphur and sulphate activation. In "Methods in Enzymology" Bergmeyer H. (editor). Academic Press, New York. 1987 Volume 143, pp 329-34

Scott G.B.D., Keyman I.F.
Ulcerative colitis in apes; a comparison with the human disease.
J. Pathol 1975 115; 241-44

Sealy L., Chalkley R.
The effect of sodium butyrate on histone modification.
Cell 1978 14; 115-21

Sedghi S., Keshavarzian A., Klamut M., Eiznhamer D., Zarling E.
Elevated breath ethane levels in active ulcerative colitis; evidence for excessive lipid peroxidation.
Am. J. Gastroenterol. 1994 89; 2217-2221

Segal A.W., Loewi C.,
Neutrophil dysfunction in Crohn's disease.
Lancet 1976 2; 219-21

Segal I., Walker A.R.P., Lord S., Cummings J.H.
Breath methane and large bowel cancer risk in contrasting African populations.
Gut 1988 29; 608-13

Seldenrijk C.A., Drexhage H.A., Meuwissen S.G.M., Pals S.T., Meijer C.J.L.M.
Dendritic cells and scavanger macrophages in chronic inflammatory bowel disease
Gut 1989 30; 484-491

Senagore A.J., MacKeigan J.M., Scheider M., Ebrom J.S.
Short chain fatty acid enemas; a cost effective alternative in the treatment of nonspecific proctosigmoiditis.
Dis. Colon Rect. 1992 35;923-27

Sharrat M Grasso P., Carpanini F.
Carrageenan ulceration as a model for human ulcerative colitis
Lancet 1970 (2); 932

Shaw L., Engel P.C.
CoA persulphide; a possible in vivo inhibitor of mammalian acyl CoA dehydrogenase.
Biochim. Biophys. Acta. 1987 919; 171-4

Shepherd N.A., Jass J.R., Duvall I., Moskowitz R.L., Nicholls R.J., Morson B.C.
Restorative proctocolectomy with ileal reservior; pathological and histchemical study of mucosal biopsy specimens.
J.Clin. Pathol. 1987 40; 601-607

Shepherd N.A., Hulten L., Tygat G.N.J.
Pouchitis
Int J Colorect Dis 1989 4;205-229

Shorter R.G., Huizenga K.A., Spencer R.J.
A working hypothesis for the aetiology and pathogenesis of non specific inflammatory bowel disease.
Am. J. Dig. Dis. 1972 17; 1024-32

Singh Kler R., Jackson S., Bartlett K., Bindoff L.A., Eaton S., Pourfazam M., Frerman F.E., Goodman S.I., Watmough N.J., Turnbull D.M.
Quantitation of acyl-CoA and acylcarnitine esters accumulated during abnormal mitochondrial fatty acid oxidation.
J. Biol. Chem. 1991 266; 22932-938

Slomiany B.L., Slomiany A.
Mechanism of Helicobacter pylori pathogenesis; focus on mucus.
J. Clin. Gastroenterol. 1992 142; S114-121

Small H., Stevens T.S., Bauman W.C.
Novel ion exchange chromatographic method using conductimetric detection.
Anal. Chem. 1975 47; 1801-1809

Smiddy F.G., Gregory S.D., Smith I.B., Goligher J.C.
Faecal loss of fluid, electrolytes and nitrogen in colitis before and after ileostomy.
Lancet 1960 1; 14-19

Smith A.C., Podolsky D.K.
Biosynthesis and secretion of colonic mucin glycoproteins
J Clin Invest 1987 80; 300-307

Smith C.M.
The effect of metabolic state on incorporation of [$^{14}$C] pantothenate into CoA in rat liver and heart.
J. Nutr. 1978 108; 863-73

Stein J., Schroder O., Milovic V., Caspary W.
Mercaptopropionate inhibits butyrate uptake in isolated apical membrane vesicles of the rat distal colon.
Gastroenterol. 1995 108; 673-79

Stenson W.F.
Role of ecosanoids as mediators of inflammation in inflammatory bowel disease
Scand J Gastroenterol 1990 (supp 172) 13-18

Stewart H.L., Jones B.F.
Pathologic anatomy of chronic ulcerative caecitis;A spontaneous disease in the rat.
Arch Pathol. 1941 31; 37-54 (quoted by Pfeiffer 1979)

Stout C., Snyder R.L.
Ulcerative colitis like lesion in Siamang gibbons.
Gastroenterology 1969 57; 256-61

Strande A., Sommers S.C., Petrak M.
Regional enterocolitis in cocker spaniel dogs.
Arch Pathol 1954 57; 357-69

Strickland R.G., Korsmeyer S., Soltis R.D.
Peripheral blood T and B cells in chronic inflammatory bowel disease.
Gastroenterology 1974 67; 569-77

Strober W.
Animal models of inflammatory bowel disease- an overview
Dig Dis Sci 1985 (supp) 30; 3S-10S

Strocchi A., Furne J.K., Ellis C.J., Levitt M.D.
Competition for hydrogen by human faecal bacteria; evidence for the predominance of methane producing bacteria.
Gut 1991 32; 1498-1501

Strocchi A., Furne J., Levitt M.D.
A modification of the methylene blue method to measure bacterial sulfide production in faeces.
J. Microbiol. Methods. 1992 15; 75-82

Strocchi A., Furne J., Ellis C., Levitt M.D.
Methanogens outcompete sulphate reducing bacteria for H2 in the human colon.
Gut 1994 35; 1098-1101

Sorbo B.
On the mechanism of sulphide oxidation in biological systems.
Biochim. Biophys. Acta 1960 38; 349-53

Susman J.L., Hornig J.F., Thomas F.C., Smith R.P.
Pulmonary excretion of hydrogen sulphide, methanthiol, dimethyl sulphide and dimethyl disulphide in mice.
Drug. Chem. Toxicol. 1978 1; 327

Takagi K., Okabe S., Saziki P.
A new method for the production of chronic gastric ulcer in rats and the effect of several drugs on its healing.
Jap. J. Pharmacol. 1970 19; 418-26

Tallgren L.G.
Inorganic sulphates in relation to the serum thyroxine level and in renal failure.
Acta. Med. Scand. 1980 Suppl 640; 1-100

Tangerman A., Nagengast F.M.
A quantatative analysis of free hydrogen sulphide and bound sulphide in human faeces.
Gastroenterology 1994 106; A634

Tansy M.F., Kendall F.M., Fantasia J., Sandin W.E., Oberly S.R., Sherman W.
Acute and subchronic toxicity studies of rats exposed to vapours of methyl mercaptan and other reduced sulphur compounds.
J. Toxicol. Environ. Health 1981 8; 71-76

Thauer R.K., Jungermann K., Decker K.
Energy conservation in chemotrophic anaerobic bacteria.
Bacteriol. Rev. 1977 41; 100-80

Thayer W.R., Charland C., Field C.E.
The subpopulations of circulating white blood cells in inflammatory bowel disease.Gastroenterology 1976 71; 379-84

Thiede C., Bayerdorffer E., Thiede H.M., Ochsenkuhn T., Neubauer A.
Quantification of the essential methyl group donor S-adenosyl methionine in human colorectal mucosa of patients with inflammatory bowel disease (IBD) and colorectal adenomas.
Gastroenterology 1995 108; A546

Thompson E.M., Price A.B., Altman D.G., Sowter C Slavin G
Quantitation in inflammatory bowel disease using computerised interactive image analysis
J Clin Pathol 1985 38; 631-638

Tobey N.A., Orlando R.C., Schreiner V.J., Powell D.W.
Cytoprotective effect of sulphate ions in acid exposed rabbit oesophagus.
Amer. J. Physiol. 1986 251; G866-69

Toofanian F., Targowski S.
Experimental production of rabbit mucoid enteritis.
Am. J. Vet. Res. 1983 44; 705-08

Tsai H.H., Hart C.A., Rhodes J.M
Production of mucin degrading sulphatase and glycosidase by Bacteroides thetaiotamicron
Letters in Applied Microbiology 1991 13; 97-101

Tsai H.H., Sunderland D., Gibson G., Hart C.A., Rhodes J.M.
A novel mucin sulphatase from human faeces; its identification, purification and charcaterisation.
Clin. Sci. 1992 82; 447-54

Tsai H.H., Dwarakanath A.D., Hart C.A., Milton J.D., Rhodes J.M
Increased faecal sulphatase activity in ulcerative colitis; a potential target for treatment.
Gut 1995 36; 570-76

Tsang M.L.S., Schiff J.A.
Studies of sulfate utilisation by algae. 14. Distribution of 3'phosphate5'phosphosulfate (PAPS) and adenosine 5'phosphosulfate (APS) sulfotransferases in assimilatory sulfate reducers.
Plant. Sci. Lett. 1975 4; 301-07

Tsang M.L.S., Schiff J.A.
Sulfate reducing pathway in Eschericia coli involving bound intermediates.
J.Bacteriol. 1976 125; 923-33

Tsang M.L.S., Schiff J.A.
Studies of sulfate utilisation by algae. 18. Identification of glutathione as a physiological carrier in assimilatory sulfate reduction by Chlorella.
Plant. Sci. Lett. 1978 11; 177-83

Tsao D., Shi Z.R., Wong A.
Effect of sodium butyrate on carginoembryonic antigen production by human adenocarcinoma cells in culture.
Cancer Res. 1983 43; 1217-22

Turvey J.R.
Sulfates of the simple sugars. In "Advances in Carbohydrate Chemistry" Wolfrom M.L., Tipson R.S. (editors) Academic Press, New York, 1965. pp 183-218

Tytgat G.N.J., Mulder C.J.J.
The aetiology of Crohns disease.
Int J Colorect Dis 1986 1;188-192

Van Kruiningen H.J.
Granulomatous colitis of boxer dogs; comparative aspects.
Gastroenterology 1967 53; 114-122

Van Kruiningen H.J.
Canine colitis comparable to regional enteritis and mucosal colitis of man.
Gastroenterology 1972 62; 1128-1142

Veerkamp J.H., van Moerkerk H.T.B.
Peroxisomal fatty acid oxidation in rat and human tissues. Effect of nutritional state, clofibrate treatment and postnatal development in the rat.
Biochim. Biophys. Acta 1986 875; 301-310

Veress B., Reinholt F.P., Lindquist K., Lofberg R., Liljeqvist L.
Longterm histomorphological surveillence of the pelvic ileal pouch; dysplasia develops in a subgroup of patients
Gastroenterology 1995 109; 1090-97

Vernia P., Gnaedinger A., Hauk W., Bruer R.I.
Organic anions and the diarrhoea of inflammatory bowel disease.
Dig Dis Sci 1988a 33; 1353-1358

Vernia P., Caprilli R., Latella G., Barbetti F., Magliocca F.M., Cittadini M.
Faecal lactate and ulcerative colitis.
Gastroenterology 1988b 95; 1564-68

Vetter R.D., Bagarinao T.
Detoxification and exploitation of hydrogen sulphide by marine organisms.
Proceedings of the International Conference on Hydrogen Sulphide Toxicity Banff 1984. (Prior M.G., Roth S.H., Green F., Hulbert W., Reiffenstein R. editors)

Waku K.
Origins and fates of fatty acyl CoA esters.
Biochim. Biophys. Acta 1992 1124; 101-11

Ward M.
The pathogenesis of Crohn's disease.
Lancet 1977 ii; 903-5

Warenycia M.W., Steele J.A., Karpinski E., Reiffenstein R.J.
Hydrogen sulphide in combination with taurine or cysteic acid reversibly abolishes sodium currents in neuroblastoma cells.
Neurotoxicology 1989 10; 201-10

Waring R.H., Steventon G.B., Sturman S., Heafield M.T.E., Smith M.G.C., Williams A.C.
S methylation in motor neuron disease and Parkinsons disease.
Lancet 1989 2; 356-57

Watt J., Marcus R.
Ulcerative colitis in guineapigs caused by seaweed extract.
Gut 1971 12; 164-171

Watt J., Marcus R.
Ulceration of the colon in rabbits fed sulphated amylopectin
J. Pharm. Pharmacol. 1972 24; 68-

Weinshilboum R.M., Sladek S., Klumpp S.
Human erythrocyte thiol metyltransferase; radiochemical microassay and biochemical properties.
Clin. Chim. Acta 1979 97; 59-71

Weinshilboum R.
Methyltransferase pharmacogenetics
In "Pharmacogenetics of Drug Metabolism" (Kalow W, editor). Permagon Press, New York 1992.
Chapter 5, pp 179-94

Weisiger R.A., Pinkus L.M., Jakoby W.B.
Thiol S methyltransferase: suggested role in detoxication of intestinal hydrogen sulfide.
Biochem Pharmacol. 1980 29; 2885-2887

Weiss S.J.
Tissue destruction by neutrophils.
N. Engl. J. Med 1989 320; 365-76

Westely J.
Cyanide and sulphane sulphur. In "Cyanide in Biology" Vennesland B., Conn E., Knowles C. et al (editors). Academic Press, New York, 19?? pp 61-67

Whitehead R.H., Young G.P., Bhathal P.S.
Effects of short chain fatty acids on a new human colon cancer cell line (LIM 1215)
Gut 1986 27; 1457-63

Widdel F., Pfennig N.
A new anaerobic, spore forming, acetate oxidizing sulphate reducing bacterium, Desulfotomaculum acetoxidans.
Archives Microbiol. 1977 112; 119-122

Widdel F.
Microbiology and ecology of the sulphur reducing bacteria. In "Biology of Anaerobic Organisms" Zehnder A.H.B. (ed.) 1988, John Wiley and Sons, N.Y. pp 469-585

Williams N.N., Brannigan A., Fitzpatrick J.M., O'Connell P.R.
Glutamine and butyric acid metabolism metabolism measurement in biopsy specimens (ex vivo); a method of assessing treatment of inflammatory bowel conditions
Gastroenterology 1992 102; A713

Williamson D., Melanby J.
D-(-)-3- hydroxybutyrate. In "Methods of Enzymatic Analysis" Bergmeyer H.U. (editor) Academic Press, New York 1974. Vol. 4 pp 1836-39

Williamson G., Engel P.C., Mizzer J.P., Thorpe C., Massey V.
Evidence that the greening ligand in native butyryl CoA dehydrogenase is a CoA persulphide.
J. Biol. Chem. 1982 257; 4313-20

Wilson D.F., Erecinska M.,
Ligands of cytochrome c oxidase. In "Methods in Enzymology" Fleischer S., Packer L. (editors) Academic Press, New York. 1978 Vol. 53; 191-201

Wilson L.G., Bierer D.
The formation of exchangeable sulphite from 3'phosphate5'phosphosulfate in yeast.
Biochem. J. 1976 1158; 255-70

Windmuller H.G., Spaeth A.E.
Intestinal metabolism of glutamine and glutamate from the lumen as compared to the blood.
Arch. Biochem. Biophys. 1975 171; 662-72

Windmueller H.G., Spaeth A.E.
Identification of ketone bodies and glutamine as the major respiratory fuels in vivo for the post absorptive rat small intestine.
J. Biol. Chem. 1978 253; 69-76

Windmueller H.G., Spaeth A.E.
Respiratory fuels and nitrogen metabolism in vivo in small intestine in fed rats
J. Biol. Chem. 1980 255; 107-112

Wintrobe M.M., Follis R.H., Alcayaga R., Paulson M., Humphries S.
Pantothenic acid deficiency in swine; With particular reference to the effects on growth and the ailmentary tract.
Bull. Johns Hopkins Hosp. 1943 73; 313

Wiseman G.
Absorption of amino acids using an invitro technique.
J. Physiol. 1953 120; 63-72

Wright S.D., Anantha Reddy P., Jong M.T.C., Erickson B.W.
C3bi receptor (complement receptor type 3) recognises a region of complement protein C3 containing the sequence Arg-Gly-Asp.
Proc. Natl. Acad. Sci. 1987 84; 1965-68

Wrong O., Metcalf-Gibson A., Morrison RB.I., Ng S.T., Howard A.V.
In vivo dialysis of faeces as a method of stool analysis
Clin Sci 1965 28 357-375

Yamada T., Sartor R.B., Marshall S., Specian R.D., Grisham M.B.
Mucosal injury and inflammation in a model of chronic granulomatous colitis in rats.
Gastroenterology 1993 104; 757-71

Zeitlin I.J., Norris A.
Animal models of colitis. In "Mechanisms of Gastrointestinal Inflammation" Chadwick V.S. (ed)
Welwyn Garden City, U.K. Smith, Kline and French 1984 pp 70-73