# Luminal and humoral influences on colorectal epithelial cytokinetics

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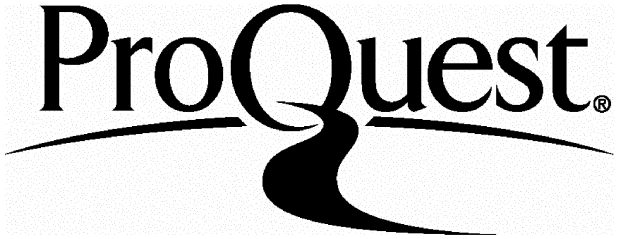
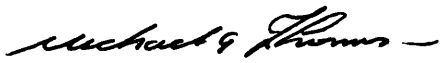


## Preface

The work for this thesis was carried out in The Department of Surgery at The Royal Postgraduate Medical School, The Hammersmith Hospital, in collaboration with The Polyposis Registry at St Mark's Hospital, London. At both institutions I was aided by helpful advice, encouragement and the friendly approach of the staff. Professor R C N Williamson supervised me during this research, and I am indebted to him for his initial ideas and constructive criticism combined with the degree of freedom he extended to me during my time at The Hammersmith Hospital.

I am also grateful for the help of Professor R Owen at Porton Down, Salisbury who performed the faecal bile acid analysis. In addition, Gary Brown in the Department of Histopathology at the Hammersmith Hospital, helped me with the proliferating cell nuclear antigen staining. The rest of the work involved in this thesis was carried out by myself. Sue Tebbutt and Morak Nohadani, however, have were of invaluable assistance in the laboratory.



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**Abstract**

Colorectal cancer may occur in individuals with a recognised high risk of developing the disease, or as sporadic cases in people with an apparently normal chance. Multiple genetic steps and environmental factors, such as diet, may be responsible for the development of colorectal neoplasia, and experimental evidence suggests that promoters of colorectal cancer also induce colorectal epithelial hyperplasia. As a corollary, dietary intervention can reduce large bowel epithelial cell proliferation. I have used the crypt cell production rate (CCPR) as my standard cytokinetic parameter to determine the effect of dietary and humoral factors on colorectal mucosal proliferation in both animals and man.

In Experiment 1, the use of a digestible fibre caused an elevation in faecal bile acids and ameliorated the hypoplasia induced by enteral feeding in rats. Neutral steroid analysis suggested this effect may be linked to bacterial degradation of bile acids. The double-blind trial reported in Experiment 2, showed that oral supplementation with calcium reduces human rectal CCPR in patients with familial adenomatous polyposis (FAP) but it had no effect on established neoplasia. In addition, vitamin $D_3$ metabolites were elevated in serum from the control patients.

In Experiments 3, 4 and 5, I have used organ and cell culture to demonstrate, for the first time, that vitamin $D_3$ and its synthetic (less toxic) analogues reduce cell proliferation in normal, premalignant and malignant human colorectal epithelial cells. I have also demonstrated that epidermal growth factor (EGF) increases CCPR in FAP tissue that expresses the EGF receptor. In addition, I have combined static and dynamic measurements of cell proliferation to determine the various phases of the cell cycle in FAP.

Dietary and therapeutic manipulations may, in the future help, decrease the incidence of colorectal cancer, and synthetic vitamin $D_3$ analogues with limited effects on calcium metabolism could possibly help treat established disease.

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# Chapter 1.

## Introduction.

Colorectal cancer is a common cause of death worldwide and the prevalence appears to be increasing (Le Fall 1981, Muir and Parkin 1985). Non-operative treatment has made little impact on overall survival, and surgery remains the most successful treatment for the disease (Reasbeck 1987). There is now increasing evidence that both environmental factors (Kune Kune and Watson 1986) and hereditary factors (Cannon-Albright et al. 1988) may be independent causative factors of large bowel cancer. This thesis explores some of the effects of environmental factors (both luminal and humoral) on the control of colorectal epithelial cell proliferation in the rodent and in groups of humans with differing risks of developing colorectal cancer.

In Chapter 2 the incidence and epidemiology of large bowel cancer is discussed. There is a higher prevalence of the disease in more affluent societies, and migrant groups assume the inherent risk of the indigenous populations (Haenszel et al. 1973); therefore geographical location and diet may influence an individual's risk of developing colorectal cancer. The study of familial adenomatous polyposis and related syndromes has highlighted the fact that nutritional factors are not solely responsible for large bowel cancer. There is now an increasing awareness that many "sporadic cases" of colorectal cancer may have an underlying hereditary component (Burt et al. 1985, Cannon-Albright et al. 1988). Investigating the polyposis syndromes has improved our understanding of the pathogenesis of colorectal carcinoma, and these syndromes might also act as a model for aetiological studies.

It has been suggested that secondary bile acids may be promoters of large bowel cancer (Aries et al. 1969). The third section of Chapter 2, therefore, deals with bile acid metabolism and the experimental evidence for the hypothesis that secondary bile acids are promoters of colorectal cancer.

Most if not all carcinomas in the large bowel are thought to develop from adenomas (Muto Bussey and Morson. 1975, Adachi et al. 1988). It has also been suggested that hyperplasia of the colonic crypts is an early event in the development of large bowel neoplasia. Chemical carcinogens combined with surgical operations provide a good model for the development of colorectal cancer in animals. In man, however, the study of intestinal hyperplasia relies more heavily on *in-vitro* experiments which utilise tissue taken from patients who are at a high risk of developing colorectal cancer. The evidence for a hyperplasia- adenoma- carcinoma progression in both laboratory animals and in man is discussed in the first section of Chapter 3.

Diet plays a major role in the development of colorectal cancer (Kune Kune and Watson 1986 and 1988a, Kune et al. 1988) . A high dietary intake of fat and protein (Armstrong and Doll. 1975) correlates with an increased incidence of colorectal cancer, whereas dietary fibre may protect against it (Burkitt. 1971). There is also epidemiological and experimental evidence to suggest that calcium, vitamin D and some of the trace elements may protect against large bowel carcinoma. Newmark has suggested that the western diet may not contain sufficient calcium to bind to toxic free fatty acids and free bile acids within the intestine (Newmark Wargovich and Bruce. 1984). Dietary supplementation with calcium might therefore help reduce the risk of large bowel carcinoma. Less is known

about the potential protective effect of vitamin D on the large bowel. This vitamin is increasingly being viewed as a steroid hormone which in addition to controlling calcium metabolism has a strong antiproliferative effect on many human cells (Chapter 3). The potential use of vitamin D metabolites as antiproliferative agents in man is limited by their profound effects on calcium metabolism. Recently, however, synthetic vitamin D analogues have been described which retain potent effects on cell proliferation but have only limited effects on calcium homoeostasis (Kissmeyer and Binderup. 1991).

Epidermal growth factor (EGF) and related small peptides can have quite profound effects on intestinal cell proliferation. Wright has suggested that EGF can stimulate cell proliferation and promote the healing of damaged mucosa throughout the gastrointestinal tract (Wright Pike and Elia. 1990a). It therefore appears that the control of cell proliferation in the colon and rectum is dependent on a multitude of luminal and humoral factors. The maintenance of normal mucosal proliferation may depend on the balance of these factors.

In order to study intestinal cell proliferation there are two main problems that must be addressed. First, what parameter of the cells life or "cycle" can we reliably and accurately measure? Second, how can we measure this parameter in man? The last section in Chapter 3 reviews the measurement of epithelial cell kinetics in the gastrointestinal tract in animals and in man. The stathmokinetic technique of metaphase arrest (Wright and Appleton 1980) is used extensively in the experiments of this thesis. Although ethical constraints limit *in-vivo* experimentation in man, combining the stathmokinetic technique with *in-vitro* organ culture produces a system that allows the proliferation of colorectal tissue to be measured in

human subjects (Allan Bristol and Williamson 1985). Chapter 4 expands the discussion of the experimental techniques used in this thesis.

The series of five experiments described in Chapter 5 were designed to investigate the effects of dietary and humoral factors on the control of large bowel proliferation in both animals and man. These experiments study the *in-vivo* effect of dietary fibre and calcium together with *in-vitro* experiments exploring the effects of vitamin D metabolites and epidermal growth factor on colorectal mucosal proliferation. The results are discussed individually with each of these experiments.

As a conclusion Chapter 6 serves to bring the findings reported in the experiments together and point to their implications on possible avenues for future research.

# Chapter 2.

## Colorectal cancer and antecedent diseases.

### 1. The extent of the problem.

Colorectal cancer is one of the most common cancers world-wide, both in the west and in developing countries. There are over 500,000 new cases reported per year, and large bowel cancer is responsible for over 300,000 deaths per year world-wide (Muir and Parkin 1985). Indeed, the overall prevalance of the disease appears to be increasing (Le Fall 1981, Hughes et al. 1982). In the USA the annual incidence of colorectal cancer increased by 9.4% between 1973 and 1986, with a 14% increase in the incidence of primary sigmoid colon cancer, whereas age-adjusted deaths from colon cancer fell by 7% (National Cancer Insitute 1989). In the U.K, colorectal cancer is newly diagnosed in approximately 27,000 individuals per year and is the cause of death in at least 16,000 people per year (Umpleby et al. 1984, Jones 1992, Stephenson et al. 1992). In Australia between 1946 and 1978, there was an almost exponential increase in deaths from colorectal cancer, and approximately 4,000 Australians now die from this cause each year (Hughes et al. 1982, Reasbeck 1987).

The operative morbidity and mortality rates for resection of colorectal cancer have improved in line with advances in surgical and anaesthetic techniques (Lockhart-Mummery Ritchie and Hawley 1976, Ohman 1982). The number of restorative procedures has increased, and this has reduced the rate of colostomy formation; thus patient comfort has improved (Raesbeck 1987). Despite these advances the overall long-term survival rates

after potential curative resections for colorectal cancer remain poor. The U.K. Large Bowel Cancer project reported 5 year survival figures of 65% for tumours of the right side of the colon, 59% for those affecting the left side and 38% for colonic tumours occuring at the splenic flexure (Aldridge et al. 1986). In Bristol, the overall 5 year survival was similar, being 59% after "curative resection" (Umpleby et al. 1984). Reported figures vary quite widely, however, with some survival rates as low as 25% (Whitticker and Goligher 1976, Gill and Morris 1978, Umpleby et al. 1984, Aldridge et al. 1986, Reasbeck 1987).

Non-operative treatment has made little impact on the mortality rate of colorectal cancer (Reasbeck 1987). Pre-operative adjuvant radiotherapy has failed to demonstrate an improvement in 5-year survival figures (MRC Working Party 1984), while adjuvant chemotherapy has produced equivocal results (Gastrointestinal Study Group 1984, Gastrointestinal Study Group 1985). Thus, early detection of the disease with early operation has been advocated as the best hope of cure (Reasbeck 1987), although there is some doubt whether this policy will have any effect on long-term survival figures (Torniqvist Ekelaund and Leander 1982). Unfortunately, at the time of presentation only a small percentage of patients (3-9%) have histologically-defined early disease, i.e. Dukes' stage A, which is associated with 5-year survival rates in excess of 90% (Umpleby et al. 1984, Aldridge et al. 1986). Although endoscopic treatment is often the preferred method of treatment for tumours confined to the mucosa (and, in some cases, submucosa), the depth of invasion can be difficult to determine in polypectomy specimens (Moreira et al. 1992); once submucosal invasion has occured there is an increased risk of metastatic disease (Coverlizza et al. 1989). Surgical resection has therefore been advocated as an

alternative method of treatment in early-stage cancers, and short-term follow-up studies have produced encouraging results (Moreira et al. 1992).

Besides tumour spread, increasing age and emergency presentation (without obstruction or perforation), are factors contributing to poor prognosis in large bowel cancer (Umpleby et al. 1984). The proportion of patients with colorectal cancer who present as an emergency lies between 15-36% (Reitmann et al. 1986, Canviet et al. 1989, Chester and Britton 1989, Mc Ardle 1990, Anderson et al. 1992). Approximately 15-20% of patients with large bowel cancer present with intestinal obstruction and 3-8% of cases with a colonic perforation (Ohman 1982, Phillips et al.1985, Runkel et al. 1991). These patients tend to be older (Phillips et al. 1985, Kyllonen 1987) and have more advanced disease than those undergoing elective operation (Runkel et al. 1991). Their perioperative mortality rate is higher (Anderson et al. 1992) and, if the patient survives operation, there is a reduction in the 5-year survival rate following presumed curative resection when compared to elective cases (Peloquin 1975, Irvin and Greanay 1977, Turunen 1983). This difference is independent of the stage of the disease, the degree of tumour spread or the age of the patient (Anderson et al. 1992). In a prospective study conducted in the west of Scotland, tumours of the splenic flexure were more common and rectal tumours less common in emergency compared to elective admissions, and the elective group of patients included more tumours that could be resected (77 vs 64%)(Anderson et al. 1992). Colon cancer at the splenic flexure is also associated with reduced survival (Aldridge et al. 1986). Rapid intra-abdominal and systemic spread may be attributable to a more aggressive biological behaviour in tumours presenting as an emergency (Willet et al. 1985).

Epidemiology.

The incidence of large bowel cancer varies widely throughout the world. Affluent poulations, such as those in North America and Western Europe have high rates, whereas the poorer countries in Eastern Europe, Asia and Africa have lower rates (Haenszel 1961). Foreign-born nationals in America have rates of large bowel cancer that diverge from those seen in their countries of origin and approach those of native born Americans. Likewise, migrants to California and Hawaii from Japan, China, Norway, and populations moving from rural to urban areas within the USA and Polish immigrants to America and Australia, all assume the same risk as the indigenous population within one-to-two generations (Haenszel 1961, Haenszel and Dawson 1965, Haenszel et al. 1973, Staszewaski Mc Gall and Stenhouse 1971). This trend is further illustrated by the steady rise in the incidence of colorectal cancer seen amongst slaves imported into the USA and likewise amongst prisoners brought into the Melbourne population in Australia (Doll 1969).

It appears that geographical location may largely dictate the individual's risk of colorectal cancer. Epidemiological observations provide some explanations. In many countries the incidence increases pari passu with affluence, for example in Colombia and Hawaii (Stemmermann 1970). Affluence cannot be solely responsible, for different religous groups have different incidences. Case-control studies in The USA show that Seventh-Day Adventists, whose diet is lacto-ovo-vegetarian and who forego cigarettes and alcohol, have an incidence that is only 65% of that of the general population. Their incidence is similar to that of Mormons, who are abstinent and also predominantly vegetarian (Phillips 1975). In contrast, Jewish and Catholic communities in the USA have a much higher incidence

(Wynder and Shigematsu 1967, Phillips 1975, Weisberger and Horn 1984). Similarly, individuals with a Jewish background in Melboune have double the rate of colon cancer compared to the overall rate for the area (Kune Kune and Watson 1986). Variations in incidence rates have also been noted between religious sects in India (Paymaster Sanghvi and Gangadharan. 1968). It therefore appears that it is an individual's life-style and not his geographical location that plays a crucial role in the effect of the enviroment on the development of colorectal cancer.

Wynder's case-control studies in Japan suggested that large bowel cancer patients consume a predominently western style diet (Wynder and Shigematsu 1967). In addition, Burkitt has suggested that a diet rich in refined carbohydrates and deficient in fibre (typical of a Western Style diet) may account for some interpopulation difference in the prevalence of colorectal cancer (Burkitt 1971). This argument is strengthened by Haenszel's findings that a decline in the daily practice of taking Japanese style meals among Hawaiian Japanese is associated with an increased prevalence of colon cancer.

The lowest reported cancer rates for a given site can be considered as the natural rate of occurence (Doll 1969). Variations from this rate may be related to those environmental factors chosen by individuals such as food, tobacco, alcohol and hygiene. Indeed, it has been estimated that over 90% of human cancers might be influenced by the environment and about 35% by diet (Willet et al.. 1984). The overall conclusions of the Melbourne colorectal cancer study group were that previous diet is an important aetiological factor, and that it is the whole dietary pattern, rather than individual foods or food groups, which

is important in the aetiology of large bowel cancer (Higginson 1966, Kune Kune and Watson. 1986, 1988a, 1988b, 1989; Kune et al. 1988).

Non-nutritional findings indicate that environmental factors are not the whole story with respect to the development of colorectal cancer. The discovery of multiple colonic polyps in a patient and the recognition that this condition was familial was made in the 1880s (Cripps 1882). Smith reported the presence of adenocarcinoma of the colon occuring within such a condition, and Bickersteth strengthened the case for a familial condition associated with multiple polyps of the colon (Smith 1887, Bickersteth 1890). A Mendelian dominant inherited genetic condition, now known as familial adenomatous polyposis (FAP), had thus been recognised as far back as the turn of the century (see page 12).

Inherited causes of colorectal cancer were initially thought to account for only a very small percentage of large bowel cancers, i.e. those associated with FAP. In sporadic colorectal cancer, however, possessing one or more first degree relatives with a history of colorectal cancer is associated with a two to three-fold risk for the subsequent development of colorectal cancers (Kune Kune and Watson. 1989). In a case-control study of 100 patients presenting with apparent sporadic colorectal cancer, no less than 23 gave a history of large bowel cancer in a first-degree relative (Stephenson et al. 1991). Also, patients who have undergone polypectomy for benign colonic polyps have a six-fold increase in risk of developing colorectal cancer (Kune Kune and Watson. 1987), a fact that strongly supports the argument for regular surveillance after polypectomy.

Family studies examining the frequency of large bowel cancer amongst first degree relatives in Utah in the 1950s and later in Ohio have also shown a strong hereditary predisposition and have suggested that second degree relatives exhibit excessive numbers of large bowel cancers (Woolf 1958, Macklin 1960, Burt et al. 1985, Cannon-Albright et al. 1988). In London, a four year study amongst first degree relatives of patients admitted with colorectal tumour showed an increase in deaths due to colorectal cancer amongst fathers, mothers, brothers and sisters. There was also an observed increase in risk if the proband exhibited a previous history of multiple benign or malignant neoplasms, a previous colorectal cancer, or early age of onset of colorectal cancer (Lovett 1976). Similarly, in Scotland the medical records of first degree relatives of 50 patients with colorectal cancers were compared to age-and sex-matched controls; eight of the cases had affected relatives compared to only one of the controls (Duncan and Kyle 1982). A large case-control study in Australia likewise, suggested that in sporadic colorectal cancer the relative risk associated with a positive family history was about 2-3 fold, and this effect was more pronounced in the colon compared to the rectum (Kune Kune and Watson. 1989). It was concluded that in sporadic colon cancer, diet and family history are separate and independent factors. It can be argued therefore that asymptomatic individuals with a positive family history should all be screened for large bowel cancer. Migrant studies suggest that this hereditary effect is more pronounced in the right colon (Kune Kune and Watson 1986, 1988a, 1988b, 1989; Kune et al. 1988,). This finding raises the possibility that the environmental factors may play an increasing role in the aetiology of colorectal cancer from the proximal to distal large intestine.

Summary

Familial polyposis and familial cancer syndromes now have a well documented genetic basis to their aetiology. There also appears to be an hereditary effect that accounts for a small percentage of sporadic colorectal cancers (approximately 20%) and this effect appears to be more pronounced in the colon than in the rectum. It follows that dietary and environmental factors, have separate and independent effects and both would appear to be the predominant aetiological factors in most cases of sporadic colorectal cancer.

## 2. Familial adenomatous polyposis and related syndromes.

### Introduction

Familial adenomatous polyposis (FAP) is a genetically determined condition characterised by the presence of multiple gastrointestinal adenomas. These adenomas initially occur in small numbers, but there is an increase in size and number of polyps with age. In the colon and rectum there is a natural and inevitable progression towards the development of adenocarcinoma.

### Historical considerations

In 1881 Woodward divided polyposis conditions into primary, i.e with no antecedent disease, and secondary, following previous inflammation or ulceration of the colon (Woodward 1881). The following year, two cases of polyposis coli were reported occuring within the same family, the disease affecting a 17-year-old girl and her 19-year-old brother

(Cripps 1882). This familial inheritance was also noticed by Bickersteth who reported polyposis coli affecting two family members in successive generations (Bickersteth 1890). Thus, by 1890 a familial condition associated with the presence of multiple colonic polyps was well recognised.

At St Mark's Hospital, Dukes and Lockhart-Mummery reported the occurence of familial adenomatous polyposis in ten families, over three or four generations, and clearly established the autosomal dominant mode of its inheritance (Lockhart-Mummery 1925, Dukes 1930, Lockhart-Mummery and Dukes 1939). A link between polyposis coli and colorectal adenocarcinoma had been recognised since 1887 (Smith 1887). In the early part of this century about two thirds of the polyposis patients presenting at St Mark's Hospital already had colorectal cancer at the time of their first visit (Bussey 1990). Attention began to turn to an interruption of the progression of adenomas to carcinoma in the colon of polyposis patients. Advances in diagnostic aids, together with an increase in the number of patients being referred, allowed the first register of polyposis patients to be set up at St Mark's Hospital. Dr Cuthbert Dukes interviewed patients' relatives and invited them to attend for examination. In the children of a polyposis patient, Dukes suggested annual sigmoidoscopy commencing at about the age of puberty. It became apparent that cancer was rare before the age of twenty but that it increased in frequency from about twenty-five years upwards, to reach an incidence of around 50% in the fourth decade. A call-up policy was therefore introduced to identify and treat affected family members. The effect of this policy was to reduce the incidence of colorectal cancer on the first examination in patients with FAP to approximately 5% (Bussey 1990). The surgical preference at St Mark's was for total colectomy and ileorectal anastomosis with outpatient follow-up of the rectal stump.

After more than 200 such operations at St Mark's, twelve patients subsequently developed colorectal cancer (a 13% cumulative risk) and four of these patients have died from the condition (Bussey et al. 1985. Bussey 1990).

Bussey examined colectomy specimens and found that the range of adenomas present varied between 104 and over 5,000 (average 988). These adenomas were occasionally discrete but sometimes confluent, in which case an accurate count was difficult to obtain. He concluded that the colon contained at least 100 adenomas in FAP (Bussey 1975), and this value was initially used as a diagnostic criterion. Many of the colectomy specimens examined by Bussey derived from relatively old patients, the average age being 39.2 years. It has since been emphasised that FAP is a progressive disease, polyps being absent at birth and increasing in numbers during life. If surveillance begins at puberty then the diagnosis in children who are "at-risk" can often be made when 20 to 25 polyps are present, especially when surveillance of the gastrointestinal tract is combined with other diagnostic aids (Jagelman 1990). In Bussey's earlier review of the St Marks experience in FAP, 194 of the 293 (66%) symptomatic patients initially seen had already developed cancer by the time they presented, and the average age at diagnosis was 39.2 years (Bussey 1975). Bulow reported that the median age at the time of developments of adenoma in the Danish polyposis registry was 16 years (range 5-38) and that the median age for colorectal cancer was 36 years with a range of 17 to 67 years (Bulow 1986). Rectal polyps occurred in all 247 Danish patients in this series. This Danish experience, has now been corroborated by several Swedish, British and American centres (Alm and Licznerski 1973, Bussey 1975, Bulow 1986, Jagelman 1990). Indeed, colorectal cancer has not yet been described in FAP patients in the absence of rectal polyps seen at proctosigmoidoscopy. Sigmoidoscopic

examination therefore appears to be a reliable and safe method of screening for colorectal cancer in this disease (Jagelman 1990).

The premalignant nature of FAP and the need for operative intervention is now beyond question. The nature and the age of onset of screening programmes programmes is still a matter of debate.

Extracolonic manifestations

Extracolonic lesions in familial polyposis coli had been reported by a variety of authors since 1895. In the 1950s Gardner and his colleagues published a series of articles documenting the occurrence of multiple osteomas of the cranium and mandible, epidermoid cysts and skin fibromas in a polyposis family (Gardner and Richard 1953). Since then desmoid tumours of the abdomen and abdominal wall, dental abnormalities and extended osteomas to include the entire skeleton have also been included. The occurence of these findings in patients with FAP became known as Gardner's syndrome.

Turcot described an association between familial polyposis of the colon and central nervous system tumours (Turcot Despres and St Pierre 1959). It has since been suggested that "Turcot's syndrome" is simply a manifestation of the FAP phenotype rather than a distinct inherited entity (Hamilton et al. 1989). It now appears to be increasingly difficult to make a clear-cut distinction between those patients with classical polyposis and those showing extracolonic manifestations. In a review of 280 patients from 11 families, it was concluded that Gardner's syndrome represented a complete expression of a number of extracolonic manifestations that may be present in any patient with polyposis coli (Watne Corre and Carrier 1975, Watne et al. 1977).

Utsunomiya and Nakamura reported that over 90% of Japanese patients with polyposis had occult mandibular osteomas on plain X-ray (Utsunomiya and Nakamura 1975). Further studies have shown osteoma frequencies of 76-81% (Ida Nakamura and Utsunomiya 1981, Jarvinen et al. 1982). These benign osteomas appear to precede the diagnosis of polyposis coli by approximately 17 years and are good predictors for subsequent development of adenomas in FAP families that include members affected by osteomas (Offerhaus et al. 1987).

Epidermoid cysts also occur in FAP patients, and these are often situated on the face or scalp as opposed to their usual position on the back. In children, such epidermoid cysts located on the face or extremities are rare except in association with the polyposis syndromes (Leppard 1974, Leppard and Bussey 1975). In 1950 Cronkhite and Canada described two patients were FAP was associated with gastrointestinal dysfunction, alopecia, nail atrophy and skin pigmentation (Cronkhite and Canada 1950).

Extracolonic manifestations of FAP.

| | | |
|---|---|---|
| **Gastric polyps** | Hauser | 1895 |
| **Osteomas of mandible, Sebaceous cysts, Subcutaneous lipomas** | Devic and Bussey | 1912 |
| **Adenomas of duodenum, jejunum and ileum, Bony exostosis Carcinoma of ampulla of Vater** | Cabot | 1935 |
| **Desmoid tumours** | Miller and Sweat | 1937 |
| **Dental abnormalities** | Gardner and Plenk | 1952 |
| **CNS tumours** | Turcot, Despres and St Pierre | 1959 |
| **Opthalmological abnormalities** | Blaire and Trempe | 1980 |
| **Biliary and pancreatic duct adenomas** | Jarvinen, Nyberg and Peltokallio | 1983 |
| **Hepatoblastoma** | Li et al. | 1987 |
| **Thyroid carcinoma** | Plail et al. | 1987 |

Blair and Trempe observed multiple patches of congenital hypertrophy of the retinal pigment (CHRPE) in patients with Gardner's syndrome (Blaire and Trempe 1980). No histopathological studies of CHRPE in Gardner's syndrome have yet been reported, but the retinal lesion is thought to be similar to the non-polyposis form of CHRPE, in which the epithelial pigment cells are enlarged and contain round pigment granules (Kurz and Zimmerman. 1962, Buettner 1975). Lynch has reported the presence of such lesions in four

patients with familial polyposis without extracolonic manifestations (Lynch Priluck and Fritzsimmons 1987). It is now recommended that ophthalmoscopy be used as a screening method in all first-degree relatives of patients with FAP.

Stomach

The association between gastric polyps and FAP has been recognised since 1895 (Hauser 1895). The advent of fibreoptic endoscopy has highlighted the frequency of gastroduodenal polyps. Most series report the presence of gastric lesions in over half the patients with FAP (Utsunomyia et al. 1974, Ushio et al. 1976). In Watanabe's series, six out of 22 FAP patients had hundreds of small hemispherical polyps scattered throughout the fundus of the stomach. Histological examination of these polyps showed the presence of cystic dilatation of the fundic glands without epithelial dysplasia (Watnabe et al. 1978). This non-neoplastic "fundic gland polyposis" has now been reported to occur in 23-56 % of patients with FAP as compared to 0.09% in the normal population (Ida et al. 1984). These fundic gland polyps are found in clusters of a few to hundreds of polyps, and the lesions are the same colour as the gastric mucosa. There appears to be no age-related increase in their numbers (Sarre et al. 1987). By contrast, gastric adenomas are relatively uncommon (except in Japanese FAP patients) their incidence being 2-13% (Bulow 1990). At the Cleveland Clinic, 28 out of 100 patients with familial polyposis had gastric polyps diagnosed at endoscopy, and of these only two were adenomas (Sarre et al. 1987). Gastric carcinoma is very uncommon in FAP patients, though a Japanese study reported such cases of gastric cancer in 22 FAP patients (Watanabe et al. 1978).

Duodenum

The incidence of duodenal adenomas varies between 24-73% in FAP (Bulow 1990); it is independent of sex and appears to increase with age (Sarre et al.1987). The polyps tend to spare the first part of the duodenum but cluster in the second part, where they are especially concentrated around the ampulla of Vater. Duodenal adenomas can occur as a solitary lesion, but they are often multiple and may produce a diffuse carpeting of the duodenum. The adenomas appear to be predominantly flat and have an irregular or granular surface appearance. Histologically they resemble the typical colonic lesions, being tubular or villous adenomas. If Brunner's glands are present in the biopsy these may show mild cystic dilatation. Occasionally, mucosa with a normal macroscopic appearance shows early adenomatous change, while sometimes in the region of the ampulla only minor endoscopic changes are seen in patients with histological evidence of adenomatous change (Jagelman 1990). Bulow has suggested that the frequency of duodenal adenomas is independent of other extracolonic manifestations.

Periampullary carcinoma of the duodenum in FAP was first reported by Cabot in 1935 (Cabot 1935). In reviewing the St Mark's Hospital experience, Bussey estimated the risk of developing periampullary carcinoma in patients surviving for five years or more after total colectomy as 4% (Bussey 1975). The Cleveland Clinic reported a prevalence of 2% in their 199 cases, and in Toronto six out of 414 FAP patients have died of periampullary cancer following colectomy (Cohen Berk and Mc Leod 1990). The overall estimated incidence of duodenal cancer in FAP is 2.5% of western populations and 0.6% of Japanese patients (Utsunomiya 1990). In FAP patients who exhibit extracolonic manifestations the incidence of periampullary cancer has been estimated to be 12% (Bussey 1972). In a review of 29

cases of duodenal cancer, no less than half were associated with synchronous duodenal adenomas (Bulow et al. 1985), strengthening the hypothesis that the adenoma-carcinoma sequence underlies the development of duodenal malignancy in FAP.

With an improvement in the overall prognosis in FAP due to prophylactic colectomy, the potential risk of periampullary carcinoma outlines the need for routine gastroduodenal endoscopy. This need is highlighted by follow-up studies of patients with colectomy and ileorectal anastomosis at both the Cleveland Clinic and St Mark's, in which there were more deaths from periampullary carcinoma than from rectal carcinoma (Jagelman 1987, Spigelman et al. 1989 Thompson 1990). The distribution of these gastroduodenal adenomas has been related to the presence of bile acid (see page 28).

The small intestine.

Hamilton has reported ileal adenomas in 9 FAP patients following total colectomy, and he has suggested that these may follow colectomy as a result of the altered anatomy of the gastrointestinal tract (Hamilton et al 1979). Ileal adenomas have also been seen arising within a continent ileostomy in one FAP patient (Stryker Carney and Dozois 1987), and in another a extensive villous adenoma on a ileostomy was large enough to require excision. When the terminal ileum was examined in nine patients constituting a Gardner's syndrome kindred, ileal lesions were present in all nine; three of these were lymphoid aggregates and six were adenomatous polyps (Burt et al. 1984). The entire small bowel was examined in 16 Japanese patients with FAP either by endoscopy or at post mortem; eight patients had

small adenomas of the jejunum, three had small polyps of the terminal ileum and nine had multiple lymphoid aggregates in the terminal ileum (Watanabe Enjoji and Yao 1977).

Thus the ileum is not immune to neoplasia in FAP, but the lesions are generally too small to cause trouble. In support, small bowel cancers (excluding duodenal malignancy) are distinctly uncommon. Ross and Mara in 1974 reported two cases of adenocarcinoma of the small bowel. In one patient, an adenocarcinoma of the jejunum co-existed with a 0.8 cm diameter adenomatous polyp in the same segment of bowel. The other patient had an invasive adenocarcinoma of the ileum in association with multiple adenomatous polyps showing areas of atypical hyperplasia (Ross and Mara 1974).

Polypoid lesions in Gardner's syndrome frequently occur outside the colon. Whereas duodenal cancer affects 2-3 % of patients jejunoileal cancer is rare. The question arises: why do separate areas of the gut have a differing cancer potential?

Inheritance patterns and genetics

All manifestations of FAP are inherited in a Mendelian dominant autosomal pattern, each offspring having a 50 % chance of inheriting the disease. The occasional sporadic case of FAP in which no relatives are affected may represent a new mutation. The frequency of these mutations varies widely. It is not known whether FAP, Gardner's and Turcot's syndrome are controlled at the same point in the nucleus (Leppart et al. 1987). There is an hypothesis that they represent a single molecular disease and an expression of one genetic entity (Smith 1968).

The precise chromosomal abnormality in FAP is unknown, but Herrera suggested that the FAP gene was located on the long arm of chromosome 5, and linkage analysis has confirmed this location (Herrera et al. 1986, Bodmer et al. 1987, Leppart et al. 1987). Although this gene is present in all cells of an individual with FAP, its expression may be limited to certain tissues (e.g the cells of the gastrointestinal mucosa may express markers not evident in skin or fibroblasts). Further investigation into genetic markers of adenoma and carcinoma expression may therefore help explain the steps in malignant transformation seen in FAP.

Burt has studied the inheritance of adenomatous polyps in Utah (Burt et al. 1985). A large kindred with a moderate clustering of common colorectal cancers was compared to their spouses who were used as demographic controls. The incidence of hyperplastic colonic polyps was similar in both groups, whereas adenomatous polyps were more frequent in the family members. Pedigree analysis was performed to determine whether this finding was likely to be due to random clustering or to autosomal dominant or recessive inheritance. It was suggested that autosomal dominant inheritance was more likely to explain the increased incidence of adenomatous polyps by many orders of magnitude. Thirty-three additional pedigrees were then studied, looking at the inheritance patterns in colon cancer cluster-families and in a random group of patients with adenomatous polyps found clinically. It was concluded that most adenomatous polyps apparent at proctosigmoidoscopy arise, at least in part, because of an inherited susceptibility. In addition, most of those individuals identifiable with a polyp or colonic cancer are affected because of dominant inheritance (Cannon-Albright et al. 1988, Burt 1990). The finding that patients with "non-familial" adenomatous polyps may have a genetic component to

their disease not only strengthens the adenoma-carcinoma model for colon cancer, but also support the hypothesis that genetic and environmental factors interact in the formation and transformation of polyps.

Hereditary non-polyposis colorectal cancer

The development of colon cancers in familial non-polyposis syndromes (sometimes referred to as Lynch's syndromes) was previously thought to be unrelated to the presence of a precursor colonic polyp (Lynch et al. 1985, Lynch et al. 1987, Lynch 1990). However, Muto has suggested such flat colonic adenomas can occur sporadically (Muto Bussey and Morson 1985), and Adachi observed these flat adenomas in two patients with proximal colon cancer (Adachi et al. 1988). The presence of flat adenomas has also been seen in a hereditary non-polyposis colon cancer family (Lynch et al. 1988), and this raises the possibility that such syndromes may be the extreme end of the spectrum of FAP (Lynch et al. 1988).

The situation is slightly complicated by the a host of other case reports suggesting the existence of a hereditary condition in which rare sebaceous neoplasms occur with visceral cancers and colonic polyposis, first described by Torre (Torre 1968). A year later an almost identical syndrome was reported by Muir, in which multiple primary carcinomas of the colon, duodenum and larynx are associated with keratoacanthomas of the face (Muir Bell and Barlow 1967). The co-existence of signs indicative of Muir-Torre's syndrome in the cancer family syndrome led to the idea that Muir-Torre's syndrome might represent the full expression of the gene responsible for the cancer-family syndrome (Lynch et al. 1981).

The adenoma-carcinoma sequence in polyposis coli

The occurrence of multipleadenomas, whether a few or several hundred, is a phenotypic marker in patients with FAP. This may also be true for the presence of a flat adenoma in patients at risk from "hereditary non-polyposis colon cancer". Size (> 1 cm), the proportion of villous architecture and the severity of dysplasia are all associated with a higher prevalence of adenocarcinoma within an adenoma (Muto Bussey and Morson 1975). Patients with synchronous adenomas in their resected carcinoma specimens who went on to develop metachronous carcinomas generally displayed a high grade of dysplasia in their initial synchronous adenomas (Morson and Konishi 1982). Likewise, carcinoma in-situ is more common in synchronous adenomas (in the presence of adenocarcinoma) than in isolated adenomas removed as solitary lesions (Kalus 1972). Thus, the presence of high-grade dysplasia appears to indicate the risk of developing synchronous or metachronous carcinoma. Indeed, it has been suggested that the incidence of metachronous and synchronous carcinomas increases with increasing numbers of adenomas (Muto Bussey and Morson 1975, Shinya and Williamson 1979). This suggestion is endorsed by the observation at St Mark's that more polyposis patients than non-polyposis patients develop synchronous malignancy (Bussey 1990). The principle that adenoma was the harbinger of carcinoma in FAP was the basis for Dukes' call-up policy. Such adenomas in FAP arise at least ten years before the development of carcinoma (Jarvinen et al 1990).

A fundamental question in the treatment of FAP is whether adenomas at other sites follow the adenoma-carcinoma sequence observed in the colon and, if so, whether this adenoma-carcinoma progression can be altered. Adenoma and carcinoma can certainly co-exist within the duodenum (Sinha and Williamson 1988). Villous adenomas were

detected adjacent to the carcinoma in four FAP patients with periampullary cancer treated at the Cleveland Clinic (Sarre et al. 1987). Endoscopic evidence from the original Gardner's syndrome kindred showed that 8 out of the 11 patients had duodenal adenomas, and several specimens of mucosa showed isolated foci of adenomatous glands similar to the colonic microadenomas observed in FAP (Burt et al. 1984). Similarly, in the terminal ileum, microadenomas and the occurrence of adenomatous change were seen within the apparently normal mucosa (Burt et al. 1984). Kozuka found adenoma in association with duodenal carcinoma in 18 of 22 non-polyposis patients, and he observed a transition from adenoma to carcinoma in several of these patients (Kozuka et al. 1981). Although circumstantial, this evidence supports the concept that all gastrointestinal adenomas can progress to carcinoma.

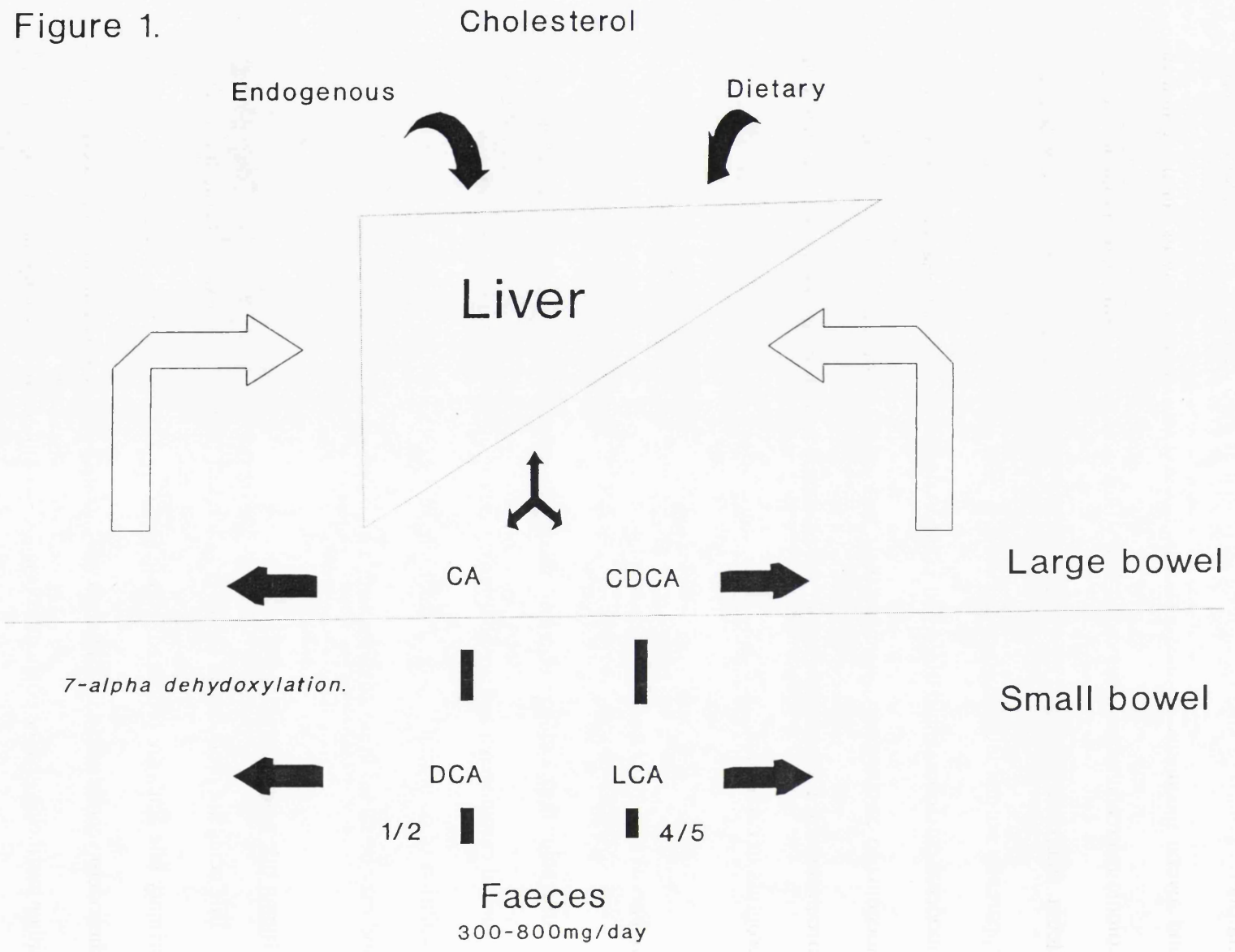
**3. Faecal bile acids and neutral steroids.**

Bile acids bear a close steric resemblance to the polycyclic hydrocarbons (well-known chemical carcinogens) and are thus strong candidates as potential co-carcinogens in the evolution of colorectal cancer. Faecal bile acid excretion reflects the amount of fat eaten and may be raised in populations with a high incidence of large bowel cancer.

Human bile acid metabolism.

Bile acids are produced in the liver as a result of the metabolism of cholesterol. Two primary bile acids are produced, cholic acid (3,7 12-trihydroxy-5-cholan-24-oic acid) and chenodeoxycholic acid (3-7-dihydroxy-5-cholan-24-oic acid), and these are secreted as either taurine or glycine conjugates (via a C-24 amide bond). Cholic acid (CD) is mainly derived from endogenous cholesterol, whereas chenodeoxycholic acid (CDCA) comes from

Figure 1.




CA = Cholic acid, CDCA = Chenodeoxycholic acid.

the metabolism of dietary cholesterol. The conjugates are poorly absorbed in the small intestine, where they aid fat digestion, and are recovered by an active transport system in the distal ileum. In normal health, less than 5% of the bile acids are lost into the colon with each passage through the small bowel, and there are about eight cycles per day (Heaton 1969, Hoffmann 1977). Normally urinary bile acid concentration is minimal and systemic levels are low (Figure 1).

The large intestine possesses a rich bacterial flora with a predominance of anaerobic non-sporing baccilli, such as *Bacteroides* sp., *Bifidobacterium sp.*, *Eubacterium sp.*and *propionibacterium sp.* These organisms deconjugate bile acids by digestion of the amide bond and allow further absorbtion of free bile acids (approximately 30%). Since deconjugation is usually highly efficient, nearly all the bile acids are released within a few hours.

The enzyme cholanyl-7-alpha-dehydroxylase is produced by bacteria within the colon and is responsible for the hydroxylation of primary bile acids to the secondary bile acids lithocholic (LCA) and deoxycholic acid (DCA). This reaction will only occur in highly anaerobic conditions. Hydroxyl-oxidoreductase, another bacterial enzymic reaction, produces oxo-bile acids and bile acids with beta-hydroxyl groups. Because anaerobic conditions are unfavourable to the action of hydroxyl oxioreductase, oxo and beta-hydroxyl bile acids account for only 1-20% of faecal bile acids; thus LCA and DCA are the major human faecal bile acids (300-800 mg/day). 7-alpha-hydroxylation probably occurs mainly in the right colon, as DCA and LCA faecal levels are unaffected by left hemicolectomy and

ileostomy fluid contains no secondary bile acids.(Fadden Owen and Hill 1984, Fernandez et al. 1985).

Whether synthesised and secreted by the liver, or present in the diet, cholesterol is poorly absorbed in the small bowel and therefore most of it enters the colon. In the large intestine it is reduced to coprostanol (60-70%), coprostanone (5-15%) or cholestenone (0-5%), while a proportion remains undigested (10-25%). These products of bacterial degradation along with the plant sterols account for the neutral steroid contents of human faeces (Owen Thompson and Hill 1984).

The degradation of primary bile acids by the colonic microflora has been implicated in the production of co-carcinogens or tumour promoters in the human colon (Aries et al. 1969, Hill et al. 1975). Comparing the faecal bacteria content of 40 Ugandans (with a low incidence of colon cancer) to the faecal bacteria content of 40 Londoners (with a high risk of colorectal cancer), Aries found that the English specimens contained 30 times or more *Bacteroides sp,* than the Ugandan specimens. Also, the bile salts present in the English faeces were degraded to a greater extent than the bile salts in the Ugandan samples. It was postulated that the difference in bacterial flora might account for the varying geographical incidence of colorectal cancer (Aries et al. 1969). Further epidemiological evidence suggests that there is a correlation between faecal free bile acids and an increased risk of colorectal cancer (Owen et al. 1987, Thompson 1984). Americans consuming a western diet excrete high levels of bile acids, compared to Japanese, Chinese and Seventh Day Adventists (Reddy and Wynder 1973). Also, there is an increase in the faecal bile acids and neutral steroids in populations with high rates of colon cancer and in patients with colorectal neoplasia compared to the normal population (Hill et al. 1971, 1975, Reddy

Weisburger and Wynder 1975, Reddy and Wynder 1977, Reddy et al. 1980). The raised faecal bile acid levels seen in patients with adenomatous polyps could not, however, be attributed to an altered diet when compared to American controls (Reddy et al. 1976). Moreover, patients with familial adenomatous polyposis and ulcerative colitis do not show an elevation in total faecal bile acids compared to controls. (Reddy et al. 1976, Watne et al. 1976).

In a series of 44 patients in London, 82% had high faecal bile acid concentrations (greater than 6 mg per gram dry weight) as opposed to 17% of 90 patients with other diseases. A high proportion of the colorectal cancer patients had *Clostridia sp* capable of performing nuclear dehydrogenation reactions, which produce unsaturated sterols from bile acids (Hill et al. 1975). Nuclear dehydrogenating clostridia (NDC) are present in greater concentrations in the faeces of patients at "high-risk" from colorectal cancer, compared to "low-risk" individuals (Mastromarino Reddy and Wynder 1978), and these bacteria can produce polycyclic aromatic hydrocarbon carcinogens from bile acids. Also, healthy individuals in countries with a high incidence of large bowel cancer have higher faecal bile acids and NDC levels than individuals in "low risk" countries (Hill 1974). In man, high-fat low-protein diets increase the activity of beta-glucuronidase (a bacterial enzyme) and the number of anaerobic bacteria (Reddy and Wynder 1973, Reddy Weisburger and Wynder 1975, Reddy et al. 1976).

In a prospective study of 102 patients with ulcerative proctocolitis of more than 10 years standing, faecal bile acids were higher in the 14 patients with proven dysplasia or carcinoma at operation than they were in 88 patients without such changes (Hill et al.

1987). However, in another study, faecal bile acid levels were not elevated either in patients at risk from large bowel cancer (namely those with ulcerative proctocolitis, a previous adenoma or carcinoma) or in the pre-and post-colectomy samples from patients with colorectal cancer (Mudd et al. 1980). Thus there appears to be no simple relationship between concentration of total bile acids in the faeces and risk of carcinoma.

Owen suggests that the ratio of LCA to DCA in the faeces correlates well with the risk from colorectal neoplasia. A high serum cholesterol, which is associated with a low incidence of colorectal cancer, will lower the LCA/DCA ratio (Rose et al. 1974, Owen Thompson and Hill 1984). Bile acid receptors have been reported in colorectal cancers but not in the adjacent normal colonic tissue, and the study of bacterial mutagenesis suggests that bile acids may promote malignant transformation (Wilpart et al. 1983, Summerton et al. 1983). Bile acids may promote the development of malignancy in benign adenomas but may not be responsible for their initial formation (Hill et al. 1987).

In summary the role of bile acids in the initiation of promotion of colorectal neoplasia is still a subject for debate.

Bile acids in FAP

In familial adenomatous polyposis where the risk from colorectal cancer approaches 100%, there should be clear-cut changes in faecal bile acid metabolism if these play an important role in carcinogenesis. The metabolism of cholesterol to copostranol is lower than expected (approximately 25%) in FAP patients (Bone Drasar and Hill 1975, Reddy et al. 1976, Watne et al. 1976), as is the degradation of bile acids (Reddy et al. 1976, Bone

Drasar and Hill 1975). In FAP patients a high proportion of bile acids are hydroxylated primary bile acids (especially cholic and chenodeoxycholic acid) (Watne et al. 1976). Similarly, their faecal flora metabolise tyrosine, lysine, arginine and ornithine to only a limited extent. Bone (1975) concluded that this limited metabolism was not due to an abnormality of the transit time, pH, redox potential or composition of the bacterial flora (Bone Drasar and Hill 1975). Using an *in-vitro* model, faecal bacteria were capable of metabolising substrates that they dealt with only poorly *in-vivo*. These findings may reflect underlying biochemical abnormalities inherited with the FAP phenotype.

Foregut adenomas are almost as common as colonic polyps in FAP and probably of similar importance as indicators of an individual's risk of cancer (Perzin and Bridge 1981, Spigelman et al. 1989). Duodenal bile in FAP has a higher concentration of total bile acids, and in particular of primary bile acids, than control subjects (Spigelman et al. 1991b). In a prospective study of 102 FAP patients, 88 where found to have duodenal polyps, and in these patients the greatest concentration of adenomas were in close proximity to the papilla of Vater (Spigelman et al. 1989). Also, primary epithelial tumours of the upper GI tract in FAP have been noted in the duodenum, biliary tree and liver, and this has linked adenoma and carcinoma formation to the presence of bile. Indeed, a patient has been reported in whom adenocarcinoma and epithelial dysplasia coexisted in the gallbladder, liver, common bile duct and pancreas (Spigelman et al. 1991a), suggesting that extensive dysplasia in association with invasive cancer could be due to an epithelial field change induced by bile (Spigelman et al. 1991a, Spigelman et al. 1991b).

Animal Studies

Intrarectal instillation of bile acids causes mucosal damage (Saunders et al. 1975, Wargovich et al. 1983), epithelial dysplasia and an increased epithelial cell proliferation rate (Rainey et al. 1984). Also, the pancreatic ducts of the dog show epithelial hyperplasia when irrigated with bile acids (O'Leary et al. 1984). A similar effect is seen in the colon following pancreatobiliary diversion to mid small bowel (Deschner and Raicht 1979, Williamson et al. 1979). The oral administration of primary bile acids increases the tumour yield in dimethylhydrazine-treated mice (Martin et al. 1981), and the direct rectal instillation of primary and secondary bile acids similarly promotes contact carcinogenesis by N-methyl N-nitroso-guanidine (Reddy et al. 1977). Per-rectal instillation of sodium deoxycholate also increases colonic CCPR and enhances azoxymethane induced colonic tumour yields, and this tumour promoting effect is reduced by the concomitant administration of metronidazole (Rainey et al. 1984). Aanaerobic bacteria may therefore be required to allow primary bile acids to act as promoters of colorectal carcinogenesis (Rainey et al. 1984). Since bile acids have little effect on the defunctioned (and hence hypoplastic) colorectum, it would appear that either luminal contents or the colonic microflora are required for their action (Rainey et al. 1983). Likewise, chemical carcinogenesis is reduced both in germ free rats (Reddy et al. 1977) and after the administration of erythromycin or tetracyclines (Goldin and Gorbach 1981). Metronidazole and a germ-free environment reduce but do not abolish chemical carcinogenesis, so the presence of anaerobic bacteria is clearly not essential for their action. (Reddy et al. 1977, Rainey et al. 1984).

Bile acids directly damage epithelial cells. Lithocholic acid can cause breaks in DNA strands (Kulkarni Cox and Yielding 1982). In addition, bile salts increase colonic cell proliferation (as shown by increased ornithine decarboxlase activity), and this effect is associated with the release of arachodonic acid and the products of lipoxygenase activity such as prostoglandins (De Rubertis Craven and Saito 1984). In support, inhibitors of cycloxygenase and lipoxygenase prevent bile acid induced colonic hyperproliferation (De Rubertis Craven and Saito 1984, Craven Pfanstiel and De Rubertis 1986). Superoxide dismutase ($O_2$-) and its subsequent reduction products $H_2O_2$ and HO are reactive intermediaries of arachadonic acid metabolism by either the cycloxygenase or lipoxygenase pathways (Marnet Wlodawer and Samuelsson 1974, Lilus and Laakso 1982, Slater 1984). Craven measured superoxide dismutase ($O_2$-) sensitive chemiluminescence and the $O_2$-inhibition of cytochrome C reductase, together with thymidine incorporation into colonic explants and their ornithine decarboxlase activity. He found that the intermediaries of prostaglandin synthesis such as reactive oxygen, rather than more distal products of arachodonate oxygenation, played a central role in the induction of colonic epithelial cell proliferation and DNA synthesis by bile acids (Craven Pfanstiel and De Rubertis 1986).

Summary

Bile acids increase epithelial cell proliferation and are co-carcinogenic in the large intestine. The levels of faecal bile acids are related to the extent of bacterial degradation in the proximal colon and appear to have some correlation with colorectal cancer incidence, though the exact nature of this relationship is unclear. The postulated environmental influence on the development of cancer in FAP may, at least in part, be due to the elevated primary bile acids seen in the duodenum and colon in these patients.

# Chapter 3.

## Cell proliferation and its relevance to colorectal cancer.

### 1. Large bowel hyperplasia and neoplasia

The adenoma-carcinoma sequence

An adenoma is a circumscribed area of intestinal epithelium showing characteristic cellular dysplasia which represents unequivocal neoplastic epithelial proliferation, as opposed to the mature epithelial cells seen on the surface of metaplastic or hyperplastic polyps (Fenoglio Kaye and Lane 1973). Histologically, this dysplasia is confined to the mucosa, and does not breach the muscularis mucosa. Adenomas are therefore non-invasive or benign. The absence of lymphatics in the muscularis could account for their inability to metastasise (Vatn and Staslberg 1982, Williams and Balasooriya 1982, Morson and Bussey 1983). Colorectal adenomas occur in approximately 4% of the population and are more common in the elderly and in males (Morson and Konishi 1982, Morson and Bussey 1983). There is some geographical correlation between the incidence of adenomas and carcinomas, both being low in Africa and more abundant in the U K and the U S A (Vatn and Stalsberg 1982, Williams and Balasooriya 1982). This correlation is by no means absolute, however (Morson and Konishi 1982, Morson and Bussey 1983).

The occurrence of adenomas in the gastrointestinal tract is well known to be a definite phenotypic marker for familial adenomatous polyposis (Bussey 1990) and, untreated, these patients will go on to develop colorectal cancer. In hereditary non-polyposis cancer, the malignant lesion was thought to develop de-novo without the presence of a precursor

adenoma, but some of these patients do develop adenomas and these grow in a plaque-like fashion, known as flat adenomas (Lynch et al. 1988). Thus, the occurrence of flat adenomas in a member of a non-polyposis cancer family is also a phenotypic marker indicating that the individual is at risk from colorectal cancer. The presence of an adenoma is also a risk factor in patients with no obvious family history of colorectal cancer, and some sporadic colorectal cancers may also have a hereditary component (Burt et al. 1985, Cannon-Albright et al. 1988). This fact suggests that adenomas act as possible phenotypic markers of large bowel malignancy in most individuals.

Macroscopically, 75% of adenomas are tubular (with tightly packed epithelial tubules), 15% tubulo-villous and 10% villous (consisting of finger-like projections of neoplastic epithelium). Adenomas are evenly distributed through the colon and rectum, whereas carcinomas occur more frequently in the distal large bowel (Day 1984). Large adenomas are however more common in the sigmoid and rectum ((Morson and Konishi 1982), and this distribution matches the left-sided predominance of large bowel cancer.

The severity of dysplasia, an increasing size and a greater proportion of villous architecture have been linked with the incidence of adenocarcinoma within an adenoma (Mutto Bussey and Morson 1975). Likewise, in those patients with synchronous adenoma and carcinoma the patients who later went on to develop metachronous carcinoma exhibited higher grades of dysplasia in their initial adenoma (Morson and Konishi 1982). The observation of varying degrees of dysplasia within an adenoma can be used as further evidence of a natural progression from a benign adenoma to a malignant carcinoma (Fenoglio Kaye and Lane 1973, Day 1984, Hagget et al. 1985). Severe dysplasia has been

termed carcinoma in-situ; it is almost seven times more common in synchronous adenomas excised with carcinomas than it is in adenomas excised on their own (Kalus 1972). Similarly, patients with adenomas exhibiting carcinoma-in-situ have higher rates of synchronous cancers compared with those that have adenomas showing less severe dysplasia (Rittenhouse and Copeland 1978). Thus the presence of high-grade dysplasia within an adenoma seems to imply a greater risk of developing metachronous and synchronous carcinoma. Retrospective studies suggest that the development of synchronous and metachronous carcinoma is related to the number of adenomas present (Mutto Bussey and Morson 1975, Shinya and Wolff 1979), while adenomas also occur more frequently in patients with single and multiple carcinomas. In a large study at St Marks (1961 cases of colorectal cancer), adenomatous tissue was only found in 14% of the carcinomatous lesions (Mutto Bussey and Morson 1975). The presence of adenomatous tissue within a large bowel carcinoma has been correlated with the stage of the lesion, suggesting that adenomatous tissue is replaced by carcinoma (Morson 1966). Indeed, 3% of colonic polyps removed at endoscopy contain carcinoma (Gillespie et al. 1979).

In the rat, injections of the chemical carcinogen hydrazine is followed by microadenoma formation in the colon (detected by electron microscopy) at 5 weeks. Macroscopic adenomas develop at 15-20 weeks and carcinoma at 20-30 weeks after hydrazine (Gilbert 1987, Cooke et al. 1984). In a similar way, adenoma formation mirrors the distribution of intestinal carcinomas when the related carcinogen azoxymethane is used (Rainey et al. 1983, Rainey Davies and Williamson 1984). Conflicting evidence in experimental models suggests no obvious relationship between invasive tumour formation and either tumour size or degree of dysplasia (Izibicki et al. 1985).

In summary, most (perhaps all) colorectal cancers develop as an area of dysplasia within the epithelium and progress to cross the muscularis mucosa, thereby becoming invasive tumours. These areas of dysplasia initially give rise to a macroscopically localised area of abnormal epithelium, and this manifestation may be in the form an adenomatous polyp.

Hyperplasia

The turnover of cells in the intestinal tract is rapid, exceeding that seen in any other body tissue, and this allows the gut to compensate quickly for the loss of mucosal mass (Williamson Bucholtz and Malt 1978, Sarraf et al. 1991). The large bowel differs from the small bowel in both structure and function. Structurally it possess crypts but no villi and it has an incomplete longitudinal muscle coat and mesentery. Functionally, it appears to act mainly to reabsorb fluid and electrolytes whereas the small bowel is mainly concerned with the absorption of nutrients (Bristol and Williamson 1984). In the small bowel the functional unit is the villus with its associated crypt or crypts (Clarke 1972). The colonic crypt with its surrounding mucosal cuff is the equivalent metabolic and proliferative unit in the large bowel (Bristol and Williamson 1984).

Before the turn of the century it was postulated that intestinal epithelial cells were replaced from cells within the intestinal crypts that migrated towards the lumen (Bizzozera 1888). The presence of a single precursor cell that can give rise to all subsequent cells has been postulated (Copson 1966). Wright suggests that, at least in the small bowel, these cells are slowly cycling and positioned at the bottom of the crypt (Wright and Alison 1984). The luminal migration of these cells appears to be accompanied by functional and biochemical differentiation (Imondi Balis and Lipkin 1969). After migration to the crypt

surface, cells are shed into the lumen (Cheng and Leblond 1974, Sunter 1984). Stathmokinetic and labelling techniques indicate that the colonic epithelium is completely replaced in approximately 2-5 days in the rodent (Obertrop et al. 1977) and 4-8 days in man (Lipkin Sherlock and Bell 1962, Lipkin Sherlock and Bell 1963, Williamson 1982). In the colonic crypt, proliferative activity varies from a low level at the base (stem cell zone) to a maximum level just above it, tailing off almost to zero at a point two-thirds of the way up the crypt (Sunter et al. 1978, Sunter Wright and Appleton 1979, de Roderiguez et al. 1979). Wide variations in intestinal cell kinetics have however been reported within the same species and at the same sites in the gastrointestinal tract, factors that effect small bowel proliferation may account for these differences, such as Circadian rhythm (Hamilton 1980), sex (Hoff Chang and Mak 1981), and diet (Stragand and Hageman 1978, Ryan et al. 1979).

Postoperative adaptation in animals

The loss of functioning intestinal tissue is a powerful stimulus for small bowel growth and the same may be true in the large bowel (Williamson Buchholtz and Malt 1978, Williamson et al. 1982). By measurements of the wet weight, DNA and protein content of the mucosa, have suggested a modest hyperplasia of the right colon of the rat 40 weeks after caecectomy or left hemicolectomy, but no equivalent effect was seen in the left side of the colon (Williamson 1982). In one study an increase in weight of the remaining colon was observed three months after right hemicolectomy (Masesa and Forrester 1977). When caecectomy is combined with distal ileal resection, an increased mucosal depth is seen throughout the colon (Scarpello Cary and Sladen 1978). The formation of a colostomy that diverts faeces from the distal colon has a more profound effect in the oppsoite direction.

Mucosal DNA, RNA and protein content declined to about half their normal values in one study, and this hypoplasia quickly reversed when the colostomy was closed (Terpstra et al. 1981); although no immediate effect was seen proximal to the colostomy, transient hyperplasia followed colostomy closure. The distal colon showed intense proliferation within one week of colostomy closure, whereas the hyperplasia in the proximal bowel segment was delayed. A systemic effect was postulated to account for the response of the proximal bowel and a direct action of the faeces for the distal effect (Bristol and Williamson 1984). The distal colonic hypoplasia seen on colostomy formation produces a reduction in total crypt cell number and CCPR, but this effect is not associated with a change in the size of the proliferative compartment (Rijke Gart and Langendon 1979, 1980, Rainey et al. 1983). Foster and Whithead have confirmed that faecal diversion by colostomy quickly and reliably produces hypoproliferation in the distal colon (Foster and Whitehead. 1990). Likewise, the diversion of faeces from a viable segment of colon by the formation of a Thiry-Vella fistula (TVF) will reduce CCPR, while the instillation of faeces into the fistula partially restores mucosal mass (Rainey et al. 1983). Diet also has a strong effect on the colon. Non-absorbable, non-nutritive bulk can preserve mucosal integrity (Dowling et al. 1967, Ryan et al. 1979), wheras low-residue diets, even with adequate calorific content, cause mucosal hypoplasia and reduced cell proliferation (Janne Carpenter and Willems 1977, Morin Ling and Bourassa 1980, Storme and Willems 1981).

Small bowel operations can affect the large bowel. An increase in caecal weight, mucosal thickening and surface area has been observed after small bowel resection in the rat (Scarpello Cary and Sladen 1978, Williamson 1982). Structural and cytokinetic adaptation is seen after both ileal resection (Nundy et al. 1977, Obertrop et al. 1977, Tilson

1980) and resection of the mid small bowel (Williamson 1982). Jejunoileal bypass also increases colonic CCPR, and this effect is maintained for up to 30 weeks postoperatively (Rainey et al. 1983, Bristol Wells and Williamson 1984). It has been suggested that a critical mass of small bowel needs to be lost in order to provoke this colonic adaptive response (Bristol Wells and Williamson 1984). Pancreatobiliary diversion to mid small bowel likewise causes colonic hyperplasia, but this response is short lived (Williamson et al. 1979, Williamson and Rainey 1984).

Adaptive responses in man.

Information on postoperative adaptation in man is sparse. Shaw and Dunn demonstrated a reduced colonic mucosal and muscle mass in the rectal stump 35 years after the formation of a defunctioning ileostomy. The number of cells was reduced but their size was unchanged (Shaw and Dunn 1981). Using an in-vitro organ culture technique to measure CCPR, Appleton has shown a reduced CCPR (58%) in the rectum of patients with a defunctioning colostomy compared to controls (Appleton et al. 1987). By contrast, patients who had undergone jejunoileal bypass for morbid obesity had rectal proliferation rates (CCPRs) that were more than double those seen in controls. This rectal hyperplasia was not associated with an altered crypt morphology or mucosal histology, and the effect was maintained for up to 11 years following the operation (Appleton et al. 1988).

From the limited evidence available in man, thereore, it appears that both small bowel resection and bypass will stimulate colorectal epithelial proliferation and that defunctioning colostomy will reduces it. Thus the human data consolidate those seen in animals.

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Epithelial hyperplasia and chemical carcinogenesis.

Cell proliferation is central to the initiation of carcinogenesis in epithelial tissue and may also play a role in tumour promotion (Williamson 1984). Since conditions that promote intestinal cancer also increase epithelial cell proliferation, it might also hold true that epithelial hyperplasia predisposes towards malignancy.

Spontaneous gastrointestinal tumours are rare in animals (Wells Slye and Holmes 1938, Roberts Rowlands and Lawson 1980), and colorectal tumours are particularly rare in the rodent (Mc Coy 1909). Nevertheless, chemical carcinogens readily induce the growth of large bowel neoplasms in rodents and can therfore provide a good model for gastrointestinal carcinogenesis. Cycasin, a natural plant product, is a potent carcinogen when fed to rats (Laquer 1968). This observation lead to the synthesis of methylmethoxymethane, azoxymethane and 1, 2 dimethylhydrazine, which are all specific carcinogens in the rodent. Dimethylhydrazine is oxidised via azoxymethane to methyl-azoxymethanol, which spontaneously decomposes to produce carbonium ions (an active methylating agent). Exposure of the intestinal epithelium to these carcinogens causes irreversible cellular damage, with the subsequent development of epithelial hyperplasia, dysplasia and neoplasia. 1,2 dimethyl hydrazine and azoxymethane are effective by either subcutaneous or intraperitoneal injection. Intestinal tumours occur after a latent period of 15-20 weeks, and these are almost entirely confined to the intestinal tract (apart from the occasional tumour of the auditory canal or the kidneys). The adenomas and carcinomas that occur are similar to those seen in man and cause weight loss, bleeding and intestinal

obstruction (Nigro 1981). All histological changes are seen from adenoma through dysplasia to invasive carcinoma, mucinous cancer being especially aggressive as in man.

Carcinogen-treated rats subjected to proximal small bowel resection display colonic epithelial hyperplasia in advance of macroscopic tumour development. The distribution of these tumours is very similar to that seen in spontaneous human gastrointestinal adenocarcinomas. Nearly all the small bowel tumours occur close to the duodeno-jejunal flexure (Williamson Welch and Malt 1983), and the ileum remains relatively resistant to carcinogenesis despite the induction of hyperplasia (Williamson Buchholtz and Malt 1978, 1984, Oscarson et al. 1979, Scudmore and Freeman 1983). Proximal small bowel resection promotes chemical carcinogenesis in the colon (Scudmore and Freeman 1983), and distal small bowel bypass has an even more profound effect (Bristol and Williamson 1984, Olubuyide et al. 1984). In a similar fashion, distal small bowel resection, jejunoileal by-pass, and pancreatobiliary diversion to mid small bowel will all induce colonic hyperplasia and promote chemical carcinogenesis (Williamson Buchholtz and Malt 1978, Williamson et al. 1979, Bristol and Williamson 1984, Olubuyide et al. 1984).

In the rat, partial colectomy produces surprisingly little adaptive hyperplasia and does not appear to increase tumour yields, except at the site of an anastomosis (Williamson and Rainey 1984). A low dose of carcinogen (in the rat) produces a low incidence of tumours, with a relatively even distribution throughout the large bowel. As the dose of the carcinogen is increased, a left-sided predominance occurs for the large bowel tumours (Williamson Buchholtz and Malt 1978, Oscarson et al. 1979). When the colon is taken out of continuity as a Thiry-Vella fistula, these differences disappear. Similarly a defunctioning

transverse colostomy reduces both the CCPR and the median number of tumours in the distal colon (Rainey Davies and Williamson 1983). The resulting hypoplasia and impaired response to azoxymethane is not reversed by the stimulus of a small bowel resection.

In contrast to man, the rectum of the rat is relatively resistant to tumour formation. If subtotal colectomy with ileorectal anastomosis is performed, this relative sparing of the rectum is overcome (Izbicki et al. 1983), possibly due to the presence of a suture line. Terpstra found that the presence of a colostomy did not promote chemical carcinogenesis in the proximal or distal colon, but colostomy closure promoted carcinogenesis in the distal colon and at the anastomoses. Thus the administration of a carcinogen during a period of intense cell proliferation enhances subsequent neoplastic growth (Terpstra et al. 1981). It appears that intestinal anastomoses are susceptible to carcinogenesis if at least one bowel end is a potential site of malignancy (Gennaro 1973, Blake et al. 1980, Celik et al. 1981, Filipe Scurr and Ellis 1982, Williamson 1982).

Primary and secondary bile acids instilled into the rectum of the rat in continuity with the faecal stream will promote chemically induced carcinogenesis. This effect is seen even in the germ free animal (Narisawa et al. 1974). Similarly, bile acids given orally increase tumour yields in animals (Cohen et al. 1980, Martin et al. 1981). When the colon is defunctioned as a Thiry-Vella fistula, irrigation of the fistula with deoxycholate has no effect on cell kinetics or carcinogenesis, implying that the luminal environment is important for the promotion of carcinogenesis by bile acid in the colon (Williamson and Rainey 1984). Also, the co-carcinogenic effects of intra-rectal deoxycholate in the rat are reduced

by oral metronidazole, suggesting that anaerobic bacteria have a role in allowing bile acids to act as tumour promoters (Rainey Davies and Williamson 1984).

In summary, it appears that any operation or procedure that leads to compensatory hyperplasia in animals will promote chemical carcinogenesis. This effect appears to be restricted to those segments of the gut that are susceptible to tumour formation. Luminal factors can also play a part in the promotion of neoplasia in the colon and rectum.

Large bowel hyperplasia and malignancy in man.

In experimental models of colorectal carcinogenesis epithelial hyperplasia precedes the development of malignancy in the large bowel. Hyperplasia could also be one of the first steps in the development of adenomas and carcinomas in man, producing either a general field change affecting the large bowel or a localised area of intense hyperproliferation (e.g at a suture line). Direct (*in-vivo*) measurements of epithelial cell proliferation are difficult to obtain in man for obvious ethical reasons (see later). Therefore, information about cell growth in man has relied heavily on *in-vitro* techniques.

Using an *in-vitro* method to label cells with thymidine, an increase in cell proliferation has been found in the rectum of patients with large bowel adenomas and carcinomas (Deschner and Lipkin 1966, Maskens and Deschner 1977, Deschner and Maskens 1982, Bleiberg Buyse and Galand 1985). Terpstra used a similar method to measure the labelling index in mucosal biopsies taken at various points throughout the colon. An increase in the labelling index at all sites throughout the colon was seen in patients with colorectal neoplasia compared to control patients (Terpstra et al. 1987). The increased labelling index

was greater for large adenomas and carcinomas than for small adenomas. This finding strengthens the suggestion that patients who are susceptible to sporadic large bowel tumours exhibit a pre-neoplastic epithelial hyperplasia throughout the colon and rectum. It could also account for the well recognised development of synchronous and metachronous tumours seen in man (see Chapter 2). Using the accurate metaphase arrest technique, Barsoum has shown increased rectal CCPR in patients with adenomas and carcinomas compared to controls (Barsoum et al. 1992).

In patients with a clear hereditary risk of colorectal cancer (e.g. FAP and the familial cancers), there is an expansion of the proliferative zone in the epithelium of the colon and rectum (Deschner Lewis and Lipkin 1963, Bleiberg Mainguet and Galand. 1972, Deschner and Lipkin 1975, Iwana Utsunomiya and Sasaki 1977, Lipkin et al. 1983, Lipkin et al. 1984). Indeed, in adenomatous tissue from patients with FAP there is a shift in the entire proliferative region towards the surface of the adenoma (Lightdale Lipkin and Deschner 1982). By contrast, Seventh-Day Adventists (whose diet renders them at a low risk of developing colorectal cancer) have labelling indices that indicate a slower rate of epithelial proliferation compared to both normal controls and patients at a higher risk of colorectal cancer (Lipkin and Newmark 1985, Lipkin 1988). The defunctioned rectum after proximal colostomy or ileostomy also has a reduced rate of epithelial proliferation (CCPR) compared to controls. This effect appears to be sustained for at least 5 years following creation of the stoma and could be directly related to the lack of faecal stream (Appleton and Williamson 1989).

Patients with ulcerative proctocolitis are well recognised as being at increased risk of colorectal cancer. Measurements of cell turnover time, labelling index and size of proliferative compartment all indicate the presence of a hyperplastic epithelium in this disease (Bleiberg et al. 1970, Eastwood and Trier 1973, Serafini et al. 1985). Likewise, the crypt cell production rate is increased in both active and quiescent disease (Allan Bristol and Williamson 1985). Thus, in ulcerative proctocolitis the intestinal crypts generate more cells at their base in response to the premature loss of surface epithelial cells. The hyperplasia seen during remission is intermediate between the "normal" colon and that in active disease, and this finding could explain the continuing susceptibility to colorectal cancer seen in patients with quiescent disease (Allan Bristol and Williamson 1985, Lipkin 1988).

Suture line cancers.

The presence of a suture line in the gastrointestinal tract favours tumour formation both in experimental animals and in man after colectomy for colorectal cancer (Williamson. 1982, Rubio et al. 1984, Phillips et al. 1984, Umpleby and Williamson 1987). Anastomotic "recurrence" following resection for cancer in man could also be due to incomplete primary resection or implantation of live exfoliated cells (Phillips and Cook 1986, Umpleby and Williamson 1987 O'Dwyer and Martin 1989). Indeed, the presence of sutures appears to aid the implantation of tumour cells (Vink. 1954, Cohen 1967, Yu and Cohen 1968). In at least 30% of cases anastomotic recurrence is a late effect, occurring well after the increase in cell proliferation that accompanies the formation of an anastomosis has subsided to normal, i.e. after approximately six weeks (Pozharisski 1975,

O'Donnell et al. 1991). Therefore, simple surgical injury may not explain the preferential tumour formation seen at suture lines.

The type of suture used to create an anastomosis also appears to be important. Braided and non-absorbable sutures provoke a greater increase in CCPR and are associated with more perianastomotic tumours than monofilament or absorbable sutures (Phillips and Cook 1986, Calderisi and Freeman 1984, Roe Fermor and Williamson 1987, Mc Gregor et al. 1989 ). In addition seromuscular sutures that do not traverse the full thickness of the bowel wall induce a smaller increase in CCPR than transmural sutures, and they also appear to reduce the incidence of perianastomotic tumours after chemical carcinogenesis in animals (O'Donnell et al. 1991). The formation of an anastomosis without sutures in rats also prevents a proliferative response and may help reduce anastomotic recurrence (Mc Cue and Phillips 1992.)

It is possible that the intensity of mucosal proliferation at the time of tumour induction favours the development of cancer at the suture line. If this is true, then the evidence suggests that sutures that promote the least kinetic response (such as seromuscular sutures with monofilament or absorbable fibres) should be used in man.

## Summary

There is very good evidence in animals to suggest that hyperplasia is an early event in the promotion and development of intestinal neoplasia. In man, hyperplasia has been observed in many conditions that carry an increased risk of colorectal cancer. Thus

circumstantial evidence indicates that hyperplasia may precede the development of colorectal cancer in man.

## 2. The influence of dietary and humoral factors on large bowel cancer and colorectal epithelial cell proliferation.

Dietary Fibre.

The incidence of colorectal cancer is low in rural African communities that have a high dietary fibre intake, with associated increase in stool bulk and reduced colonic transit time. Burkitt therefore suggested that dietary fibre may protect against colonic cancer (Burkitt 1971). That the type of fibre is more important than the mere presence of dietary bulk is illustrated by a study conducted in 6 healthy volunteers, comparing different fibre preparations from dehulled soya beans. The mean excretion of bile acids was increased with soya pulp but not with a purified soya diet. The increase in total faecal bile acids was due to increased faecal deoxycholic acid (DCA), but faecal levels of neutral steroids and cholesterol conversion were unaffected (Schweizer et al. 1983). It is well known that dietary fibre can elevate faecal bile acids, and this effect has been related to either trapping of bile acids within the faecal bulk or to changes in bacteriological and physiological conditions within the large bowel. Quite small variations in fibre preparation appear to have marked chemical and physiological effects (Schweizer et al. 1983).

In animal studies, fermentable fibres increase the length and area of the caecum and reduce its luminal pH (Lupton Coder and Jacobs 1988, Lupton and Marchant 1989). These effects are greatest with the most fermentable fibres. Thornton has suggested that a high colonic pH can promote the development of colorectal cancer, and that the acidification of the colon, either by bacterial degradation of fibre to short chain fatty acids (SCFA) or by milk (in lactose intolerant individuals), may prevent this action (Thornton. 1981). In

support, black South Africans eating a high-fibre low-fat diet have a faecal pH of 6.5, whereas white South Africans eating a western diet have a higher pH (Walker Walker and Segal 1979). Likewise, Seventh-Day Adventists, who have a low risk from large bowel cancer, have a lower faecal pH than colorectal cancer patients (Mc Donald Webb and Mahory 1978). The major action of dietary fibre may be to reduce colonic pH by the production of SCFA (Cummings et al. 1976). By contrast Walker (Walker Walker and Segal 1979)), suggests that dietary fat and protein intake do not affect faecal pH and are thus unlikely to have an affect on the degradation of bile acids. A reduction in caecal pH has been associated with an increased epithelial proliferation. Characteristic of this acid fermentation by colonic microflora is the generation of volatile fatty acids, especially butyrate and proprionate (Deminge and Remesy 1985, Levrat et al. 1991).

These SCFAs are usually present in the lumen of the colon and comprise the main solute fraction of faecal water. They are readily taken up by the colonic mucosa and are the preferred respiratory fuel of colonocytes (Roediger 1982). Cellular proliferation, as determined by ornithine decarboxlase activity, thymidine kinase activity and the proportion of cells in the S-phase of the cell cycle, has been correlated with luminal pH (Lupton Coder and Jacobs 1988) and volatile fatty acid production (Levret et al. 1991). The rate of absorption of these volatile acids or SCFAs is proportional to increasing chain length. Some of these fatty acids may also be metabolised in the colonic wall (Deminge and Remesey 1985). Faecal concentrations of SCFAs are similar in patients with colonic adenomas and carcinomas to those seen in control patients (Clausen Bonnen and Mortensen 1991), while the ratio of butyrate to total SCFAs is reduced in patients with colorectal carcinoma or adenoma (Weaver et al. 1988, Clausen Bonnen and Mortensen 1991).

Butyrate is an important fuel for colonocytes (Roediger 1980, Roediger 1982), It promotes colonic cell proliferation but inhibits neoplastic growth (Clausen Bonnen and Mortensen 1991). In human-derived colorectal cancer cell lines, butyrate reduces cell growth, increases the potential doubling time and induces changes indicative of differentiation (Kim et al. 1980, Whitehead Young and Bhanthal 1986). It has therefore been suggested that dietary fibre exerts its effect via the production of butyrate, which in turn induces colonocyte differentiation. (Clausen Bonnen and Mortensen 1991). In support of this hypothesis, butyrate levels are low in the sigmoid and descending colon where the incidence of neoplasia is high (Cummings et al. 1987).

Dietary protein intake is associated with an increased incidence of colon cancer in humans and promotes carcinogenesis in rat colon (Armstrong and Doll 1975). Ammonia has also been implicated in the stimulation of cellular proliferation (Topping and Visek 1977). Lupton suggests that fibre may dilute ammonia by bulking the stool, speeding its passage through the colon or, in the case of fermentable fibres, by lowering proximal colonic pH (Lupton Coder and Jacobs 1988, Lupton and Marchant 1989). Since the diffusion of ammonia is mainly non-ionic, the more acidic the lumen becomes, the less absorpton of ammonia will occur (Swales Tange and Wrong 1970). Bacteria may also play a role in scavenging ammonia for protein synthesis. The choice of an appropriate fibre could therefore ameliorate the potential harmful effects of protein metabolism (Lupton and Marchant 1989). In support, protein diets in the rat increase the caecal concentration of ammonia, while dietary fermentable pectin fibre has the opposite effect. In rat colon, fermentable fibre increases colonic luminal ammonia levels, perhaps due to a greater

protein load reaching the colon or to an increased bacterial activity (Lupton and Marchant 1989). Studies in germ-free animals would tend to support the suggestion that bacteria are responsible for the production of luminal ammonia and urea (Evrard et al. 1964, Warren and Newton 1959).

In summary, the amount of fermentable fibre in the diet appears to be important in the control of colonic epithelial cell proliferation, but the mechanism of this action is not as yet fully understood.

Dietary fat.

Diets that are rich in total fat and have a low fibre content have been correlated with an increased incidence of colorectal cancer (Wynder et al. 1969, Armstrong and Doll 1975, Burkitt 1978, Reddy 1981), and Kune's extensive case-control studies in Australia support this relationship (Kune Kune and Watson 1988a, Kune et al. 1988). One explanation for the mode of tumour promotion is that dietary fat alters the composition of the bile and modification the gut microflora, allowing greater production of tumour promoters from bile acids within the colonic lumen (Hill et al. 1971, Weisburger Reddy and Wynder 1977). In support, a significant increase in the faecal secondary bile acids and neutral steroids is seen in populations consuming a high-fat mixed "western diet" (Reddy 1981). According to Thornton, although a high-fat diet increases bile acid excretion the provision of fibre in such a diet could protect against carcinogenesis by reducing colonic pH and therby preventing 7 alpha-hydroxlation of bile acids to co-carcinogens (Thornton 1981).

Animals given a high-fat diet have increased labelling with tritiated thymidine and an accumulation of mitotic figures in the colonic crypts (Bird et al. 1985, Bird and Stamp 1986). In a similar way, high fat (corn oil) diets increase thymidine labelling indices in human rectal biopsies, a finding that correlates with increased lithocolic, deoxycholic and total bile acid levels in the faeces (Stadler et al. 1988). The effect of dietary fat could therefore be secondary to an increase in those faecal bile acids that are known to promote experimental carcinogenesis. If healthy human volunteers are given a high-fat, high-beef diet (compared to a customary western diet), they also show an increase in faecal levels of total bile acids and secondary bile acids (Reddy 1981). Similarly, increasing the fat content in rat chow will increase biliary excretion of bile acids and raise secondary bile acid levels in the faeces (Reddy Watanabe and Weisburger 1977).

It appears that the fatty acid composition of the fat is also important for carcinogenesis (Reddy Watanabe and Weisburger 1977, Reddy 1981, Reddy and Maeura 1984, Reddy Tanaka and Simi 1985). Eskimos consuming large amounts of marine oils in their diets but have lower cancer rates than North American and other western populations. These fish-oil high-fat diets have no enhancing effects on azoxymethane-induced colonic carcinogenesis (Reddy and Sugie 1988, Reddy Burill and Rigotty 1991), whereas corn oil will enhance colonic carcinogenesis in the post-initiation phase (Bull et al. 1981, Reddy and Maruyama 1986). When dietary fish oil is given in conjunction with corn oil, experimental colorectal carcinogenesis is reduced. This effect is seen at both the initiation and promotional stages and could imply that fatty acid composition may also exert an effect during initiation of cancer (Reddy, Burill and Rigotty 1991).

Both eicsosapentaenoic and docosahexaenoic acid (w-3 fatty acids present in fish oils) can inhibit cycloxygenase activity, and eicsosapentaenoic acid also inhibits the lipoxygenase pathway of prostaglandin synthesis (Reddy et al. 1980, Reddy et al. 1990, Reddy Burill and Rigotty 1991). The prostaglandin synthesis inhibitors aspirin (Kune Kune and Watson 1988), indomethacin and piroxicam (Craven Pfanstiel and De Rubertis 1986, Reddy et al. 1990) also protect against colorectal cancer. The observed lack of tumour promotion with high fish-oil diets may be due to an effect on prostaglandin synthesis, whereas the effect of these diets on initiation of chemical carcinogenesis might be explained by altered metabolism of azoxymethane (Reddy Burill and Rigotty 1991).

Calcium

In Scandinavian countries the risk of colorectal cancer has been correlated with the intake of dairy produce. A high dietary intake of dairy produce (and hence calcium) in Finland is associated with a reduced incidence of colorectal cancer. Denmark has a higher incidence of large bowel cancer than Finland, and the intake of calcium is much lower (IARC Working Party 1982). Similary, Japanese men living in Hawaii have low calcium diets and are at greater risk from colon cancer than their counterparts in Japan, whose diet is comparatively rich in calcium (Garland and Garland 1985). However, a separate study failed to expose this correlation between calcium intake and colorectal cancer in Hawaiian men of Japanese extraction (Heilbrun et al. 1986). A 19-year study of 1954 electricity workers in Chicago showed a strong inverse relationship between both dietary calcium and calcium-vitamin D product and the incidence of colorectal cancer (Garland et al. 1985). Likewise, Seventh-Day Adventists have a high calcium intake (Philips 1975).

Animal Studies

Increased dietary calcium may therefore protect against colorectal cancer in the community. The mechanism of this action is unknown but an interaction with bile acids has been postulated to account for the protection it affords man. Fry and Staffeldt in 1964, reported that bile acids were toxic to the colonic mucosa of the mouse. This mucosal damage is accompanied by an increase in the rate of cell proliferation of the colonic epitheliam of the rat (Bull et al. 1983). Bile acids given both orally and by intrarectal instillation increase colorectal tumour yields after exposure to carcinogens (Cohen et al. 1980, Rainey Davies and Williamson 1984). Similarly, dietary fat promotes tumour formation in the colon of the rat after 1,2 dimethylhydrazine (Reddy et al. 1977), or azoxymethane (Reddy and Maeura 1984). The toxic effects of deoxycholate on the rat colon (Wargovich et al. 1983) and fatty acids in the mouse colon are ameliorated by the addition of calcium to the drinking water (Wargovich Eng and Newmark 1984). These observations led Newmark to suggest that calcium protected against colon cancer indirectly by binding to free fatty acids (FFA) and free bile acids (FBA) within the colonic lumen (Newmark Wargovich and Bruce 1984). The resultant non-irritant calcium soaps might reduce the colonic mucosal damage and thus prevent any compensatory hyperproliferation. In support of this hypothesis, the calcium salts of fatty acids and bile acids appear in the faeces if sufficient calcium is present in the diet of children (Telfer 1930), and faecal free fatty acids are elevated when rats are given supplementary calcium (Appleton et al. 1991).

The tumour-promoting effect of high dietary fat in the rat is reversed by doubling the daily intake of calcium (Pence and Buddingh 1988). Likewise, small bowel resection enhances azoxymethane induced colonic and duodenal carcinogenesis in the rat yet this effect is negated by simply doubling the daily calcium intake (Appleton et al. 1987). To the

contrary, Mc Sherry failed to show a protective effect of a three-fold increase in dieatary calcium on tumour yields in N-methyl-N-Nitrosourea-treated rats receiving oral cholic acid (Mc Sherry et al. 1989). Similarly, in rats given 1,2 dimethyl hydrazine, supplemental calcium failed to reduce the overall incidence of colorectal tumours (Sitrin et al. 1991). Oral calcium in this experiment did decrease both the number of rats with multiple tumours and the individual size of tumours, an effect that was abolished in vitamin D-deficient animals.

In animals there is good evidence that an increase in colorectal epithelial proliferation predisposes the mucosa to malignant change and that dietary calcium supplements appear to have a protective effect. In the rat, N-methyl-N-nitroso N-nitrosoguanidine-induced hyperproliferation of colonic crypts can be reduced by dietary supplementation with calcium (Reshef et al. 1990). In a similar fashion, jejunoileal bypass powerfully stimulates colonic crypt cell production rate (CCPR) and promotes chemical carcinogenesis. Doubling the daily intake of calcium (by the addition of calcium lactate to the drinking water) reduces CCPR and abolishes the adaptive response to small bowel resection (Appleton Bristol and Williamson 1986).

The induction of colonic ornithine decarboxylase (ODC) and tyrosine kinase activity by azoxymethane (indicative of epithelial hyperplasia) is likewise suppressed by modest calcium supplements. This effect is also seen using an in-vitro organ culture system (Arlow et al. 1989). *In-vitro* CCPR in rat colorectal mucosa can be reduced by addition of calcium lactate to the organ culture fluid, suggesting that calcium has a direct action on the epithelium (Appleton et al. 1991). Analysis of faecal fatty acids and bile acids in the rat

with or without calcium supplements has shown that calcium may bind to free fatty acids but not bile acids within the lumen of the gut (Appleton et al. 1991).

In animals it appears that calcium can suppress epithelial proliferation and reduce chemical carcinogenesis. These effects may be in part due to an "indirect" acidic lipid binding to form calcium soaps, but in addition there appears to be some "direct" action on the colonic epithelium.

Human studies.

The evidence for a potential beneficial effect of calcium against the development of colorectal cancer in man relies mainly on experiments that indirectly assess an individual's risk of developing colorectal cancer. The rate of colorectal epithelial proliferation has been correlated with this risk. An increase in the thymidine labelling index is seen in patients with large bowel neoplasms, in asymptomatic patients with familial adenomatous polyposis and in the hereditary non-polyposis syndromes (Lipkin et al. 1984, Bleiberg Buyse and Galand 1985). As a corollary, Seventh-Day Adventists have reduced labelling indices (Lipkin Uehara and Winawer 1985). In addition, the labelling index is raised throughout the large bowel in patients with colorectal neoplasms compared to control patients (Terpstra et al. 1987). Likewise, CCPR is raised in ulcerative proctocolitis (Allan Bristol and Williamson 1985) and in patients with colonic adenomas and carcinomas (Barsoum et al. 1992).

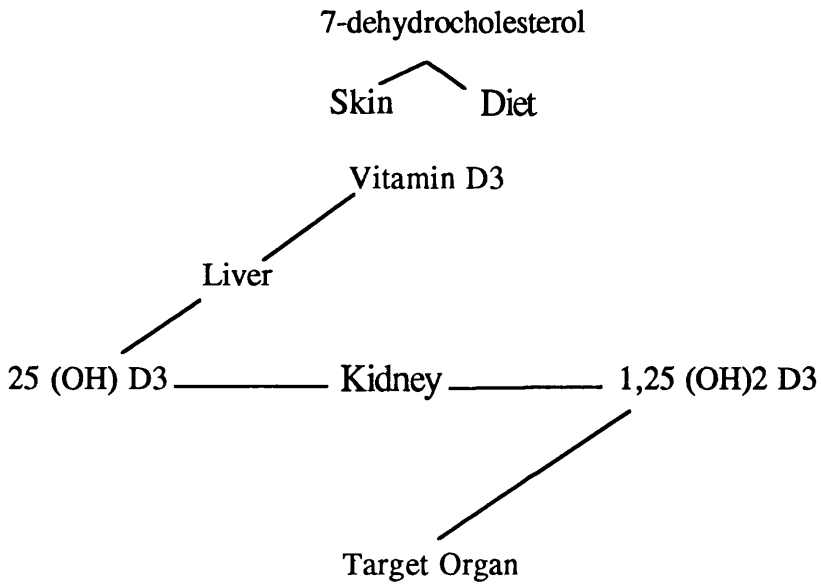
Cell kinetic parameters can be used to assess the effects of supplemental dietary calcium on the colorectal epithelium. In an uncontrolled trial, ten individuals from colon cancer families were given 125g of oral calcium carbonate for 2-3 months, and this reduced the *in-vitro* thymidine labelling profiles to a pattern similar to that seen in a low risk group (Lipkin and Newmark 1985). Two further uncontrolled trials of oral calcium in patients with familial adenomatous polyposis (Lipkin et al. 1989) and familial colon cancers (Rozen et al. 1989) suggested that only those individuals with initially elevated labelling indices will respond to dietary supplements. Gregoire et al. (1989) found no response in a double-blind trial of oral calcium in postoperative patients, but another double-blind trial in patients with villous adenomas has recently shown a reduction in the CCPR compared the placebo and pre-treatment patients (Barsoum et al. 1992). Buset has compared in-vivo thymidine incorporation after oral calcium with *in-vitro* response of biopsies to calcium in seven patients at a high risk from colon cancer. He concluded that calcium could reduce epithelial proliferation by a direct action on the epithelium (Buset et al. 1986). *In-vitro* labelling could not accurately predict the outcome of dietary supplements, since only six out of the nine "responders" in the supplemental group showed a reduction in their labelling index.

In conclusion, oral supplements of calcium in animals reduce epithelial cell proliferation inhibit chemical carcinogenesis within the large intestine. There is growing evidence that oral calcium may also reduce epithelial cell proliferation in man. Further work is required to explore this effect and determine whether optimal calcium intake will reduce an individual's chance of developing large bowel cancer.

Vitamin D

The seco-steroid hormone 1,25 $OH_2$ $D_3$ is the biologically active metabolite of vitamin $D_3$. Seco-steroids possess a ring that has undergone fission, and in vitamin D this is the B ring (Reichel Koeffler and Norman 1989). Vitamin $D_3$ is either synthesised in the skin from 7-dehydrocholesterol by a reaction that is catalysed by ultra-violet light (Holick 1981), or it is taken up into the bloodstream from dietary sources within the intestine. Vitamin $D_2$, which has similar actions to $D_3$, is only provided from dietary sources. Vitamin D metabolites in the blood are transported via vitamin D-binding proteins similar to alpha-fetoprotein and albumin (Gibbs and Dugaiczyk 1987).

In the liver, the carbon bond at position 25 is hydroxylated to yield 25-hydroxy-vitamin $D_3$ (25 OH $D_3$), and this enzymic reaction is tightly autoregulated by 25 OH $D_3$ (Henry and Norman 1984). The kidney can also produce the metabolite 24 R 25 $(OH)_2$ $D_3$, if the supply of vitamin D is adequate, but the exact role of this steroid is presently unknown.



The production of 1,25 $(OH)_2$ $D_3$ is closely controlled and is related to the calcium needs of the individual (Fraser 1980, Henry and Norman 1984). Parathyroid hormone stimulates 1-alpha-hydroxlase activity by a direct action on the cell, and 1,25 $(OH)_2$ $D_3$ inhibits this enzyme (Fraser 1980, Henry and Norman 1984). Phosphate also controls the production of 1,25 $(OH)_2$ $D_3$, possibly by a direct action on the renal tubule cells (Reichel Koeffler and Norman 1989). Hypophosphatemia stimulates the production of 1,25 $(OH)_2$ $D_3$, whereas a high dietary intake of phosphate reduces its production. Vitamin $D_3$ is known to be a mediator of calcium balance and a regulator of bone and mineral metabolism, but it may also have a wider biological role.

The discovery of a high affinity nuclear receptor for the active metabolite of vitamin D (1,25 $OH_2$ $D_3$) suggests that this hormone may act in an analogous way to the other steroid hormones. Some of the effects of vitamin D are now believed to be mediated via specific binding to this nuclear receptor, which belongs to the family of nuclear receptors for glucocorticoids, oestrogen, thyroxine and retinoic acid. (Baker et al. 1988, Mingehetti and Norman 1988, De Luca Krisiner and Darwish 1990). The presence of the vitamin D receptor (VDR) has been demonstrated in a wide range of tissues not previously considered as target organs, again suggesting a more widespread role for the hormone (De Luca 1988). Indeed, a property of 1,25 $OH_2$ $D_3$ that had not previously been appreciated is its ability to induce the differentiation of promyelocytic cells into monocytes (Abe et al. 1981, Tanaka et al. 1982). By measuring VDR and mRNA levels, the induction of differentiation in these cells has been correlated with an up-regulation of the receptor at the transcription level. A reduction in the protoncogene c-myc levels also occurs, with inhibition of proliferation but without inducing differentiation in these cells (Taoka Collins and Norman

1991). Indeed, the expression of many genes are now known to be regulated by 1,25 $(OH)_2$ $D_3$ at the level of messenger RNA (Russell Leittieri and Sherwood 1986, Naveh-Many and Silver 1988, Rowe and Kream 1982, Franceshi et al. 1987, Fraser Otawara and Price 1988).

Altered cellular activity is seen when the active metabolite of vitamin D is added to osteogenic sarcoma cells, colorectal cancer cells and breast carcinoma cells grown in culture. In addition, 1-alpha hydroxy vitamin D causes regression of mammary tumours induced by nitrosomethylurea (Colston Berger and Coombes 1989). The presence of VDR (detected by immunohistochemistry) in tissue from 136 breast carcinomas, was correlated with a longer disease-free interval, when compared with VDR-negative tumours (Berger et al. 1991).

Full length DNA coding for the rat and human VDR has now been sequenced. Thus the entire structure of the 1,25 $OH_2$ $D_3$ is known. The human receptor has 427 amino acids and possesses two "zinc finger" binding sites. It is the smallest in the family of steroid receptors (Baker et al. 1988). The genes that are activated by the receptor-ligand complex are largely unknown, although a vitamin D-responsive element has been reported in the osteoclastin gene (Kerner Scott and Pike 1989) and vitamin D may control pre-proparathyroid gene expression (Russell Leittieri and Sherwood 1986) and insulin secretion (Chetow et al. 1986). 1,25 $OH_2$ $D_3$ also inhibits cellular proliferation in activated T-lymphocytes, a function thought to be cytokine-mediated (Kissmeyer and Binderup 1991).

The therapeutic potential of both 1,25 $(OH)_2$ $D_3$ and 1-alpha hydroxycholecalciferol has been limited by their potent effects on calcium metabolism, since they cause hypercalcaemia and hypercalciuria (Kissmeyer and Binderup 1991). Biochemical research has therefore been directed towards the production of analogues that retain the growth regulatory properties but have little effect on calcium metabolism. The concentration of 1,25 $OH_2$ $D_3$ required to suppress growth and induce differentiation in many cell culture experiments would generally have dramatic effects on calcium metabolism in patients. It has been suggested that increasing the side chain of the molecule by two carbon groups would eliminate the calcium mobilising effects of the vitamin and produce a 10-fold increase in the induction of differentiation of HL-60 cells (Perlman et al. 1989). Kissmeyer and Binderup (1991) have also found that altering the stereochemistry at the carbon-20 position produced a group of analogues (the 20-epi-vitamin D analogues) with extremely potent cell regulatory properties compared to 1,25 $OH_2$ $D_3$, with limited effects on serum calcium (Kissmeyer and Binderup 1991). In addition, the analogue MC-903 (calcipotriol), synthesised by the same group, retains its ability to alter cell growth and differentiation with little effect on calcium metabolism (Binderup and Bramm. 1988). Clinical trials using MC-903 suggest that it is a safe and effective topical treatment for psoriasis (Kragballe and Beck 1988, Kragballe 1989, Kragballe et al. 1991).

The presence of nuclear receptors for vitamin D in normal human colonic tissue, its reduced expression in colorectal adenocarcinomas (Lointier et al. 1991) and the ability of the colon to absorb calcium after small bowel resection (Hylander Ladefoged and Jarnums 1980) would all suggest that the human colon may be a potential target for the action of 1,25 $OH_2$ $D_3$. This hypothesis correlates well with epidemiological observations made by

Garland, who noticed that the mortality rate from colorectal cancer was inversely related to exposure to sunlight (Garland et al. 1985). His study of electricity workers in Chicago followed up for 19-years showed a high incidence of colorectal cancer in workers with low intakes of calcium and vitamin D and vice versa. Antiproliferative effects of vitamin D in colorectal cancer cell lines are now well documented. The colorectal cell lines LOVO (Lointier et al. 1987), Caco2 (Giuliano and Wood 1991) and HT-29 (Brehier and Thomasset 1988) all respond to 1,25 $OH_2$ $D_3$ by a reduced proliferation and increased cell differentiation. Likewise, the active metabolite of vitamin $D_3$ inhibits the growth of xenografts derived from colonic tumour cells in immunosuppressed mice (Eisman Barkala and Tutton 1987).

Selenium.

In animals, dietary selenium inhibits experimentally-induced colonic tumours (Fiala 1977). Colonic carcinogenesis induced by bis (2-oxopropyl) nitrosamine, 1,2 dimethylhydrazine and methylmethoxymethane, is reduced by dietary selenium supplements (Jacobs Forst and Beams 1981), an effect that appears to be due to its action at several stages in the initiation and promotion of experimental carcinogenesis (Ip 1981). Indeed, selenium now appears to be an essential trace element. It has a specific role in the prevention of degeneration of the chick exocrine pancreas and is a component of the enzyme glutathione reductase (Rotruck et al. 1973). In children multifocal myocarditis (Keshans disease) and disoders of cartilage development (Kashin-Beck disease) occur in regions of China where the dietary selenium intake is low (Chen et al. 1980, Yang et al. 1983).

Low levels of selenium have been observed in the blood of patients with gastrointestinal cancer (Shamberger et al. 1973, Broghamer McConnell and Blotcky 1976). The antineoplastic effect of selenium may be due to a reduction in the DNA damage caused by a carcinogen (Thompson and Becci 1980, Wortzman Besbris and Cohen 1980) or an increased rate of DNA repair (Birt et al. 1982). Inhibition of protein phosphorylation via an action on protein kinase C or a potentiation of the immune response have also been advocated as possible methods of action (Spallholz et al. 1975).

Protein kinase C was originally dicovered in the rat brain and it plays a key role in protein phosphorylation, cell membrane signal transduction and cell replication (Su et al. 1986). Some promoters of experimental carcinogenesis such as tumour-promoting phorbol ester and mezerein stimulate protein kinase C activity (Castagna et al. 1982, Miyake et al. 1984). Similarly, antineoplastic agents such as adriamycin, lipoidal amine CP-46,665-1 and tamoxifen inhibit the enzyme (Katoh et al. 1981, Shoji Volger and Kuo 1985, Su et al. 1985). Inorganic, but not organic, selenium compounds inhibit the activity of protein kinase C in human leukaemic cells and in tissue from the pig heart and brain (Su et al. 1986). This appears to be a non-competitive effect, and because the enzyme is calcium-dependent, it has been suggested that selenium acts as a general inhibitor of $Ca^{2+}$ dependent enzymes.

Ascorbic Acid

Residual rectal adenomatous polyps may disappear in patients with FAP following total colectomy with ileorectal anastomosis (Hubbard 1957, Cole and Holden 1959, Cole Mc

Kalen and Powell 1961, Shepherd 1972). The exposure of the retained rectum to ileal contents appears to be a prerequisite for this spontaneous regression (De Cosse et al. 1975). It has been suggested that an antioxidant (or reducing agent) such as ascorbic acid may be responsible for this effect (De Cosse et al. 1975). In support, ascorbic acid inhibits chemical carcinogenesis in the colon of mice (Reddy and Hirota 1979, Logue and Frommer 1980). In an uncontrolled trial, oral ascorbic acid (3 gramms/day) reduced the number of rectal polyps in four of five patients with FAP. In a much larger double-blind control trial conducted at St Mark's Hospital in London, this effect was seen to be transient and was greatest when the total polyp area was compared as opposed to the actual number of polyps (Bussey et al. 1982).

Epidermal growth factor and related peptides.

Epidermal growth factor (EGF) is a small polypeptide with 53 amino acid residues. It was first identified and isolated from the mouse submandibular gland (Cohen 1962). The human peptide is structurally and biologically very similar to mouse EGF (Cohen and Carpenter 1975, Huang et al. 1991). The receptor for EGF is a 17,000 mw protein which spans the cell membrane and mediates the initial response of the cell to EGF. This receptor has an extracellular component which binds to EGF and an intracellular component which has tyrosine kinase activity (Carpenter 1983). The EGF receptor (EGFR) and a related protein C-erb B-2 are both members of the type 1 family of receptor tyrosine kinases, which are involved in the regulation of cell growth of many different kinds of cells (Hanks Quinn and Hunter 1988).The receptor (EGFR) is present in a wide range of normal epithelial tissues and EGF is present in normal human plasma and in almost all body fluids

(Gusterson et al. 1984, Kasselberg et al. 1985). The peptide appears to be cleared from the bloodstream very rapidly (Byyny et al. 1974, Jorgensen Poulsson and Nexo 1988). It is secreted into the gastrointestinal tract in saliva, duodenal and pancreatic secretions and in bile (Heitz et al. 1978, Elder et al. 1978, St Hildaire Hradek and Jones 1985). In animals it has been identified throughout the gastrointestinal tract in the luminal contents and within the mucosa (Reeves Richards and Cooke 1991). As a result of EGF binding to its specific receptor there is an increase in DNA synthesis, cell proliferation and repair of damaged tissues (Schlessinger et al. 1983, King et al. 1985). The human homologue of EGF, EGF/urogastrone, has little effect on cell proliferation when the gastrointestinal surface is intact, but it will heal and prevent experimentally produced ulcers (Kikegaard et al. 1983, Konturek et al. 1988). Indeed, Wright suggests that mucosal ulceration anywhere in the gastrointestinal tract can induce the formation of a cell lineage from multipotential stem cells within the intestinal crypts. This lineage ramifies to produce a new gland which delivers EGF/urogastrone to the luminal surface, where it can stimulate cell proliferation, regeneration and ulcer healing (Wright Pike and Elia 1990a). In addition to stimulating growth in the gastrointestinal tract (Farber 1981), EGF can produce mitogenic effects in a wide range of cell types after binding to a specific transmembrane receptor (Cohen 1983). Likewise the peptide may play a role in carcinogenesis and the growth of malignant cells (Stoscheck and King 1986, Singletary et al. 1987).

Intravenous EGF (but not luminal EGF) in the rat can prevent the colonic hypoplasia induced by defunctioning colostomy (Foster and Whithead 1990) and also reverses the hypoplasia caused by total parenteral nutrition (Goodlad et al. 1987). High doses of EGF

given by rectal instillation can similarly increase rectal CCPR in the rat, and this effect may be due to an interaction with apical EGF-receptors on the colonocytes.

Ornithine decarboxylase (ODC) is the rate-limiting step in the production of the polyamines putrescine, spermidine and spermine, which are intracellular mediators of cell growth and differentiation (Peg 1988). Rat colonic explants maintained in organ culture show an increase in ornithine decarboxlase activity with EGF (Arlow et al. 1990). The increased proliferation induced by EGF in colorectal epithelial cells is therefore mediated, at least in part, by ODC activity.

The abnormal production of growth stimulatory factors or the abnormal expression of their receptors may allow cells to escape from the normal constraints of growth control (Todaro 1978). The autocrine model of growth control suggests that the acquisition of malignant properties is due in part to the tumour cell's ability to produce and respond to its own growth factors. (Sporn and Todaro 1980). The EGF receptor is most commonly overexpressed in squamous cell carcinomas (Gullick et al. 1991), whereas adenocarcinomas tend to over-express the C-erbB-2 protein (Gullick and Venter 1989). Many large bowel carcinomas, however, express the EGF-receptor (Bradly Weiss and Salem 1986). Transfection of the EGF-gene into moderately differentiated colonic carcinoma cells (MOSER cells) results in expression and secretion of EGF and an acceleration of their growth rate. This appears to be an EGF-stimulated autocrine effect. Highly malignant cells (the KM12SM cell line) showed a reduction in cell growth and a loss of the ability to respond to EGF after transfection with EGF, and this may be an intracellular effect (Huang et al. 1991). It therefore appears that both autocrine and intracellular effects determine how a cell responds to epidermal growth factor expression. Human colon carcinoma cells in

culture produce and respond to a variety of polypeptide growth factors (Coffey et al. 1987). Anzano analysed 18 human colon carcinoma cell lines for the production of growth factors, 17 produced transforming growth factor (TGF-alpha), 16 produced TGF-beta and 6 produced platelet derived growth factor. It was concluded that the ubiquitous expression of growth factors was consistent with some type of autocrine control, although there was no clear association between production and phenotypic traits of malignant behaviour (Anzano et al. 1989).

In conclusion, it would appear that in man EGF/urogastrone plays a dominant role in the healing of damaged tissue, whereas related polypeptide growth factors seems important in the growth of malignant human colonocytes.

Summary

The rate of proliferation of colorectal epithelial cells is influenced by a host of luminal and humoral environmental factors. Various types of fat and fibre, together with the oral intake of calcium and trace elements such as selenium, seem to be the most important dietary factors. In addition, both vitamin D and epidermal growth factor have profound effects on epithelial cell proliferation. The aim of this thesis is to further investigate the action of dietary fibre, oral calcium, vitamin D metabolites and epidermal growth factor on cell proliferation in colorectal epithelium.

## 3.Epithelial cell kinetics in the gastrointestinal tract.

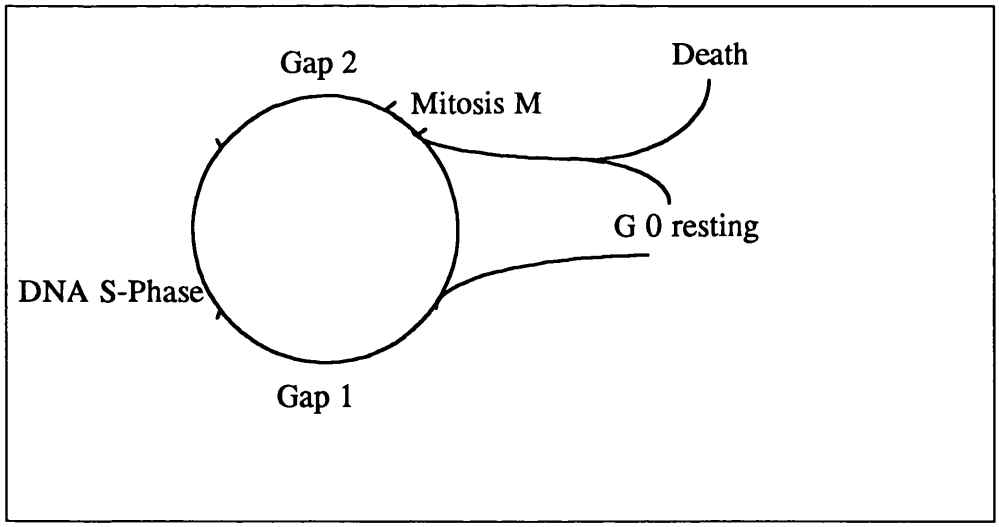
### Introduction

The cells in the gastrointestinal tract have the fastest turnover of all body tissues and these cells are constantly renewed by cell division. Enterotropic factors, such as epidermal growth factor (EGF) and enteroglucagon, stimulate cell proliferation, and may therefore be important in the healing of mucosal tissue damage (Wright Pike and Elia 1990a, Sarraf et al. 1991). Also, in disorders that predispose to intestinal malignancy such as familial polyposis coli and ulcerative proctocolitis, there is an increased size of the crypt/villus proliferation compartment compared to controls, together with an accelerated cell birth rate (Bleiberg et al. 1970, Allan Bristol and Williamson 1985, Lipkin et al. 1984). Likewise, groups of individuals with differing rates of cancer incidence have varying rates of cell proliferation (Lipkin 1988). Indeed, the exposure of animals to chemical carcinogens causes an acceleration in the rate of epithelial cell proliferation in the gastrointestinal tract (Williamson Welch and Malt 1983, Bristol and Williamson 1984, Appleton Bristol and Williamson 1986). An accurate measurement of both the length of the phases that make up the cell cycle and the rate of cell birth may therefore provide a greater understanding of disease processes. There is consequently a need for techniques that can accurately and reliably measure cell kinetics in the fields of clinical practice and research.

### The cell cycle.

Howard and Pelc in 1953 described the mammalian cell cycle as a series of phases and this concept is now well accepted (Howard and Pelc 1953, Aherne Camplejohn and Wright 1977). The period of DNA synthesis is known as the S phase and lasts for 7 to 19 hours. It is followed by a gap, or $G_2$ phase, before the onset of the next mitosis, the M phase. The

period between mitosis and and the S phase is known as $G_{1.}$. During $G_1$ there appears to be a critical point at which the cells commit themselves either to further mitosis or to enter a resting phase $G_0$. As a third alternative, cells can progress to differentiation and then eventually to death. If the cells continue in the cycle, then late in $G_1$ there is intense biosynthetic activity in preparation for the coming S phase. Variations in the duration of $G_1$ and $G_2$ will determine the cell cycle time ($t_c$) for a particular cell. Not all cells within the intestinal crypts traverse the cell cycle at the same time (Wright and Alison 1984), and therefore kinetic studies only estimate average parameters of the cell cycle.




Diagramatic representation of the cell cycle.

Thymidine autoradiography

Before the introduction of radioisotopes the study of cell renewal relied heavily on the counting of mitoses to give a static measure of the tissue's growth potential (Bertalanffy and Nagy 1961, Padykula et al. 1961, Yardly et al. 1962). The introduction of

radioisotopes, and in particular tritiated thymidine, provided an approach to the study of cell kinetics that allowed a detailed analysis of the dynamics of the cell cycle.

Thymidine autoradiography combines the incorporation of tritiated thymidine($^3$HTdR) into DNA with micro-autoradiography. The resulting "picture" of the tissue is due to the release of low energy $\beta$-particles, thereby allowing a precise localisation of newly formed DNA. The duration of the S, $G_2$ and M phases of the cell cycle can be estimated by counting the labelled mitoses on an autoradiographic slide after the in-vivo injection of tritiated thymidine ($^3$HTdR). Thymidine is taken up by the cell during S-phase and incorporated into the DNA. Serial mucosal biopsies (in man) or serial sacrifice of animals allows the accumulation of labelled DNA to be studied, and the fraction of labelled mitoses can then be determined. Cells heavily labelled at the time of injection would have been in S-phase during the few minutes that the label is available. When the interval between injection and biopsy is short, some of the cells in mitosis will have been in $G_2$ phase at the time of injection, and these mitoses will therefore be unlabelled. After a longer period between injection and biopsy there will be a greater proportion of labelled mitoses. If the number of labelled mitoses is plotted against the time from injection, then the S-phases appear as waves of labelled mitoses. This graph can be used to estimate the duration of the various phases of the cell cycle (Lipkin 1965). Using this technique Lipkin and his co-workers have estimated the duration of $G_2$, S and M phases in man. Although there is regional variation throughout the gastrointestinal tract, $G_2$ was estimated to be between 1 and 7 hours, S 10-14 hours and M about 1 hour. It was also noted that the S and $G_2$ phases were longer in man than in comparable segments of the rodent gut (Lipkin 1965). The appearance of the second wave of labelled mitoses can give a rough idea of the duration of

the $G_1$ phase. In the large bowel in man this appears to vary from 4-13 hours. A few colorectal cells however appear to remain in interphase (or $G_0$) for long periods of time before dividing again (Lipkin. 1965).

The position of proliferating cells within the intestinal crypt column can also be assessed using $^3$HTdR, and the proportion of labelled proliferating cells (with $^3$HTdR) to mature unlabelled cells can be determined. This ratio or labelling index can be used as a cytokinetic parameter allowing comparisons to be made between individuals and throughout the gastrointestinal tract. In the small intestine the zone of proliferation is sharply defined, whereas in the stomach and large bowel there is a greater tendency for the proliferative zone to blend into the zone of differentiation (Lipkin 1965).

Both mutagenic and carcinogenic effects have been described after $^3$HTdr administration, and this limits the use of *in-vivo* labelling with $^3$HTdR in man to patients who have a limited life expectancy (Lisco Baserga and Kisieleski 1961, Bateman and Chandley 1962). The problem was circumvented when Deschner developed a technique by which thymidine could be incorporated into human biopsy material maintained in a viable state in organ culture (Deschner Lewis and Lipkin 1963). In normal human rectal mucosa a similar distribution of $^3$HTdr labelling was seen using this *in-vitro* system to that observed after *in-vivo* labelling. Again, rectal tissue from patients with familial adenomatous polyposis (FAP) showed an expansion of the proliferative compartment, with labelling of cells extending into the upper level of the crypt. Tissue from patients with FAP and from controls showed a gradual increase in the number of cells labelled with increasing time in organ culture. In addition, a three-to-four day generation time (or cell cycle time) was

estimated in normal rectal mucosa using this technique (Deschner Lewis and Lipkin 1963). The use of biopsy material combined with *in-vitro* organ $^{3}$HTdR labelling has allowed the labelling index to be determined in several different groups of patients. For example, the colonic crypt labelling index is increased in patients at high risk from colorectal cancer as opposed to either a control population or a group of individuals who have a low incidence of large bowel cancer (Seventh-Day Adventists). An increase in the labelling index is also seen in the rectosigmoid and throughout the large bowel in patients with large bowel neoplasia when compared to controls (Deschner et al. 1977, Deschner 1982). Indeed, Lipkin has used the $^{3}$HTdR labelling index extensively to characterise groups of individuals according to their risk of colorectal cancer (Lipkin 1988).

There have been some critics of the $^{3}$HTdR technique. It is argued that because $^{3}$HTdR uptake is usually assayed in whole tissue, including isotope taken up by the lamina propria, muscle and serosa, it does not reflect epithelial activity alone. Also, thymidine is not a precursor of the de-novo pathway of DNA synthesis but is taken up and used by the salvage pathway. This route is dependent on the activity of several enzymes, transport mechanisms and on the size of the endogenous thymidine pool (Goodlad and Wright 1984). Thymidine can also be taken up, stored and recycled by bacteria (Maurer 1981). In addition to these methodological problems, the technique is laborious (Wright and Appleton 1980). Wright also argues that many epithelial responses occur far too quickly to be analysed by the fraction of labelled mitoses method, thus limiting its use in the study of dynamic epithelial events. Thymidine autoradiography relies on the determination of the labelling index to measure dynamic events, but the labelling index is affected by changes in the phase durations as well as the proliferation rate in the mucosa (Wright and Alison 1984). The use

of the exclusive S-phase marker bromodeoxyuridine (BrdU) can overcome some of these technical problems because it relies on immunolocalisation to measure the labelling index (page 77).

The metaphase arrest technique.

Thymidine-uptake autoradiography is time-consuming both technically and quantitatively. The labelling index and mitotic rate measured using $^{3}$HTdR techniques reflect changes in the rate of cell proliferation and the duration of the phases of the cell cycle. As an alternative the stathmokinetic (or metaphase arrest) technique can avoid some of the inaccuracies inherent in $^{3}$HTdR autoradiography. This method relies on the the arrest of cells as they pass through mitosis by means of a suitable stathmokinetic agent. The amount of kinetic information may be limited when compared directly with thymidine autoradiography, but it does allow estimation of one useful parameter, namely the cell birth rate ($k_b$). This parameter ($K_b$) also has the advantage that it is relatively independent of the age distribution of the cells within the tissue (Wright and Appleton 1980). In addition, the cell birth rate can be described as a cell production rate when it is expressed as a function of a unit of the cell population, for example per 1,000 cells or per intestinal gland or crypt. The strength of the technique lies in its reproducibility and accuracy, and also its ability to permit direct comparisons of a useful kinetic parameter between different tissues. The technique can be adapted to give an estimate of the cell cycle time in epithelial tissue and of the potential doubling time ($t_{pd}$) in tumours (Wright and Alison 1984).

Metaphase arrest results from either a failure of assembly of the mitotic spindle or disruption of the spindle. The accumulation of arrested metaphases is measured sequentially

after administration of the stathmokinetic agent. The number of arrested metaphases is plotted against the time from administration of the agent, the slope of the line giving an estimate of the cell birth rate($K_b$).

The earliest agent used to produce arrest of metaphases was colchicine. Its toxicity lead to its subsitution by a less hazardous derivative, Colemid (*N*-desacetly-*N*-methylcolchicine). Colemid has been used to determine the mean $t_{pd}$ in tumours of the oral cavity and larynx. It has also been used in patients with vesical carcinoma (in whom a negative correlation between differentiation and cell proliferation was found) and in neuroblastomas. In 1967 Tannock outlined the ideal requirements that needed to be fulfilled by metaphase arresting agents. He concluded that the *vinca* alkaloids were the most suitable agents. Indeed the choice of the stathmokinetic agent and the dose used in a specified tissue are of crucial importance in reducing the experimental error implicit in determining the cell birth rate (Tannock 1967, Nome 1975). If the dose is too low, there is both a long delay period before metaphase arrest is complete and a failure to arrest all metaphases, allowing some cells to "escape" into anaphase (Clarke 1971, Tannock 1967 Wright and Appleton 1980). If the dose is too high, then some of the arrested metaphases undergo degeneration and a reduced metaphase count is obtained (Taylor 1965, Tannock 1967, Fitzgerald and Brehaut 1970).

Tissue exposed to metaphase-arresting agents will show arrested prophases and metaphases. Both can be used to calculate the birth rate, but prophases, are difficult to count. If only the metaphases are counted, then the duration of mitosis $t_m$ can also be determined, and for this reason counting of metaphases alone is often preferred (Wright

and Appleton 1980). Because the intestinal crypts act as closed systems, arrested metaphases are unable to escape from the crypts during most experiments. The microdissection of intestinal crypts and the counting of metaphases allows the cell birth rate to be expressed per functional unit as the crypt cell production rate (CCPR) (Wimber and Lamerton 1963, Clarke 1971). The use of this parameter avoids the need to employ correction factors used with histological sections (Tannock 1967, Simmett 1968). The CCPR of a tissue is sensitive to changes in the number of cells proliferating (the growth fraction), the cell cycle time ($t_c$) and the size of the crypt. (Wright and Appleton 1980, Wright and Alison 1984). Indeed, estimates of the cell cycle time using metaphase arrest techniques compare favourably with those determined by the fraction of labelled mitoses method with tritiated thymidine (Wright and Appleton 1980).

The use of an appropriate dose of a suitable metaphase arresting agent is important in stathmokinetic experiments. Also the timing of subsequent samples or biopsies must take place during the period in which the accumulation of arrested metaphases is linear. *In-vivo* stathmokinetic studies involve taking biopsies after a bolus injection of vincristine and then embedding the tissue in paraffin prior to histological sectioning. Such a technique can be used to calculate the mitotic index at time zero, ie the native mitotic index ($I_{mo}$), and after vincristine ($I_{mt}$). From these two values ($I_{mo}$ and $I_{mt}$) the cell birth rate can be calculated. If the proportion of cells that are proliferating in an epithelial compartment is known (the growth fraction), an estimate of the cell cycle time can also be determined (Wright et al. 1973a, Wright and Alison 1984).

Wright used an *in-vivo* stathmokinetic technique in man to show that the cell cycle time in the avillous mucosa of untreated gluten-sensitive enteropathy was about half that seen in controls. There was no apparent lag-period before the onset of metaphase arrest following injection of vincristine. He concluded that even if there was no increase in the proliferative population, cell production rate would be at least doubled (Wright et al. 1973a). In a further experiment, Wright demonstrated an increase in the number of proliferative cells per crypt in gluten-sensitive enteropathy despite a lowering of the growth fraction. The conclusions drawn from these *in-vivo* experiments were similar to studies using an *in-vitro* thymidine technique, suggesting that the two methods can give comparable results (Wright et al. 1973b).

Vincristine will cause mitotic spindle disruption in all rapidly dividing tissue in the body, and this limits its *in-vivo* use in man to patients who are terminally ill. The metaphase arrest technique has therefore been adapted to measure cell kinetics in tissue maintained in a viable state outside the body using an *in-vitro* system. Human gastrointestinal tissue can now be successfully maintained in organ culture for many hours. Duodenal mucosal biopsies can be maintained for up to 22 hours (Wheeler and Challacombe 1987, Challacombe and Wheeler 1991), and rectal mucosa for up to 24 hours in organ culture (Eastwood and Trier 1973), with a good preservation of the tissue architecture and minimal evidence of tissue necrosis. Allan used an organ culture system to measure the crypt cell production rate in rectal mucosal tissue from patients with ulcerative proctocolitis (Allan, Bristol and Williamson 1985). In this study the optimum dose of vincristine needed to produce complete metaphase arrest was between 0.5 and 1 $\mu$g/ml of

culture medium. After an initial lag period there was a linear accumulation of metaphase figures between 16 and 19 hours.

Organ culture techniques are now widely used to enable the crypt cell production rate to be measured as a cytokinetic parameter in human tissue. A problem that has been observed when explants are used in organ culture is the disparity between the labelling of cells at the periphery of the tissue compared to the centre (Shorter Spencer and Hallenbeck 1966). This "edge labelling" can been avoided by using small pieces of tissue and by rocking the culture plates on a platform to improve overall tissue oxygenation (Allan Bristol and Williamson 1984, Autrup et al. 1987, Appleton et al. 1991).

Immunohistochemistry

Thymidine autoradiography and the metaphase arrest techniques give high quality kinetic information about the cells in the gastrointestinal tract, but both these techniques are laborious. The immunohistochemical labelling of cells using antibodies is a much quicker method of gaining kinetic information. It also has the theoretical advantage of preserving the histological architecture of the tissue.

Bromodeoxyuridine

The non-radioactive pyrimidine, 5-bromo-2-deoxyuridine (BrdU) is taken up by proliferating cells in S phase and has been used to label dividing cells in a similar way to classical thymidine autoradiography. Like $^{3}$HTdR, BrdU is incorporated into DNA using the salvage uptake pathway (Gratzner 1982, Chwalinski Potten and Evans 1987). The use of a highly specific monoclonal antibody for the nucleotide provides a rapid technique for

the *in-vivo* and *in-vitro* labelling of proliferating cells (Graztner 1982, Morstyn et al. 1986, Kamata et al. 1989).

In mice, rapidly dividing cells can easily be detected by their incorporation of BrdU after an *in-vivo* infusion, and the technique gives a similar estimate of labelling index to that of thymidine autoradiography (de Fazio et al. 1987). The use of a sophisticated *in-vivo* double labelling technique with BrdU and $^{3}$HTdR has emerged as a very accurate method for studying cell kinetics in animals (Chwalinski Potten and Evans 1988). In man, where the aim is to develop a quick, reliable and safe method of determining quantitative parameters of cell kinetics, this double labelling technique has little advantage over more conventional methods.

BrdU may have cytocidal or mutagenic effects with prolonged administration (Goz. 1978). The *in-vitro* labelling of endoscopically obtained tissue is thus a preferred technique. In a large Japanese study, in-vivo labelling with BrdU was compared to a high-pressure rapid hyperbaric *in-vitro* labelling technique. The *in-vitro* and *in-vivo* labelling of tissue from gastric cancer patients gave similar values for labelling index (Kamata et al. 1989). Indeed, when tissue from the colon of the mouse is labelled with BrdU using organ culture, the resulting histological slides show less background staining and the labelling of cells is better than that seen with thymidine autoradiography (Morstyn et al. 1986).

Ki-67 monoclonal antibody

Bromodeoxyuridine requires either in-vivo injection or in-vitro organ culture, and its incorporation into the cells may also be influenced by limited diffusion of the compound

into the tissue. The use of monoclonal antibodies directed against naturally occuring antigens expressed in proliferating cells may avoid some of these problems. One such antibody is Ki-67, which is directed against an as yet undefined proliferating cell antigen (Gerdes et al. 1983). Ki-67 will label proliferating cells in all but $G_0$ and early $G_1$ phases of the cell cycle, whereas BrdU is exclusively an S-phase marker (Gerdes et al. 1984). Yet Ki-67 reactivity correlates with other measures of cell proliferation (Sasaki et al. 1988, Isola et al. 1990,), as demonstrated in breast carcinoma tissue where the labelling index with BrdU is lower than that seen with Ki-67. The use of Ki-67 to measure the growth fraction suffers from the drawback that it is reliant on the use of frozen sections of histological tissue (Isola et al. 1990). The technique involves the labelling of tissue sections with a murine-derived monoclonal antibody, then using a labelled rabbit-antimouse antibody, followed by labelling with a swine-antirabbit antibody to amplify the staining (Gerdes et al. 1983, Gerdes et al.1984). The result is usually expressed as a labelling index, i.e. the proportion of labelled cells labelled to unlabelled cells (Porschen et al. 1989).

The potential usefulness of the technique is illustrated in a study in which tissue from patients with ulcerative proctocolitis was labelled with Ki-67 using the three-stage peroxidase method. Labelled nuclei were clearly visible, and the labelling was significantly greater in both active and quiescent disease than that seen in control. In tissue from patients with active disease, there was an extension of the proliferative compartment into the upper quarter of the colonic crypt (Franklin et al. 1985). These results confirmed those previously available from thymidine autoradiography and metaphase arrest, and to an extent serve to validate the method.

Porschen used Ki-67 to study the proliferatve activity of large bowel tumours. When cryostat sections of 61 colorectal carcinomas were stained with Ki-67, positive nuclei were clearly seen. There was a marked variation in the labelling index (7.7% -75.3%, mean 38.7%), and patterns were similar in the colon and rectum irrespective of age or sex. This figure is similar to growth fraction estimates calculated with the aid of thymidine-labelled mitosis in human colorectal xenografts (Porschen et al. 1989).

Ki-67 immunohistochemistry can therefore be used to determine the growth fraction in histological sections, but the disadvantage of its use as a marker of cell proliferation is the need to use frozen sections which are associated with a loss of tissue architecture.

Proliferating cell nuclear antigen (PCNA).

Proliferating cell nuclear antigen, also known as DNA polymerase delta associated antigen or cyclin (Waseem and Lane 1990), is an acidic nuclear protein (molecular weight 50 Kd) that is essential for DNA synthesis (Mathews et al. 1984). DNA polymerase delta exists in two forms: one form is independent of PCNA and is implicated in DNA repair, and the other form depends on PCNA for its action and is implicated in DNA replication (Fairman 1990). PCNA is expressed in all proliferating human cells. It was first identified due to the autoantibody activity seen in the serum from patients with systemic lupus erythematosus (Miyachi Fritzler and Tan 1978). The gene encoding for PCNA has now been localised to the short arm of human chromosone 20 (Webb Parsons and Chenevix-Trench 1990). Using recombinant DNA, Wassem and Lane (1990) developed a series of monoclonal antibodies against human PCNA. The antibody PC 10 appeared to be the most useful antibody because of its tight binding to the antigen and also its ability to

stain formalin-fixed histological tissue. Labelling using monoclonal antibodies directed against PCNA has the advantage over many other methods that it is a non-invasive technique and can label tissue that has been processed by standard histological techniques (Sarraf et al. 1991).

The PC 10 antibody labels cells both during and outside the S-phase of the cell cycle. Throughout the gastrointestinal tract of the rat, the labelling index determined using PC 10 is significantly higher than that seen with BrdU (Coltera and Gown 1991). Thus PCNA immunolocalisation provides a useful index of cell proliferation, and the antibody PC 10 demonstrates the antigen from the late $G_1$ phase throughout the cell cycle to the M phase (Sarraf et al. 1991). Indeed, the relatively long half-life (about 20 hours) of the PCNA protein results in PCNA expression in some cells that have recently ceased to proliferate (Hall et al. 1990, Sarraf et al. 1991, Scott et al. 1991). The immunoreactivity seen following PC 10 staining therefore frequently extends to label cells in the upper aspects of the crypt. It also shows weak staining of cells above the proliferative zone (Goodlad 1989, Sarraf et al. 1991). Despite this increased sensitivity, estimates of the growth fraction compare very well with those obtained by more established kinetic techniques (Hall et al. 1990, Sarraf et al. 1991, Woods et al. 1991). The antibody 19A2 also immunoreacts with PCNA and has activity restricted to the S phase of the cell cycle. The use of 19A2 does however require methanol fixation, however which has limited its routine use (Galand and Degraef 1989).

In summary, the labelling of proliferating cells using antibodies directed against PCNA appears to yield useful kinetic information. In addition, the use of the PC 10 antibody has

many advantages over more classical kinetic techniques because it can be used on routine histological tissue. One disadvantage is that the use of the PC10 antibody may slightly over estimate the size of the growth fraction.

Flow cytometry

The quantity of abnormal DNA within a tissue can be measured using flow cytometry. The cells in the $G_0$ or $G_1$ phase of the cell cycle have two copies of their chromosomes and are therefore diploid. Once the cells have completed DNA synthesis they are tetraploid ($G_2$ and M phases), and while synthesizing DNA they possess a varying amount of DNA. Cells that contain an abnormal amount of DNA are aneuploid. The measurement of the DNA content of a tissue by flow cytometry relies on the formation of a single cell suspension (Ahnen 1987). The nuclei are labelled with fluorescein, and the amount of scintillation in the tissue is determined using a flow cytometer (Quirke and Dyson 1987). A large number of cells can be rapidly analysed, and the resultant data are expressed as a DNA histogram to determine the ploidy of the tissue. Flow cytometry can also be used to measure the S-phase fraction of a tissue (Ahnen 1987, Quirke and Dyson 1987). The kinetic information is limited, however, and only represents a static measurement (Rew et al. 1991). DNA aneuploidy has been seen in up to 60% of colorectal adenocarcinomas using flow cytometry (Quirke et al. 1986, Quirke et al. 1987, Armitage et al. 1987).

Flow cytometry combined with BrdU labelling will increase the amount of kinetic information obtained by using either technique in isolation (Rew et al. 1991). Using this combination, values for the potential doubling time ($t_{pd}$), labelling index and S-phase

duration can be determined for a tissue (Rew et al. 1991, Rew et al. 1992). Rew (1991) determine $t_{pd}$ for colorectal tumours and suggested that their proliferative activity was greater than had previously been estimated. There are some important limitations of the technique, however, BrdU delivery to the cell nucleus is influenced competitively by thymidine, and labelling of non-epithelial cells will occurs.

Although flow cytometry allows a quick and relatively simple method of measuring kinetic parameters, the inaccuracy inherent in the methodology makes it unattractive as a potential experimental tool. It may be used to give a rough estimate of the potential doubling time in tumours.

## Summary

There is now a wide variety of methods available to gain information about the kinetics of gastrointestinal cells. Most of these techniques measure an index of proliferation or the growth fraction. All have their own limitations. Recently introduced, monoclonal antibodies to proliferating cells are an exciting discovery that require further evaluation. At present, the metaphase arrest technique and thymidine autoradiography, appear to give the most accurate and reliable results and therefore act as standards against which other methods must be compared.

# Chapter 4.

## General materials and methods.

### 1. Organ culture.

Background.

Organ culture is an *in-vitro* technique in which fragments of organs are cultured with a minimum of outgrowth (Howdle 1984). The aim is to maintain the tissue in a viable state with preservation of the physiological functions of the cells and the architecture of the tissue. The first record of successful organ culture was by Harrison in 1907 (cited by Carrell 1911), who demonstrated the growth of neural tissue from frog embryos cultured in lymphatic fluid. Carrel extended this work and was able to keep a wide range of tissues (both benign and malignant) proliferating in culture for long periods (Carrell 1911, Carrell 1912). Adult human tissue was first successfully cultured in 1959 by Trowell, and his general methodology has been adapted to allow different gastrointestinal tissue to be maintained in organ culture (Trowell 1959, Browning and Trier 1969). The technique involves taking mucosal biopsies from the gastrointestinal tract while the subject (or animal) is alive or immediately after death. The mucosa is gently separated from the submucosa and is then divided into small explants (usually less than 2 mm in size). These explants are placed mucosal surface uppermost on a supporting structure within a culture dish. The supporting structure can be either sterile grids (Browning and Trier 1969, Allan and Jewel 1983, Howdle 1984), filters (Pritchett et al. 1982), gelfoam (Senior et al. 1982) or fibrin foam (Schiff and Moore 1980). Culture fluid is added to the dish so that the undersurface of the biopsy is in contact with the fluid but the mucosal surface is left exposed (Howdle 1984). The tissue is then cultured in a moist warm atmosphere, i.e. at

$37^0$C in a gas mixture of 95% $O_2$ and 5% $CO_2$. A rocking platform can be used to bathe the biopsy intermittently, and this technique may improve tissue survival (Autrup 1987, Challacombe and Wheeler 1985, Senior et al. 1984, Wheeler and Challacombe 1987). If tissue is cultured for long periods of time, the culture medium should be changed every 24-48 hours, thereby replenishing essential nutrients and minimising bacterial colonisation (Senior et al. 1982).

The exact recipe for the culture fluid varies, but it usually consists of commercially available solutions containing essential nutrients, electrolytes and trace elements, combined with fetal calf serum. The medium can be supplemented with hydrocortisone (Johansen 1970, Challacombe and Wheeler 1985), glucose and insulin (Eastwood and Trier 1973, Wheeler and Challacombe 1987) to aid growth and with antibiotics to prevent bacterial contamination (Johansen 1970, Allan and Jewel 1983, Allan Bristol and Williamson 1985, Challacombe and Wheeler 1985). Tissue preservation is optimal with 95% $O_2$ and 5% $CO_2$ (Schiff and Moore 1980), and this mixture is non-toxic to the cultured cells for up to 30 days (Autrup et al. 1987, Senior et al. 1982). With careful attention to detail it is possible to maintain intestinal mucosa so that after 18-36 hours of culture it is histologically and ultrastructurally indistinguishable from normal tissue. If it is assumed that physiological function is likewise maintained, then organ culture techniques can be used as an *in-vitro* model of intestinal tissue function (Eastwood and Trier 1973, Howdle 1984, Allan Bristol and Williamson 1985).

Eastwood and Trier maintained human rectal tissue in organ culture for up to 24 hours with good preservation of tissue architecture. Degenerative changes increased in frequency

after 24 hours of culture but were limited to the basal half of the crypt in the centre of the biopsy (Eastwood and Trier 1973). In this experiment the medium was supplemented with insulin and glucose, and electron microscopy revealed good preservation of epithelial cells with their intracellular organelles after 24 hours of culture, although intracellular spaces were narrowed when compared with uncultured biopsies. The rectal mucosal cells were able to incorporate tritiated thymidine ($^3$HTdR) for up to 21 hours, indicating that the cells were able to proliferate in culture; there was some preferential labelling at the edge of the explants. Eastwood and Trier used this technique to culture tissue from patients with ulcerative proctocolitis. There was slightly more degeneration seen after culture in the tissue from patients with ulcerative proctocolitis, but there was good preservation of cellular structures between 6 and 18 hours of culture time. Johansen has described viable cultures of human rectal tissue for up to 90 hours and suggests that hydrocortisone may assist tissue survival (Johansen 1970).

Deschner used a similar organ culture technique to label rectal tissue from normal controls and patients with familial adenomatous polyposis with $^3$HTdR. Survival of the entire biopsy was seen for up to 19 hours, but longer periods of culture resulted in histological evidence of degeneration (Deschner Lewis and Lipkin 1963). Serafini used a much shorter culture period (six hours) to label tissue from patients with active and quiescent ulcerative proctocolitis with $^3$HTdR (Serafini Kirk and Chambers 1981). Organ culture can also be used to flash label cells with either $^3$HTdR or bromodeoxyuridine (Morstyn et al. 1986, Lipkin. 1988).

Organ culture can be adapted to measure *in-vitro* CCPR. Allan cultured rectal tissue from normal controls and patients with ulcerative proctocolitis and determined the CCPR after the addition of vincristine. His preliminary experiments established the optimum dose of vincristine needed to produce complete metaphase arrest: 0.5 $\mu$g/ml for normal mucosa and 1.0 $\mu$g/ml for tissue from patients with ulcerative proctolocolitis in relapse. After 16 hours of culture, the medium was replaced with culture medium containing vincristine, and explants were removed one, two and three hours later. There was a linear accumulation of metaphases over this period. Transmission electronmicroscopy revealed an excellent preservation of tissue architecture after 19 hours of organ cuture (Allan Bristol and Williamson 1985). Appleton used a similar method to culture tissue from the rat colon and obtained *in-vitro* CCPR values which were similar to those obtained using an *in-vivo* technique, thus validating the use of this method to measure rectal CCPR (Appleton et al. 1991).

Methodology employed

The methodology used in Experiments 2-6 described in this thesis is similar to that used by both Allan and Appleton. Sigmoidoscopy was performed between 9.00 am and noon on the day of the study using a Lloyd-Davies sigmoidoscope (Figure 1). Biopsies were taken from the upper rectum using biopsy forceps, by closing the jaws of the forceps and rotating the instrument. The mucosal biopsies were transported in culture medium cooled on ice, to the organ culture laboratory. Explants were established in culture within 60 minutes.

Using a sterile technique within a cell culture hood, the tissue was orientated mucosal surface uppermost on filter paper and was divided into small explants. The explants were

placed on steel grids within a multi-well organ culture dish (Lux laboratories). Culture medium was added to each well so that the under-surface of the mucosa was in contact with the medium (Figure 2). The culture medium was made up as 20 ml stock solutions and consisted of 18.3 ml CMRL-1066 (Gibco), 1 ml 5% fetal calf serum, 0.2 ml of L-glutamine, 0.2 ml of Hepes buffer, 0.1 ml penicillin and streptomycin (10,000 IU/ml), 0.1 ml hydrocortisone 21-hemisuccinate and 0.1 ml insulin (all obtained from Serolabs, Crawley, U. K). In addition, 20 mg of D-glucose (Serolabs) was added to each 20 ml of culture medium. The culture dishes were then placed in an organ culture chamber (made at The Works Department, The Hammersmith Hospital, London). The culture chamber was placed on a rocking platform (Bellco, New Jersey, U.S.A.), and the tissue was rocked at 3 cycles/min in 95% $O_2$ and 5% $CO_2$ at a temperature of $37^0$C (Figure 3). During preparation, explants were kept moist at all times.

After 15 hours of culture, vincristine 0.6 $\mu$g/ml (Oncovin, Eli Lily, Basingstoke, U.K.) was added to the culture medium. Explants were removed one, two, and three hours later. Infected cultures were discarded. The tissue was fixed for 2-4 hours in Carnoy's fluid and then stored in 70% alcohol. Later, the tissue was rehydrated with serial solutions of 50%, 25% and 10% alcohol before being acid hydrolysed in 1M HCl for six minutes at $60^0$C and then stained with Schiff's reagent. Individual crypts were then microdissected using a 22g hypodermic needle and an operating microscope (Fergusson et al. 1977). A cover slip was applied and gently pressed down onto the crypts, and the slides were then transferred to a light microscope. If there was any evidence of crypt necrosis the samples were discarded (Allan Bristol and Williamson 1985).

Schiff's reagent reacts with the aldehyde groups released by acid hydrolysis of DNA and produces a magenta colour. Arrested metaphases were therefore clearly visible against the pale pink of cells not in metaphase (Figure 4). The number of arrested metaphases per crypt was counted in at least 20 microdissected crypts. The mean number of arrested metaphases was plotted against the time from vincristine addition, the slope of the line being fitted by least squares linear regression analysis to give a value for the crypt cell production rate in cells/crypt/hour.

**Animals**

Sprague-Dawley rats were used in Experiment 1. This animal is a hardy outbred laboratory strain. Rats were received into the animal house one week before the start of the experiment and were housed three to a cage. The animal house was lit and darkened in a 12 hour alternating cycle. The rats had access to chow and water ad libitum before the experiment.

**Faecal steroids**

At the time of death, faeces were collected from the caecum, ascending, descending colon and rectum of the rats in Experiment 1. The specimens were frozen to -40°C and then transported to the Public Health Laboratory, Porton Down. Faecal analysis was performed by Professor Robert Owen at Porton Down, using the method described in Experiment 1.

**Immunohistochemistry**

Immunohistochemistry was performed using either standard paraffin sections or 3 $\mu$m cryostat frozen sections. Wax sections were prepared by placing histology slides in xylene

for 15 minutes, followed by gentle agitation in 100% alcohol (methanol) to remove the xylene. Slides were then passed through serial dilutions of alcohol to rehydrate the tissue. The slides were processed using the three-stage immunoperoxidase method. Either the antibody or a control solution was first applied to the slide for one hour. The slides were washed three times in TRIS buffer (Tris-hydroxymethyl-methylamine). The second layer, a swine anti-biotinylated-rabbit antibody, was then applied (1:300 dilution in TRIS buffer) and left for one hour. The slides were again rinsed in TRIS buffer (x3). The third layer was a commercially bought label, consisting of a peroxidase conjugated swine anti-rabbit anti-serum (Dakopatts, U.K), which labels biotinylated antibodies brown. The slides were washed three times in TRIS buffer and developed with 0.0375% $H_2O$, and 250 $\mu$g DAB (3,3 Diaminobenzadine tetrachloride) in TRIS buffer. Finally, the slides were rinsed with tap water, counter-stained with haemotoxylin for a few minutes and washed with tap water. A cover slip was applied and the slides were viewed using a light microscope.

**Cell culture.**

In Experiment 4, two commercially available colorectal cancer cell lines were used. These cell lines are human-derived malignant colorectal cells which spontaneously proliferate when maintained in culture medium. The cells were grown in Dulbecco's modified essential medium (DMEM) with the addition of 10% fetal calf serum. The medium was changed every other day, and cells were incubated in a mixture of 95% $O_2$ and 5% $CO_2$.

**Ethical considerations.**

Regional and local ethical committee approval was obtained for all the studies involving the participation of patients. Fully informed written consent was obtained from all patients before rectal biopsies were taken. In Experiment 1, Home Office approval was granted.

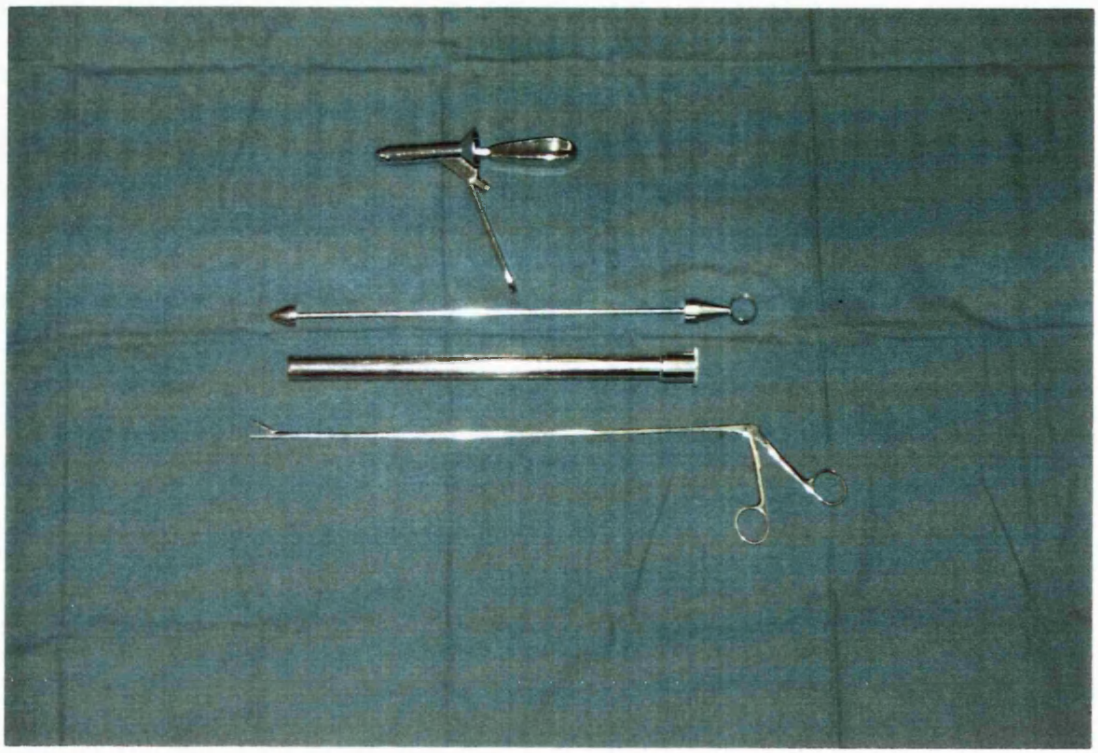


Figure 1. Sigmoidoscopic and proctoscopic instruments used to obtain rectal biopsies.

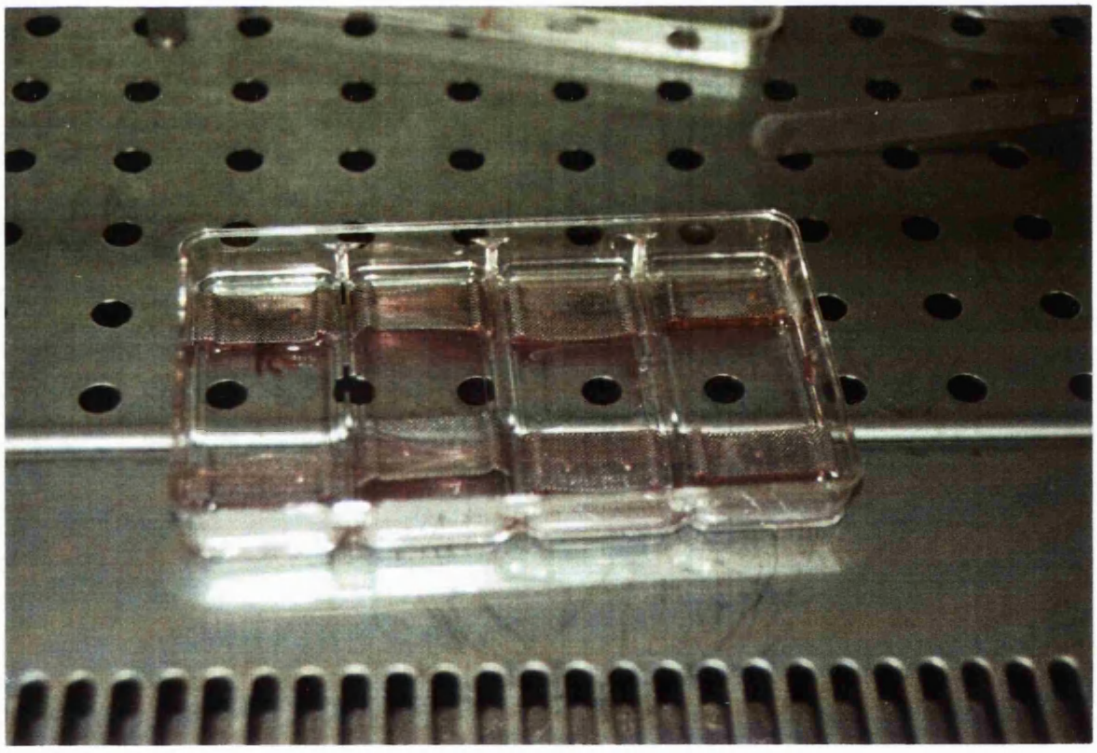


Figure 2. Rectal explants orientated mucosal surface uppermost on a metal grid within an organ culture dish.


Figure 3. The organ culture chamber sealed and placed on a rocking platform within an incubator.

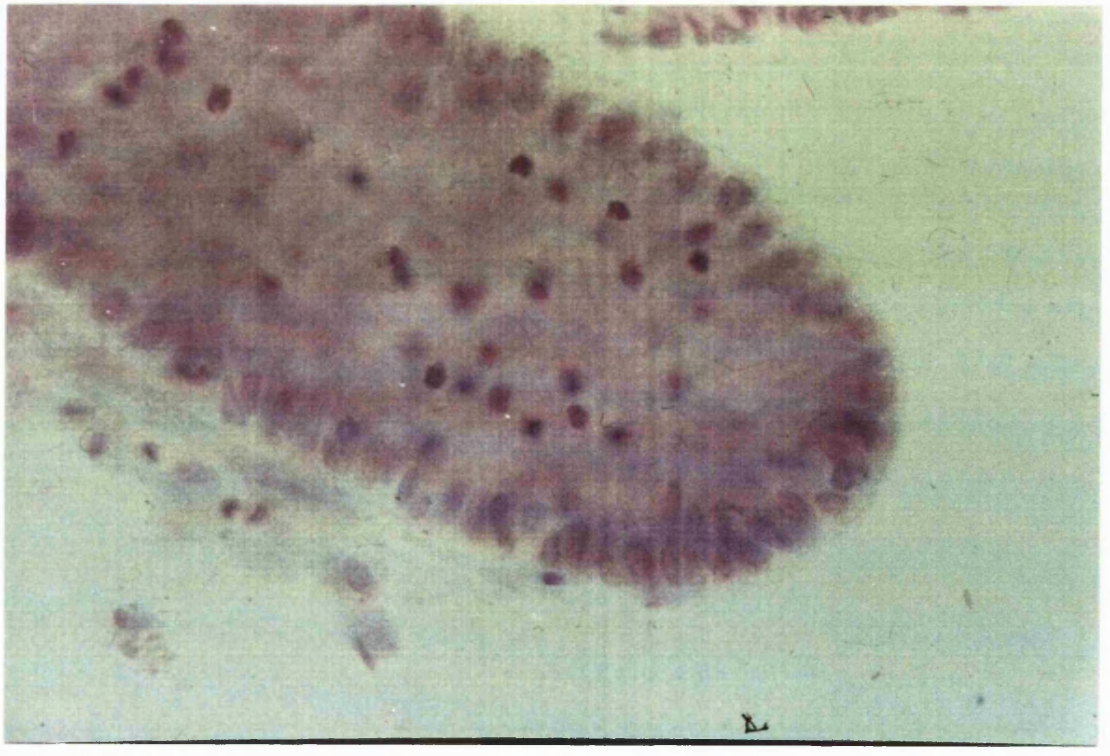


Figure 4. Metaphase arrest figures within a microdissected colonic crypt.

# Chapter 5.

## Experiments 1-5.

# Experiment 1

## The effect of enteral feeding on intestinal cell proliferation and faecal bile acid profiles in the rat.

### Background

In the colon the presence of faeces seems to be important for maintaining normal mucosal mass and sustaining cellular proliferation at its normal rate (Williamson 1984). Diversion of the faecal stream from a segment of large bowel, either by the formation of a proximal colostomy or by its exclusion as a Thiry-Vella fistula, reduces mucosal mass, nucleic acid content and crypt cell production rate (CCPR) (Terpstra et al. 1981, Rainey et al. 1983). Likewise, total parenteral nutrition and low-residue enteral diets produce colonic hypoplasia in animals (Janne Carpenter and Willems 1977, Morin Ling and Bourassa 1980, Storme and Willems 1981, Williamson 1984). Large bowel hypoplasia can be prevented by adding non-absorbable, non-nutritive bulk to the diet (Dowling et al. 1967, Ryan et al. 1979, Goodlad et al. 1987). The type of fibre present in a diet is also important, digestible fibres being more effective in preventing mucosal hypoplasia than inert bulk (Sakata 1980). This finding may be due to the action of the colonic microflora which can release fatty acids by the fermentation of fibre (Thornton 1981, Goodlad and Wright 1983, Clausen Bonnen and Mortensen 1991). Bacterial action in the colon also governs the production of secondary bile acid (Aries et al. 1969, Galloway et al. 1986). Both fatty acids and bile acids appear to have profound effects on cellular proliferation.

The clinical use of enteral feeding aims to provide an adequate nutritional intake without causing profuse diarrhoea. Enteral diets that contain fermentable soy fibre may help to maintain faecal bulk and bacterial degradation and therefore prevent mucosal hypoplasia with its attendant diarrhoea.

The aim of this experiment was to compare the effects of a standard fibre-free enteral diet (Ensure•) with those of a soy-fibre-containing enteral diet (Enrich•), measuring mucosal mass and cell proliferation of the rat intestine and analysing faecal bile acid profiles.

## Method

Twenty young male Sprague-Dawley rats weighing $310.3 \pm 1.6$ g (mean $\pm$ s.e.m) were received into the animal house one week before the start of the experiment. They were housed two or three to a cage, the animal house being lit in alternate 12-hour cycles. In a small pilot experiment, the daily intake of the fibre-free and soy-fibre enteral diets (Enrich• and Ensure• Abbot laboratories,) and the subsequent weight increase in a group of rats were compared to those seen in rats fed standard rat chow (PRD, Biosure, Cambridge, U.K.). It was found that rats of this age and strain consumed approximately 60 ml of either enteral diet per day. The standard rat chow group gained weight at a greater rate than the other two groups over a two week period. This difference was almost certainly due to an unequal calorific intake (Table 1). The control group were therefore given 20g of standard rat chow per day to achieve a similar intake of calories in all three groups. Thereafter the

percentage increase in body weights was similar in all groups (Table 1). Rats were allowed water ad-libitum and drank 25 ml of tap water per day irrespective of diet. There was no attempt to prevent coprophagia.

**Table 1.**

The energy, nutrient and fibre content of enteral diets and standard rat chow.

| | Fibre-free (60ml) | Soy-fibre (60 ml) | Chow (20 g) |
|---|---|---|---|
| Energy (KJ) | 252 | 262 | 252 |
| Protein (g) | 2.1 | 2.3 | 3.9 |
| Fat (g) | 2.1 | 2.1 | 0.6 |
| Carbohydrate (g) | 8.2 | 9.2 | 10.6 |
| Fibre (g) | 0 | 0.8 | 7.5 |

The rats were randomised into three groups, receiving either an enteral diet; fibre-free (n=7), soy-fibre (n=7) or standard rat chow (n=6) for a four week period. Rats were weighed and then killed 40-180 minutes after an intraperitoneal injection of 1 mg/kg vincristine sulphate (Oncovin. Eli Lily, Basingstoke, U.K.), which was given to cause metaphase arrest within the intestinal crypts. The gastrointestinal tract was excised, stripped of its mesentery and fat and then gently dried.

**Bile acid and neutral sterol assays**

Faecal samples from the proximal and distal large bowel were immediately frozen to -20$^0$C and later transported to the Public Health Laboratories, Porton Down. Analysis of neutral steroids and bile acids was performed by Dr Robert Owen. Faecal samples were freeze dried and ground to a fine homogeneous powder, and an extract was obtained from 500 mg using chloroform/methanol (1:1) for 12 hours in a soxhlet apparatus. Extracts were fractionated into their composite neutral sterols and free bile acids by anion-exchange chromatography on diethylaminohydoxypropyl-sephadex (DEAP-LH 20). Neutral sterols and free bile acids (in methyl ester form) were quantitatively analysed by gas-liquid chromatography (GLC) on a Pye 304 gas chromatograph fitted with a 3% OV-1 column. Either 4-androstone-3 17 dione or methyl nor-deoxycholic acid were included as internal standards for quantification of neutral and acid steroids.

**Crypt cell production rate (CCPR)**

Mucosal samples were taken from the proximal, mid and small intestine: i.e. 10%, 50% and 90% of the total length and from the proximal, mid and distal large intestine. Each sample was divided into two, half being snap frozen under liquid nitrogen to determine DNA and RNA concentrations per gram of mucosal tissue. The remaining tissue was fixed in Carnoy's fluid. The tissue was rehydrated in serial alcohol solutions acid-hydrolysed with 1 M HCl at 60$^0$ C for 6 minutes and then stained with Schiff's reagent. The mucosa was stripped from the muscle coat and crypts were microdissected. The number of metaphase arrest figures was counted in at least 20 crypts at each time point; 30, 60, 180 120,and 180 minutes in the control group and 30,60, 120 100 and 180 minutes in the enteral fibre groups. The mean number of arrested metaphase figures was plotted against

time from vincristine injection. The crypt cell production rate was determined by linear regression analysis.

**DNA, RNA and protein analysis.**

Mucosal tissue was weighed and homogenised in 7.5 ml ice-cold phosphate-buffered saline (PBS). The concentration of the homogenised solution was adjusted to 1mg of tissue per ml of fluid by further addition of PBS. To determine tissue DNA content, 200$\mu$l of tissue homogenate was incubated with 340 $\mu$l of a reaction mixture, which consisted of 100 $\mu$l Pronase (Sigma, Protease type XXV, 2 mg/ml PBS) 40 $\mu$l RNAase (Sigma, Ribonuclease A, 20 mg/ml PBS) and 200$\mu$l of PBS. This mixture was incubated for 45 minutes at 37$^0$C. Ethidium bromide (Sigma, 5.7 mg per litre of PBS) was added, and the fluorescence was read at an excitation wavelength of 536 nM and emission wavelength of 602 nM.

The RNA content was determined by a similar procedure, without the addition of ribonuclease A. The difference between the two readings was compared to RNA standards (1-10 $\mu$g RNA, Sigma RNA type III) to determine RNA content.
Protein comtent was determined by the Lowry method.

**Statistical analysis.**

One- way analysis of variance was used to compare body weights, steroid analysis and nucleic acid measurements and unpaired Student's *t*-test for CCPR values.

## Results

### Body weights

Rats thrived on each of the three diets. There was little difference in their weight gain. Controls increased their weight by 24% over the four weeks. Weight gain was similar (27.0%) with soy-fibre and only slightly less (18.6%) with fibre-free diet ($p<0.05$ compared with fibre-free).

### Crypt cell production rates (CCPR).

There was a linear accumulation of metaphases in all areas of both the large and small bowel. The mean correlation coefficient being $0.92\pm0.02$ mean$\pm$ s.e.m., median 0.92 (range 0.77-0.99). There were no significant differences between groups in any of the three areas of small bowel (Table 2).

**Table 2.**

CCPR (cells/crypt/hour) in the proximal middle and distal small bowel.
Values are mean $\pm$ s.e.m.

| | Proximal | Middle | Distal |
|---|---|---|---|
| Controls | $6.8\pm3.5$ | $15.1\pm5.4$ | $11.8\pm1.3$ |
| Soy-fibre | $16.8\pm7.1$ | $24.1\pm7.9$ | $11.5\pm6.2$ |
| Fibre-free | $10.3\pm3.9$ | $18.6\pm4.6$ | $10.6\pm4.0$ |

In the proximal large bowel, animals fed either of the enteral diets showed a reduction in CCPR compared to the control group (Table 3). Values with the soy-fibre diet were only 5% of controls and values with the fibre-free diet only 7.5%. This profound hypoplasia was also seen in the mid colon of the group fed the fibre-free diet (CCPR 2.5% of controls, whereas the soy-fibre diet produced values similar to the control group. There were no differences in the values between the groups in the distal colon (Table 3).

**Table 3.**

CCPR (cells/crypt/hour) in the proximal, middle and distal large intestine.
Values are mean ± s.e.m.

| | Proximal | Middle | Distal |
|---|---|---|---|
| Controls | 16.1±2.1 | 6.1±0.4 | 1.1±1.7 |
| Soy-fibre | 0.8±0.7* | 6.2±2.7 | 2.4±0.5 |
| Fibre-free | 1.2±0.7** | 0.8±0.3*** | 1.3±0.7 |

*Control v Soy-fibre $p<0.001$ **Control v Fibre-free $p<0.001$

***Control v Fibre-free, and Fibre-free vs Soy-fibre $p<0.05$ (Analysis of variance)

<u>Nucleic acid analysis</u>

In chow-fed rats the mean DNA concentration in the mid-small bowel was 4.86±0.30 μg/mg and the mean RNA concentration was 14.16±0.39 μg/mg. In the mid large bowel mean colonic DNA concentration was 2.69±0.24 μg/mg and RNA concentration 9.26±0.78 μg/mg. Nucleic acid concentrations were not significantly altered by either enteral diet at any site (Table 4).

**Table 4.**

Nucleic acid concentrations in the mid point of the small and large intestine.

Values are mean± s.e.m.

| | RNA (μg/mg) | DNA (μg/mg) |
|---|---|---|
| Small bowel | | |
| Control | 14.16±0.39 | 4.86±0.54 |
| Soy-fibre | 16.53±0.43 | 5.47±0.18 |
| Fibre-free | 12.82±0.27 | 4.42±0.22 |
| Large bowel | | |
| Control | 9.26±0.78 | 2.69±0.24 |
| Soy-fibre | 9.24±0.41 | 1.35±0.19 |
| Fibre-free | 9.80±0.48 | 1.26±0.09 |

**Faecal steroid analysis**

The mean weight of faeces collected from the proximal and distal colon in the three groups was not significantly different. Analysis of the various faecal bile acids and sterols was determined as the mean concentration (as mg/g dry weight) or as mean content values (in mg of sterol).

Figure 1.

# Faecal bile acids

(Concentration mg/g dry wt)

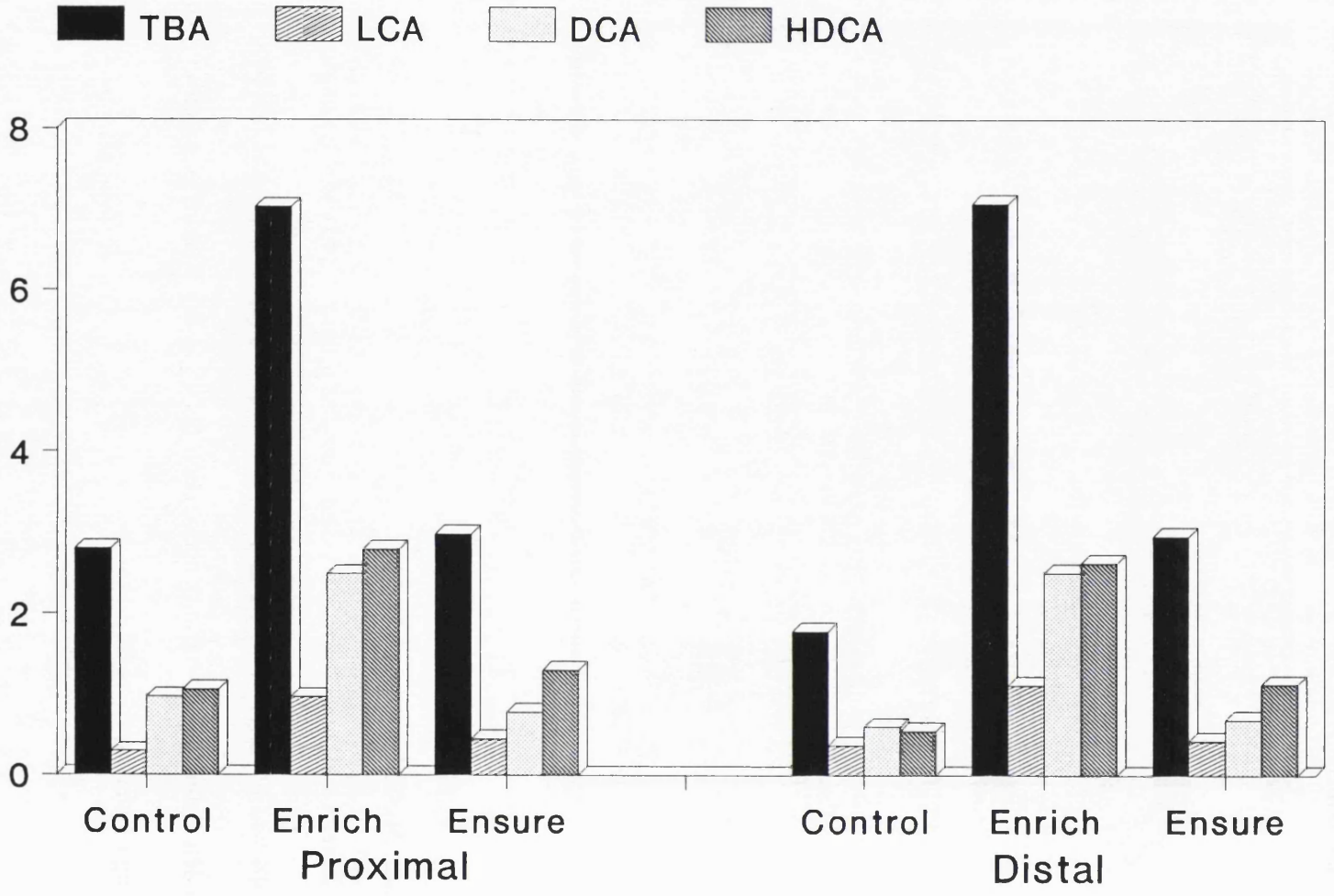


**Table 5.**

Bile acid concentrations (mg/dry wt) in faeces obtained from the proximal and distal large intestine.

Mean values are shown ±s.e.m.

| | TBA | LCA | DCA | HDCA | LCA/DCA |
|---|---|---|---|---|---|
| Proximal | | | | | |
| Control | 2.80±0.31 | 0.29±0.04 | 0.97±0.08 | 1.06±0.15 | 0.31±0.04 |
| Soy-fibre | 7.03±0.73* | 0.97±0.17 | 2.49±0.27* | 2.79±0.31 | 0.39±0.05# |
| Fibre-free | 2.98±0.64# | 0.45±0.05# | 0.78±0.15# | 1.30±0.40 | 0.64±0.06*# |
| Distal | | | | | |
| Control | 1.78±0.30 | 0.37±0.03 | 0.60±0.03 | 0.55±0.16 | 0.68±0.10^ |
| Soy-fibre | 7.07±1.25** | 1.12±0.20** | 2.50±0.48** | 2.62±0.59 | 0.48±0.07 |
| Fibre-free | 2.95±0.34## | 0.43±0.03## | 0.69±0.09## | 1.13±0.18 | 0.68±0.07 |

TBA = Total bile acids, LCA = Lithocolic acid.

DCA = Deoxycholic acid, HCDA = hydodeoxycholic acid.

Significance (Analysis of variance)

* Soy-fibre vs Control $p<0.05$ **Soy-fibre vs Control $p<0.01$

#Soy-fibre vs Fibre-free $p<0.05$, ## Soy-fibre vs Fibre-free $p<0.01$

^ Control vs Control $p<0.02$, *# Fibre-free vs Control $p<0.02$

**Faecal bile acids.**

The bile acid concentrations in the proximal and distal colon were increased in rats receiving the soy-fibre compared to either controls or rats fed the fibre-free diet (Table 5). This increase in concentration represented a greater excretion of the secondary bile acids lithocolic, deoxycholic and hydodeoxycholic acid. The overall steroid profile in the fibre-free group was similar to that seen in the control group. The trend was similar when the total contents of bile acids were compared (Table 6), but there was no significant difference in the content of total bile acids or lithocolic acid in the proximal colon. This difference reflects a large standard error in the dry weights in the soy-fibre group. The LCA/DCA ratio was elevated in the proximal enteral diets and the distal control compared to the proximal control.

**Table 6.**

Mean bile acid content (mg) in the proximal and distal large intestine.

Mean values are shown ± s.e.m.

| | TBA | LCA | DCA | LCA/DCA | HDCA |
|---|---|---|---|---|---|
| Proximal | | | | | |
| Control | 2.00±0.42 | 0.20±0.03 | 0.69±0.13 | 0.31±0.03 | 0.76±0.18 |
| Soy-fibre | 3.40±0.29 | 0.48±0.08 | 1.21±0.11* | 0.39±0.05# | 1.36±0.14 |
| Fibre-free | 1.42±0.23 | 0.22±0.02 | 0.38±0.06 | 0.63±0.06*# | 0.60±0.15 |
| Distal | | | | | |
| Control | 1.09±0.27 | 0.22±0.04 | 0.39±0.11 | 0.68±0.10 | 0.33±0.10 |
| Soy-fibre | 4.52±1.76** | 0.67±0.26* | 1.74±0.71** | 0.49±0.08 | 1.60±0.66** |
| Fibre-free | 1.66±0.29## | 0.24±0.05## | 0.40±0.08## | 0.70±0.09 | 0.58±0.01# |

Significance

* Control vs Soy-fibre $p<0.05$, ** Control vs Fibre-free $p<0.02$, *# Control vs Fibre-free $p<0.02$

# Soy-fibre vs Fibre-free $p<0.05$, ## Soy-fibre vs fibre-free $p<0.02$

## Neutral steroids.

Total animal sterol contents were raised in the soy-fibre and fibre-free groups compared to controls in the proximal and distal colon ($P<0.05$). In the soy-fibre group this increase was mainly due to an increase in the cholesterol content ($p<0.05$ compared to control). Copostranol concentrations and mean total content were raised in faecal samples from both

proximal and distal colon in rats fed one or other enteral diet. The ratio of copostranol to total animal steroids (a measure of the bacterial degradation of cholesterol) was raised in both proximal and distal colonamong rats fed the fibre-free diet and in distal colon alone among rats fed the soy-fibre diet (Table 8).

**Table 7.**

Faecal neutral steroids (mg/g dry wt) in faeces

obtained from the proximal and distal large intestine. (Mean values± s.e.m.).

| | Copostranol | Cholesterol | % Conversion |
|---|---|---|---|
| Proximal | | | |
| Control | 0.77±0.26 | 1.20±0.27 | 59.4 |
| Soy-fibre | 3.63±0.79 | 9.15±0.88 | 71.67 |
| Fibre-free | 2.29±0.47 | 7.93±2.41 | 76.86** |
| Distal | | | |
| Control | 0.78±0.19 | 1.13±0.18 | 59.4 |
| Soy-fibre | 2.63±0.48 | 7.80±1.77 | 74.67* |
| Fibre-free | 1.96±0.57 | 6.74±2.56 | 76.37*** |

*Control v Soy-fibre $p < 0.05$

**Control v Fibre-free $p > 0.01$

**Control v Fibre-free $p < 0.001$

## Comment

Enteral feeding in this experiment reduced cell proliferation (CCPR) but not DNA or RNA content in the proximal colon and mid colon; it had no such effect in the small intestine. The failure to correlate the observed reduction in CCPR with a similar reduction in nucleic acid content was a suprise and can not be easily explained. One possible explanation might be the small number of animals used in each group. The observed colonic hypoplasia (i.e. the reduced CCPR) is similar to the effect of enteral feeding seen in the mouse (Goodlad et al. 1987). By contrast the distal colon did not show any changes with standard enteral feed. The maintenance of body weight and the preservation of a normal CCPR in the mid colon amongst animals fed the soy-fibre diet suggests that fermentable fibre may help to prevent mucosal atrophy and may be useful in patients receiving enteral diets. This enteral diet contains a modest amount of fermentable fibre, but standard rat chow contains considerably more. A moderate increase in thymidine kinase activity and ornithine decarboxylase activity (linked to an increase in proliferation) has previously been observed in rats fed a soy fibre containing diet. This effect may be related to an observed increase in volatile fatty acids (Leverat et al. 1991).

Enteral feeding with the soy-fibre diet produces higher faecal concentrations of total bile acids and secondary bile acids than either standard rat chow or the fibre-free diet, which may reflect bacterial degradation of soy fibre. Both enteral diets raised neutral steroids, the soy-fibre diet causing a large increase in the cholesterol content possibly owing to a secondary effect on bile acid production. The percentage conversion of cholesterol to

copostranol suggests that bacterial activity is increased in rats fed enteral diets. Fermentable fibres reduce proximal colonic pH (Lupton Coder and Jacobs 1988) and increase ammonia production, possibly due to a greater production of urea by the colonic microflora (Lupton Coder and Jacobs 1988). Acidic colonic luminal contents may act directly to increase epithelial cell proliferation in the proximal colon (Lupton Coder and Jacobs 1988, Lupton and Marchant 1988). As an alternative explanation, both enteral diets contain more fat than standard chow, and faecal bile acids are raised when dietary fat is increased (Stadler 1988, Reddy et al. 1977). In man, diets containing soya pulp increase faecal bile acids without altering faecal neutral sterols, an effect attributed to either 7-alpha-dehydroxylation of cholate decreased absorption of deoxycholate (Schweizer et al. 1983).

The failure of the enteral diets to induce distal colonic hypoplasia might be related to bacterial activity in the caecum. A more direct assessment of faecal bacterial content or activity in the three regions of the colon might have aided interpretation of the results.

Secondary bile acids can enhance experimental carcinogenesis, and an increase in the LCA/DCA ratio has similarly been correlated with the risk of large bowel cancer (Owen et al. 1984). The elevated LCA/DCA ratio seen in this experiment correlates with our observed increase in CCPR, and suggests that care may be required with the long-term use of fermentable fibres, as they may act as co-carcinogens.

# Experiment 2.

## Oral calcium supplementation in familial adenomatous polyposis.

**Introduction.**

Many sporadic colorectal cancers may have a hereditary component to their aetiology (Burt et al. 1985, Cannon-Albright et al. 1988). Indeed, the loss of DNA sequences in the q21-q22 region on chromosome 5 has been noted in at least 20% of such tumours (Solomon et al. 1987). The familial adenomatous polyposis (FAP) gene has also been localised to this region (Herrera et al. 1986, Bodmer et al. 1987, Leppart et al. 1987). These findings, together with the widespread acceptance of the adenoma-carcinoma sequence, suggest that FAP can be used as a model for the hereditary component of colorectal cancer. The effects of dietary manipulation in patients with FAP can be used to investigate environmental influence on the genetic predisposition to colorectal cancer. There is also increasing evidence in man that epithelial hyperplasia predisposes towards colorectal malignancy. In FAP there is an expansion of the proliferative zone of the colonic crypts (Lipkin 1988), while patients with sporadic adenomas and those at risk of familial colorectal cancer have elevated labelling indices (Lipkin et al. 1983). Also, thymidine uptake and crypt cell production rate (CCPR) in colonoscopic biopsies are increased in patients with colorectal adenomas and carcinomas (Terpstra et al. 1987, Barsoum et al. 1992).

Calcium may reduce colorectal epithelial proliferation and hence protect against large bowel cancer (Chapter 3). Evidence in man suggests that a calcium intake of greater than 1500 mg/day is associated with a reduced incidence of colon cancer (Lipkin and Newmark 1985, Garland et al. 1985). In the United Kingdom 500-600 mg/day is considered

sufficient for normal health, whereas in the USA 800-1,000 mg/day is recommended (Hackett et al. 1984). The "western diet" of the U K may therefore be deficient in calcium, and thus oral supplementation with calcium could dampen epithelial proliferation and protect against colorectal cancer.

The registration of families of patients with FAP at the Polyposis Registry, St Mark's Hospital, London, began in 1925. There are now at least 350 families with familial adenomatous polyposis registered. Monitoring rectal polyp progression, together with the measurement of colorectal epithelial proliferation in patients with FAP, may allow the effects of dietary calcium on the colorectum to be assessed. A double-blind control trial was designed to study the effect of oral calcium supplementation on rectal cytokinetics and polyp progression in members of these families.

## Method.

### Subjects.

Between January 1990 and June 1991, 28 patients with FAP were recruited into the trial. There were 19 males and 9 females, and the median age was 38 years (range 16-65). As is routine practice at St Mark's for this disease, all patients had previously undergone total abdominal colectomy with ileorectal anastomosis and were attending the outpatient clinic for follow-up. The median period since colectomy and ileorectal anastomosis was 12.5 years (range 2-30, mean 12 years). Exclusion criteria included a past history of hypercalcaemia, renal failure or peptic ulceration. The trial was fully explained to each patient and, after acceptance, a questionnaire was completed including the following details: age at their first diagnosis of FAP and operation, past medical history, a full drug

history and recent gastrointestinal symptoms. At each subsequent visit the medical history was updated. Patients were randomised in a double-blind fashion to receive either 1500 mg/day of calcium carbonate for a six month period or an inert placebo (Inositol) both supplied by Dalgety Pilsbury Ltd, Birmingham, U.K; this involved taking eight tablets per day. Coding and dispensing of the tablets was performed at the pharmacy department at St Mark's Hospital. The code was broken only after completion of the trial. Compliance was assessed at subsequent visits to the outpatients by using a simple questionnaire and by examining returned medication containers. Entry into the trial did not alter the treatment of the patients. At St Mark's, the managment of polyps within the retained rectum is usually simple observation, unless the size increases greatly. None of the patients recruited were admitted for fulguration of the polyps during the trial period. Fully informed, written consent was obtained from all the patients, and local and regional ethical committee approval was granted before commencement of the trial. A contact telephone number was given to all patients.

**Procedure**

Abdominal examination and rigid sigmoidoscopy were performed at each visit to the clinic. At sigmoidoscopy, the position of the ileorectal anastomosis was first visualised, then the site, size and number of polyps were recorded systematically in all four quadrants of the rectum while slowly withdrawing the sigmoidoscope. The information was recorded on a standard sheet completed at each visit. Two rectal biopsies were taken from adjacent macroscopically normal rectal mucosa. The first biopsy was transported in culture medium, cooled on ice, to allow the determination of CCPR by in-vitro organ culture. The second biopsy was fixed in formalin, then stained with haematoxylin and eosin for routine

histological examination (to exclude the presence of microadenomas). Blood samples were taken to determine serum calcium, phosphate and vitamin D metabolites.

Crypt cell production rate (CCPR)

Rectal biopsies were divided into multiple small explants and were orientated mucosal surface uppermost on a metal grid within an organ cuture dish (Lux laboratories). The crypt cell production rate was determined as previously described ( Materials and Methods). Breifly, after 15 hours, vincristine 0.6 $\mu$g/ml (Oncovin, Eli Lily, Basingstoke U.K.) was added to the culture medium to produce metaphase arrest within the rectal crypts. Explants were removed one, two and three hours later, fixed (for 2-4 hours) in Carnoy's fluid and then stored in 70% alcohol. Later, biopsies were rehydrated and after acid hydrolysis in 1M HCL at 60$^0$C for six minutes, the tissue was stained with Schiff's reagent. Using a dissecting microscope at least 20 crypts were isolated in each specimen, and the number of metaphase figures was counted per crypt. The mean number of arrested metaphases was plotted against time from vincristine administration, the slope of this line (determined by least squares linear regresssion analysis ) giving a value for the crypt cell production rate in cells/crypt/hour.

Serum profiles

Serum levels of 1,25 $OH_2$ $D_3$ and 25 OH $D_3$ were analysed at the Regional Assay Centre, The Middlesex Hospital, London. Calcium, albumin phosphate liver function tests and electrolytes were measured at the Hammersmith Hospital, London.

Stastical analysis.

Pre-and post-treatment values were compared using the paired Student's t-test within the test groups and the Mann-Whitney U-test between groups. Polyp counts were compared using analysis of variance, Chi-squared test and the Mann Whitney U-test. Serum electrolytes were compared using the Spearman correlation test.

## Results.

Twenty-five patients completed the trial. Three violated the protocol and were therefore excluded. Pre-and post-treatment polyp counts were obtained in all 25 patients who completed the trial. Initial rectal CCPR values were obtained in 22 patients, but one of these values was later excluded owing to the presence of a microadenoma on histological examination. Paired CCPR values before and after treatment were obtained in 16 patients (nine placebo and seven calcium patients). The tablets were well tolerated in all subjects with no adverse side effects being reported.

CCPR

There was considerable variation in the 21 pre-treatment values obtained, the overall mean being $5.96 \pm 0.88$ cells/crypt/hour (mean$\pm$s.e.m.) with a range of 1.14- 19.80 cells/crypt/hour (Table1). The median control metaphase count was 11.72 cells/crypt (range 4.16-18.70) at 1 hour, 14.36 cells/crypt (range 5.11-33.46) at two hours and 17.42 cells/crypt (range 13.22-35.87) at three hours.

Calcium reduced CCPR by 49% from a pre-treatment value of $4.72 \pm 0.48$ to a post-treatment value of $2.42 \pm 0.48$ cells/crypt/hour ($p < 0.05$) There was no significant difference in the placebo group, CCPR being $5.46 \pm 1.21$ before treatment and $5.08 \pm 1.17$ cells/crypt/hour after treatment (Tables 2 and 3).

## Table 1.

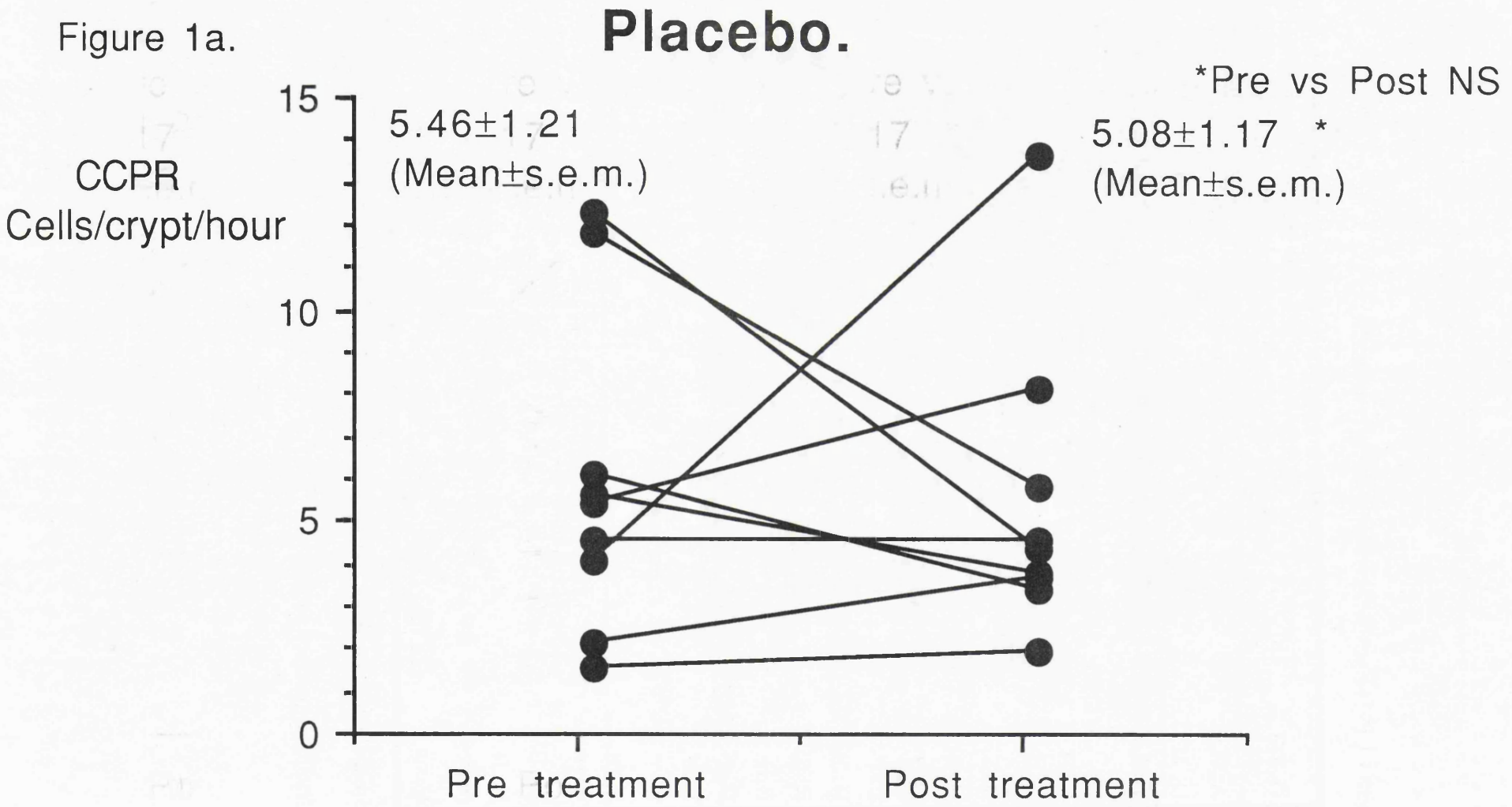
Pre-treatment CCPR values in 21 patients (cells/crypt/hour).

| Patient | IRA yrs/ago | Age | Sex | CCPR |
|---|---|---|---|---|
| A. N. | 13 | 38 | M | 5.07±0.56 |
| D. M. | 3 | 16 | M | 19.80±1.2 |
| T. A. | 37 | 65 | M | 1.14±0.02 |
| M. S. | 8 | 29 | M | 11.4±1.14. |
| R. H. | 2 | 46 | M | 11.09±0.5 |
| C. W. | 18 | 39 | F | 3.77±0.47 |
| C. S. | 29 | 53 | F | 5.18±0.54 |
| H. M. | 6 | 22 | F | 3.70±0.75 |
| M. J. | 30 | 56 | M | 7.30±1.10 |
| E. F. | 13 | 37 | F | 4.16±0.38 |
| H. B. | 12 | 29 | M | 6.11±1.88 |
| R. S. | 6 | 46 | M | 7.36±1.10 |
| R.H. | 6 | 30 | M | 5.60±1.74 |
| D. N. | 12 | 36 | M | 5.20±0.94 |
| J. M. | 14 | 40 | F | 4.47±0.24 |
| R. T. | 6 | 43 | M | 1.74±1.87 |
| S. M. | 6 | 40 | F | 3.65±2.25 |
| S. H. | 20 | 40 | M | 3.90±1.77 |
| M. B. | 10 | 29 | M | 5.47±0.14 |
| H. H. | 13 | 40 | M | 3.60±1.87 |
| P.T. | 4 | 33 | M | 5.45±2.74 |
| Mean | 12.50+1.52 | 36.70+2.87 | | 5.96+0.88 |

IRA = Ileorectal anastomosis.

CCPR = Crypt cell production rate.

Figure 1a.


Placebo.
*Pre vs Post NS
5.46±1.21
(Mean±s.e.m.)
5.08±1.17 *
(Mean±s.e.m.)
CCPR
Cells/crypt/hour
15
10
5
0
Pre treatment
Post treatment

## Table 2.

The effect of six months treatment with the placebo on rectal CCPR (cells/crypt/hour).

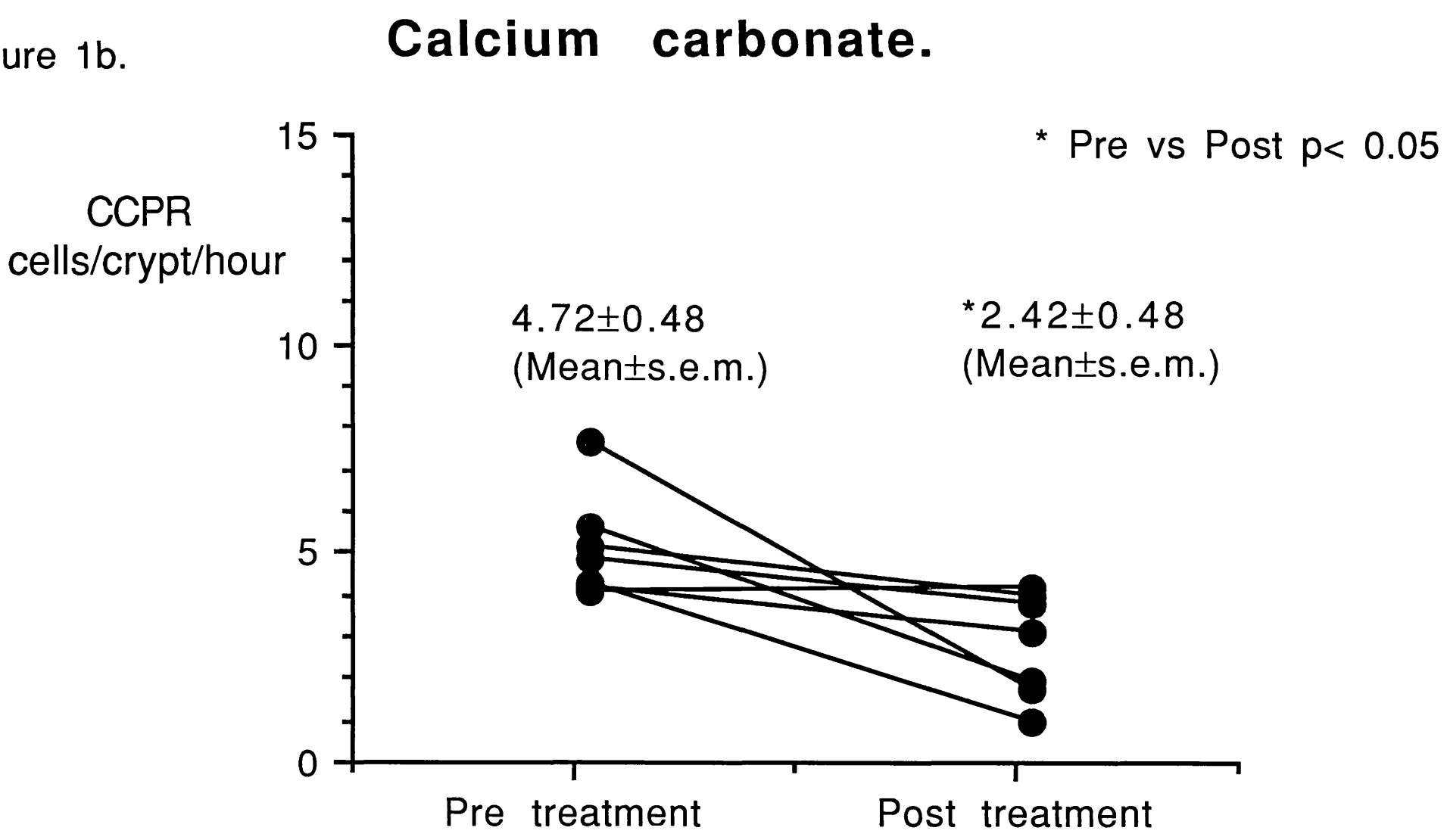
| Sex M:F | Age Years | Pre-treatment CCPR | Post-treatment CCPR |
|---|---|---|---|
| M | 38 | 5.07±0.56 | 7.70±0.22 |
| M | 65 | 1.14±0.02 | 1.55±1.19 |
| M | 29 | 11.40±1.14 | 5.43±0.25 |
| M | 46 | 11.09±0.50 | 3.99±0.15 |
| F | 37 | 4.16±0.38 | 4.15±0.32 |
| M | 30 | 5.67±1.74 | 3.00±0.50 |
| M | 36 | 5.20±0.94 | 3.40±0.02 |
| M | 43 | 1.74±1.87 | 3.28±0.76 |
| F | 40 | 3.65±2.25 | 13.24±1.41 |
| Mean | | 5.46±1.21 | 5.08±1.17 |

CCPR = Crypt cell production rate.

Values are mean±s.e.m.

(Figure 1a).

Figure 1b.


Calcium carbonate.
* Pre vs Post p< 0.05
CCPR
cells/crypt/hour
15
10
5
0
4.72±0.48
(Mean±s.e.m.)
*2.42±0.48
(Mean±s.e.m.)
Pre treatment
Post treatment

## Table 3

The effect of six months treatment with calcium on CCPR (cells/crypt/hour).

| Sex M:F | Age years | Pre-treatment CCPR | Post-treatment CCPR |
|---|---|---|---|
| F | 39 | 3.77±0.47 | 2.72±0.40 |
| F | 53 | 5.18±0.54 | 1.51±0.54 |
| F | 22 | 3.70±0.75 | 3.79±0.36 |
| M | 56 | 7.30±1.10 | 1.31±0.55 |
| M | 40 | 3.90±1.77 | 0.60±0.37 |
| M | 36 | 4.70±1.87 | 3.59±1.80 |
| F | 40 | 4.47±0.24 | 3.40±0.02 |
| Mean | | 4.72±0.48 | 2.42±0.48* |

CCPR = Crypt cell production rate.

Values are mean±s.e.m.

Significance * $p<0.05$. (Figure 1b)

**Polyp counts (Table 4)**

The number of rectal polyps varied widely from zero to almost complete confluence of polyps. Oral supplementation with calcium had no significant effect on polyp number, polyp progression or polyp distribution. The percentage increase in polyp count in the calcium group (47%) did not differ significantly from that in the placebo group (58%).

## Table 4.

The number of rectal polyps.

Values are mean±s.e.m.

| | Before treatment | After treatment |
|---|---|---|
| Placebo | 17.15±5.85<br>(Range 2-78) | 28.0±9.32*<br>(Range 0-100) |
| Calcium supplements | 16.82±7.68<br>(Range 0-68) | 24.68±10.96**<br>(Range 0-100) |

*Before vs after NS

**Before vs after NS.

Serum calcium and vitamin D (Table 5and 6).

Post-treatment serum levels of vitamin D metabolites were only obtained in 13 patients, eight receiving placebo and five receiving calcium supplements. Patients receiving placebo had elevated levels of vitamin D metabolites. The value for 1,25 $OH_2$ $D_3$ was well above the normal range (20-65 pg/ml) and significantly higher than the corresponding value in patients given calcium supplements. The value for 25 OH $D_3$ was outside the normal range (3-40 ng/ml), though the difference between the two groups did not attain statistical significance (Table 5).

By contrast, serum levels of calcium, phosphate, albumin and liver function tests were within normal limits in each group (Table 6).

## Table 5.

Post treatment serum levels of vitamin D metabolites.

Values are mean±s.e.m.

| | Placebo (n=8) | Calcium (n=5) |
|---|---|---|
| 1,25 $OH_2$ $D_3$<br>Normal range<br>20-60 pg/ml | 159.21±63.75<br>pg/ml | 77.40±13.18*<br>pg/ml |
| 25 OH $D_3$<br>Normal range<br>30-40 ng/ml | 69.37±28.35<br>ng/ml | 28.00±10.31<br>ng/ml |

Calcium vs placebo $p < 0.05$.

## Table 6.

Serum Calcium, phosphate and liver function tests.

| | Active | Placebo |
|---|---|---|
| Calcium | 2.42±0.02 | 2.43±0.03 |
| Phosphate | 2.20±1.14 | 0.95±0.05 |
| Bilirubin | 8.25±1.31 | 10.17±0.72 |
| Alkaline phosphatase | 75.75±3.33 | 94.00±7.40 |
| Aspartate transaminase | 33.75±5.17 | 24.17±1.72 |

Values are mean±s.e.m.

Normal range: Calcium 2.15-2.65 mmol/l Phosphate 0.80-1.40 mmol/l

Bilirubin 2-13 μmol/l Alkaline Phosphate 30-130 iu/l

## Comments.

The CCPR values indicate that oral calcium supplementation can inhibit rectal epithelial cell proliferation in FAP, but it has no clear effect on rectal polyps. Six out of seven patients in this trial responded to calcium, although our initial CCPR values (despite a wide scatter) were similar to previously reported values ($4.65 \pm 0.54$ cells/ctypt/hour) in control subjects (Appleton et al. 1989.). A reduction in the rectal CCPR has been reported in a double-blind trial of calcium supplements in patients with villous adenomas (Barsoum et al. 1992). In an uncontrolled trial of oral calcium in ten subjects at high risk from colorectal cancer, the tritiated thymidine labelling index was reduced to a value similar to that seen in a low risk group (Lipkin and Newmark 1985). This effect, may however, be restricted to those patients whose initial labelling index is higher than normal (Lipkin et al. 1989, Rozen et al. 1989). Indeed, in a controlled trial of calcium supplements in patients with previous colorectal cancers (but normal labelling indices), calcium had no antiproliferative effect (Gregoire et al. 1989). The observed reduction in CCPR in this trial relies on data from only 7 patients, two of these patients demonstrated a marked reduction in CCPR and whereas another showing a slight increase. A larger study with more frequent biopsies would be useful to determine if this effect is reproducible and whether calcium has a lasting or only a transient effect.

*In-vitro* exposure to calcium can also reduced the labelling index in human rectal tissue. Buset found that cells cultured from established adenomas did not respond to *in-vitro* calcium and neither did those from adenocarcinomas (Busset et al. 1986). Likewise, the

polyp count and progression of polyps in this trial were unaffected by oral calcium supplements. It therefore seems likely that epithelial cells do not respond to the regulatory effects of calcium once adenomatous change has been evoked. The failure to show a reduction in the progression of the polyp count presumably reflects the continued growth of microadenomas during the trial period. To determine if calcium supplemenatation can prevent the development of new microadenomas and their progression to adenomas would probably require a much larger trial possibly involving ingestion of calcium over a long period of time with biopsies taken at shorter intervals (for example every two months). In the U.K. a study of this size would probably need to be conducted as a multi-centre trial. In addition, prolonged trial period might run into problems with patient compliance.

Calcium may exert its action on colorectal epithelial cell proliferation by binding to free fatty acids and bile acids that would otherwise stimulate cell damage and regeneration (Wargovich et al. 1983, Wargovich Eng and Newmark 1984, Rainey Davies and Williamson 1984). Newmark has suggested that the dietary intake of calcium required to form non-toxic salts of bile acids in man would need to be doubled from a typical value of 930 mg/day to 1.5-2.0 g/day. Calcium may also act in a more complex way, as illustrated by a study in 12 healthy volunteers (Van de Mee et al. 1990), in whom calcium supplements increased the faecal excretion of bile acids and phosphate and reduced the dihydroxy bile acid concentration in duodenal bile. These data imply that calcium binds to phosphate in the small bowel and that it is this calcium-phosphate complex that binds luminal bile acids. If so, then levels of phosphate must be sufficient to allow calcium to exert a protective effect. The placebo patients in this trial had levels of vitamin D

metabolites well above the normal range, possibly owing to a overall intracellular calcium depletion in these FAP patients.

A wide range in values was observed in the mean CCPR values, despite low values for the individual standerd errors of the mean. This wide range might to an extent reflect either inadvertent sampling of microadenomas or areas of hyperplasia reflecting an overall increase in epithelial cell proliferation. This variation in mean CCPR values is less likely to be a methodological artefact as in normal individuals the method produces a very consistent mean CCPR with low standard errors (see experiment 3).

The distribution of gastrointestinal adenomas in in FAP may reflect exposure to bile (Spigelman et al. 1989), and duodenal cancer is now responsible for many deaths in patients with FAP (Jones and Nance 1975, Jagelman 1987, Sinha and Williamson 1988). If calcium were to reduce duodenal CCPR, either directly or indirectly, long-term dietary manipulation might reduce microadenoma formation and duodenal cancer in FAP.

To conclude, oral calcium supplementation reduces rectal CCPR in patients with FAP without an obvious effect on the rectal polyps. The mechanism of this action and the potential therapeutic use of dietary manipulation to protect against intestinal carcinoma in this disease need to be investigated further.

# Experiment 3.

# The effect of vitamin D metabolites on rectal epithelial proliferation.

## Background.

Calcium may protect against colorectal cancer by diminishing epithelial cell turnover (Appleton Bristol and Williamson 1986, Appleton et al. 1987). Vitamin D has been advocated as another possible chemopreventative agent (Garland et al. 1985), and there is strong epidemiological evidence to support this claim (Chapter 3). Also, vitamin $D_3$ metabolites alter cell turnover in various cancer cell lines, reducing proliferation and inducing cellular differentiation (Tanaker et al. 1983, Lointier et al. 1987, Colston Berger and Coombes 1989). Indeed, the human colon cancer cell lines HT-29 (Brehier and Thomasset 1988, Harper Iozzo and Haddad 1989), LOVO (Lointier et al. 1987) and Caco2 (Giuliano and Wood 1991) possess high affinity receptors for the active form of the vitamin, 1,25 $(OH)_2 D_3$. On addition of 1,25 $(OH)_2 D_3$ to cultures of these cells, the growth rate is reduced and changes indicative of differentiation are induced. The demonstration of the vitamin D receptor (VDR) in the normal human colon (Lointier et al. 1991) and the ability of the colon to absorb calcium (Hylander Ladefoged and Jarnums 1980) both suggest that the large bowel may be a potential target organ for the action of the active metabolite of vitamin D. In Experiment 2, the effect of oral calcium supplementation on rectal epithelial cell proliferation was investigated using an *in-vitro* organ culture technique to determine rectal CCPR. A similar method can be used to determine whether vitamin D and its metabolites affect the rate of human rectal epithelial cell proliferation.

Although the therapeutic use of vitamin $D_3$ itself is limited by its profound effects on calcium metabolism, several vitamin $D_3$ analogues have recently been introduced that retain potent cell regulatory properties while causing only limited hypercalcaemia and hypercalciuria; one such analogue is the steroid MC-903 (Binderup and Bramm. 1988, Kissmeyer and Binderup. 1991, Kragballe Beck and Sogaard 1988, Kragballe 1989, Kragballe et al. 1991). The aim in this experiment was to use stathmokinetic and immunohistochemical techniques to study the effect of vitamin D metabolites and the analogue MC-903 on cell proliferation in human rectal epithelium.

## Method.

### Clinical material

Paired rectal biopsies were taken from 17 patients (mean age 60.55, range 37-80) with macroscopically normal rectal mucosa who were attending the outpatient clinic with incidental anal conditions. One biopsy from each patient was examined histologically. The other biopsy was established in organ culture with or without the addition of vitamin D or its analogues. In four control patients, tissue was frozen to -80$^0$C after culture for subsequent immunohistochemical analysis.

### Vitamin D preparations

1,25 $(OH)_2D_3$ and ergocalciferol (vitamin $D_2$) were donated by Roche Products Ltd (Welwyn Garden City, U.K.). The vitamin D analogue MC-903 was supplied by the Department of Biology, Leo Pharmaceuticals Products (Ballerup, Denmark). Stock solutions of steroids were prepared in absolute alcohol and stored at -20$^0$C until use.

Control medium was prepared with a similar dilution of alcohol, and this produced no obvious effect on proliferation.

**Organ culture**

Rectal biopsies were divided into small explants and orientated mucosal surface uppermost on a metal grid within an organ culture dish (Lux Laboratories). Explants were cultured as paired samples in standard culture medium or in culture medium to which vitamin D analogues had been added: 1,25 $(OH)_2$ $D_3$ at concentrations of 1$\mu$M, 10 nM or 100 pM, ergocalciferol (vitamin $D_2$) at 10 nM and MC-903 at $10^{-7}$M. Hence each biopsy acted as its own control. The final concentration of 1,25 $(OH)_2$ $D_3$ in the standard culture medium was 6 pM (determined by batch-testing the fetal calf serum). As in Experiment 2, the organ culture dishes were sealed in an atmosphere of 95 % $O_2$ and 5% $CO_2$ at a temperature of 37$^0$C and were gently rocked at 3-5 cycles per minute (Appleton 1988). Crypt cell production rate was determined as previously described in Chapter 4.

**Immunohistochemistry**

After 18 hours organ culture in medium with or without 1,25 $(OH)_2$ $D_3$ (100pM), paired mucosal explants were mounted, and 3$\mu$m cryostat sections were cut and air dried before blocking in $H_2O_2$ (0.22%) and methanol for 5 minutes. The slides were washed in TRIS buffer( pH 7.3) for 5 minutes (x3). Sections were then stained using a three-stage peroxidase procedure in which Ki-67 monoclonal antibody (Gerdes et al. 1983, 1984), (1:50 in TRIS buffer for 60 minutes), biotinylated rabbit anti-mouse(1:300 in TRIS buffer for 60 minutes) and avidin-biotin complex (Dakopatts, for 45 minutes) were applied. As a negative control TRIS buffer was used without the primary antibody and positive controls

were histological frozen sections from bladder tumours previously shown to express Ki-67 immunoreactivity. Slides were developed in diamino benzadine-hydrogen peroxide (DAB) in TRIS buffer for 5 minutes, then washed in tap water. Haematoxylin was used as a counter stain. To assess rectal epithelial proliferation, the labelling index was determined in at least 15 crypts per section. Labelling index was calculated as the ratio of Ki-67 positive to negative cells per crypt (Franklin et al. 1985). The mean values of these counts were compared using a paired Student's t-test (each patient acting as its own control).

## Results.

### Organ Culture.

The overall crypt cell production rate (CCPR) in 17 normal patients was $4.74 \pm 0.25$ cell/crypt/hour (mean$\pm$s.e.m.) with a range of 2.85-7.07. This value is similar to previously reported results in control patients who had a mean CCPR of $4.65 \pm 0.54$ (Appleton et al. 1989). The median control metaphase count was 8.51 cells/crypt (range 3.5-17.9) at 1 hour, 11.53 cells/crypt (6.76-21.45) at 2 hours and 16.90 cells/crypt (11.20-32.53) at 3 hours. The mean correlation coeficient of the linear regression curves was $0.99 \pm 0.01$ (mean$\pm$s.e.m.), range 0.91-1.00. Explants showed excellent preservation of crypt architecture with an infection and crypt necrosis rate of only 1 %. The active metabolite of vitamin $D_3$ (1,25 $(OH)_2$ $D_3$) consistently halved the overall CCPR in normal tissue, irrespective of the concentration employed (analysis of variance). Thus (compared to matched controls) at 1 $\mu$M CCPR was reduced from 4.96 to 2.15 cells/crypt/hour, at 10 nM CCPR was reduced from 4.71 to 2.10 cells/crypt/hour and at 100 pM CCPR was reduced from 4.86 to 2.67 cells/crypt/hour. The data suggest a slight dose-dependent trend when the individual explants were compared to their own controls (paired Student's t-test):

**Table 1.**

The effect of vitamin D metabolites on normal human rectal CCPR (cells/crypt/hour).

Values are mean±s.e.m.

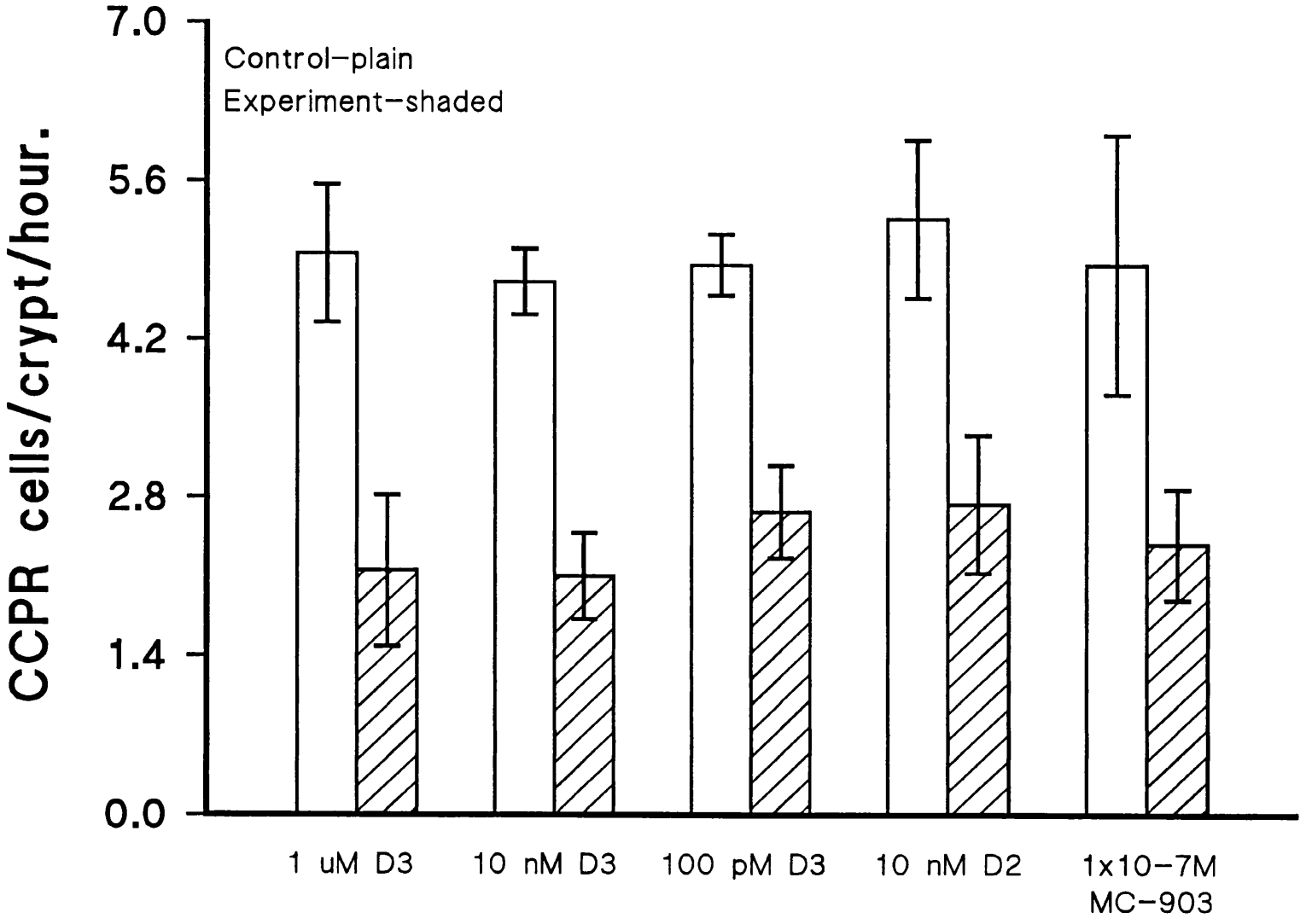
| | Control | 1 $\mu$M 1 ,25$OH_2D_3$ | 10 nM 1,25$OH_2D_3$ | 100 pM 1,25$OH_2D_3$ | 10 nM $D_2$ |
|---|---|---|---|---|---|
| 1 | 4.10±0.62 | 1.29±0.62 | 1.12±0.49 | | |
| 2 | 4.15±0.54 | | 1.32±0.85 | 2.31±0.12 | |
| 3 | 5.02±0.52 | | 2.24±0.03 | 2.62±0.44 | |
| 4 | 4.32±0.30 | | 2.16±0.05 | 2.38±1.92 | |
| 5 | 4.04±0.06 | | 3.97±0.58 | 1.68±0.43 | |
| 6 | 5.40±0.63 | | 2.67±1.87 | | |
| 7 | 5.97±0.28 | 2.25±0.65 | 1.22±0.60 | 2.72±2.03 | 1.22±0.60 |
| 8 | 7.07±1.13 | 5.15±0.77 | | | 4.42±2.07 |
| 9 | 3.27±1.46 | 0.28±0.31 | | | |
| 10 | 5.65±1.18 | 2.25±0.65 | | | 3.84±0.37 |
| 11 | 3.70±1.46 | 1.68±0.33 | | | |
| 12 | 4.80±0.50 | | | | 1.70±1.16 |
| 13 | 2.85±0.92 | | | | 2.54±2.50 |
| 14 | 5.20±0.03 | | | 3.84±0.67 | |
| 15 | 5.50±0.98 | | | 4.20±0.98 | |
| 16 | 5.20±1.14 | | | 4.00±0.89 | |
| 17 | 4.32±0.54 | | | 0.33±0.50 | |
| Mean | 4.74±0.25 | 2.15±0.67# | 2.10±0.67# | 2.67±0.41** | 2.74±0.61* |

Significance vs control (analysis of variance).

* $p<0.05$ ** $p<0.01$ # $p<0.001$

Figure 1.

The effect of vitamin D on CCPR in human rectal tissue.



vitamin $D_2$ (ergocalciferol) at a dose of 10 nM reduced CCPR in normal rectum from 5.27 to 2.74 cells/crypt/hour (Table 1). While the vitamin D analogue MC-903 (1 $x10^{-7}$ M) also reduced CCPR in 7 patients from 4.86 to 2.38 cells/crypt/hour (Table 2, Figure 1).

**Table 2.**

The effect of MC-903 ($1x10^{-7}$M) on rectal CCPR (cells/crypt/hour)

| Patient | Control | MC-903 ($1x10^{-7}$M) |
|---|---|---|
| 1 | 3.60 | 1.84 |
| 2 | 4.15 | 3.78 |
| 3 | 1.55 | 1.34 |
| 4 | 11.09 | 3.68 |
| 5 | 5.43 | 3.76 |
| 6 | 3.00 | 1.15 |
| 7 | 5.18 | 1.14 |
| Mean | 4.86±1.15 | 2.38±0.49* |

Values are mean ± s.e.m.

* Significance vs control $P<0.05$.

Immunohistochemistry

The labelling index with Ki-67 was reduced from a control value of $7.28 \pm 0.68$ (mean $\pm$ s.e.m.) to $3.74 \pm 0.64$ ($n=4$ $p<0.01$) in the presence of 100 pM 1,25 $(OH)_2$ $D_3$ (Figure 2).

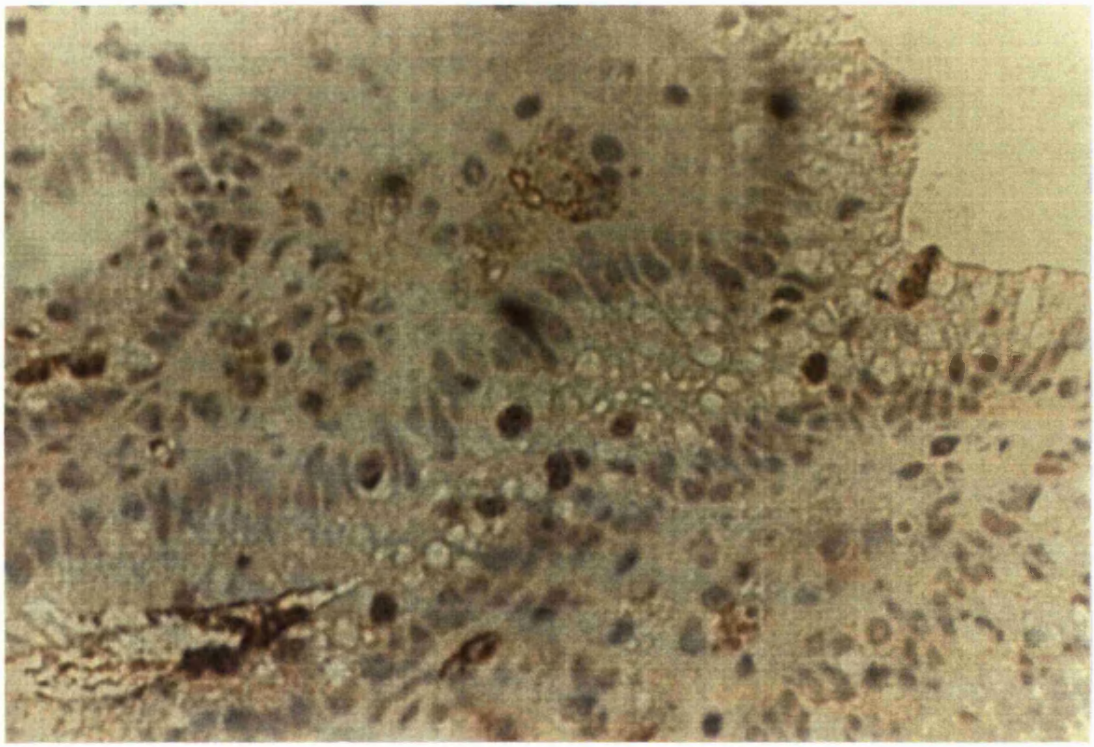


Figure 2. Ki-67 labelling in cryostat sections after 18 hours organ culture.

**Comment.**

This study is the first to demonstrate an inhibitory effect of vitamin $D_3$ and its analogues on human colorectal crypt cell production rate. Normal rectal tissue showed a reduced epithelial proliferation (CCPR), at least *in-vitro*. This finding has been confirmed histochemically using the monoclonal antibody Ki-67 to measure the crypt labelling index. The Ki-67 antibody binds to an antigen present in proliferating cells. The data therefore suggest that along with the birth rate of cells (i.e. CCPR), the growth fraction is also reduced by vitamin D. The presence of vitamin $D_3$ receptors in normal and malignant colorectal tissue (Lointier et al. 1991), together with the recovery of calcium absorption after small bowel resection (Hylander Ladefoged and Jarnums 1980), had previously suggested that the colon might be responsive to 1,25 $(OH)_2$ $D_3$. It is possible that some of the proliferating cells in the crypt have been induced into terminal differentiation by the addition of the vitamin D metabolites.

The use of a rocking platform to improve tissue oxygenation was probably one of the reasons the CCPR results in normal tissue were very consistent, with a very low level of crypt necrosis and virtually no "edge labelling" seen in the explants. Indeed, the values for normal controls were only slightly lower than previously reported values (Allan, Bristol and Williamson.1985) using a similar technique.

The therapeutic use of vitamin D metabolites is limited by its profound effect on calcium metabolism. The analogue MC-903 has limited effects on calcium metabolism while it retains potent cell regulatory properties (Binderup and Bramm 1988). Clinical trials using

MC-903 as a topical agent in psoriasis have been encouraging, with good patient tolerance and few side effects (Kragballe Beck and Sogaard 1988, Kragballe 1989, Kragballe et al. 1991). The present study suggest that MC-903 also has an effect on rectal epithelial proliferation (at least *in-vitro*).

There is now substantial evidence to show that 1,25 $(OH)_2$ $D_3$ acts as a differentiating agent (Tanakar et al. 1983, Brehier and Thomasset 1988, Studzinski Bhandal and Brelvi 1985, Colston Berger and Coombes 1989, Harper Iozzo and Haddad 1989), and that some of these actions are associated with modulation of receptor concentrations (Bhalla et al. 1983, Studzinski, Bhandal and Brelvi. 1985). It is tempting to postulate that the observations represent a genomic effect, but proof would require the demonstration of a measurable gene product that could be correlated with inhibition of proliferation (Lointier et al. 1987). Some differentiated cells have shown a reduced vitamin D receptor expression (Provvedini Deftos and Manolagas 1984), for example HT-29 cells (Brehier and Thomasset 1988), and modulation of receptor expression may also depend on the state of differentiation, as in Caco2 cells (Giuliano Franceschi and Woods 1991).

The was no-clear cut dose response effect seen with reducing doses of 1,25 $OH_2$ $D_3$ (only a dose-dependent trend). Moreover, vitamin $D_2$ (a less potent form of vitamin D) had almost an equal effect on CCPR. There is thus no good evidence to suggest that the effect is a pure receptor-ligand reaction. An alternative hypothesis is that the the observed inhibition of proliferation may be a non-genomic effect related to calcium ion transport. In support, verapamil and glucocorticoids (both of which influence calcium transport) effect the morphological changes induced by 1,25 $(OH)_2D_3$ in LOVO cells (Lointier et al. 1987).

Irrespective of the mode of action, vitamin $D_3$ and its analogues appear to inhibit *in-vitro* colonic epithelial proliferation and hence may offer a therapeutic option in patients at high risk of developing colorectal carcinoma.

## Experiment 4.

## The effect of synthetic vitamin D analogues on the growth of colon cancer cell lines.

Background

The intestine is a target for the potent active metabolite of vitamin $D_3$ - 1,25 $OH_2$ $D_3$, and its mode of action in the gut appears to be similar to that of other steroid hormones (Haussler 1986, Giuliano Franceschi and Wood 1991). On entering the cell 1,25 $OH_2$ $D_3$ binds to a high affinity receptor, and this hormone-receptor complex binds to chromatin and initiates transcription of mRNA (Feldman et al. 1979, Giuliano Franceschi and Wood 1991). Autoradiographic studies have localised the vitamin D receptor (VDR) to the nuclei of epithelial cells along the villi and crypts of the small intestine and to the luminal surface of the cells in the colonic crypts (Chan Chim and Atkins 1984, Chan and Atkins 1984, Giuliano Franceschi and Wood 1991). Many colon-derived adenocarcinoma cell lines express the vitamin D receptor (usually in their undifferentiated state) and show an inhibition of proliferation and induction of differentiation with vitamin D metabolites (Lointier et al. 1987, Brehier 1988, Giuliano and Wood 1991, Giuliano Franceschi and Wood 1991). The human colon cancer cell line HT-29 loses expression of VDR after induction of differentiation by the active metabolite 1,25 $OH_2$ $D_3$ (Brehier and Thomasset 1988, Giuliano Franceschi and Wood 1991).

Caco2 cells differ from most other colon cancer lines in that they spontaneously differentiate when in culture (Chung et al. 1985). The initial log-phase of growth is associated with minimal functional differentiation, whereas in the later stationary-phase of growth functional differentiation is maximal (Pinto et al. 1983). This effect is parallelled

by an increase in VDR expression, and the receptor appears to be functional in both undifferentiated cells and the more quiescent differentiated cells (Giuliano and Wood 1991 Giuliano Franceschi and Wood 1991). In the rat intestine the concentration of vitamin D receptors is 2.4 fold greater in more differentiated non-proliferating crypt cells than in activelproliferating cells. (Chan Chim and Atkins 1984). Caco2 cells may therefore reflect the effects of vitamin D on the differentiated colonic cell.

The control of cell proliferation by vitamin D is limited by its potent effects on calcium metabolism (Vieth 1990). Hypercalcaemia is induced by systemic doses of only a few $\mu$g per day. The synthetic vitamin D analogue MC-903 when administered to rats either by intraperitoneal injection or orally, was at least 100 times less active than either 1,25 $OH_2$ $D_3$ and 1- alpha-OH $D_3$ in inducing hypercalciuria, hypercalcaemia and bone calcium mobilisation. Nevertheless, it retains potent effects on cellular proliferation (Binderup and Bramm 1988).

The aim of this experiment was to determine the effect of two synthetic vitamin D analogues on the growth of colon cancer cell lines. The two cell lines Caco2 and HT-29 (which have different levels of vitamin D receptor expression after differentiation) were cultured with the synthetic vitamin D analogue MC-903 and a related compound EB-1089.

## Method.

Chemicals

MC-903 and EB-1089 were gifts from Leo Pharmaceuticals, Ballerup, Denmark. These vitamin D analogues were stored in isopropyl alcohol at -20$^0$C until use. Fresh stock solutions containing the different concentrations of the analogues were prepared each week.

Between changes of culture medium the stock solutions were kept at 20$^0$C in the refrigerator. Control culture medium contained a small amount of isopropyl alcohol added (approximately 0.2%).

Cell culture.

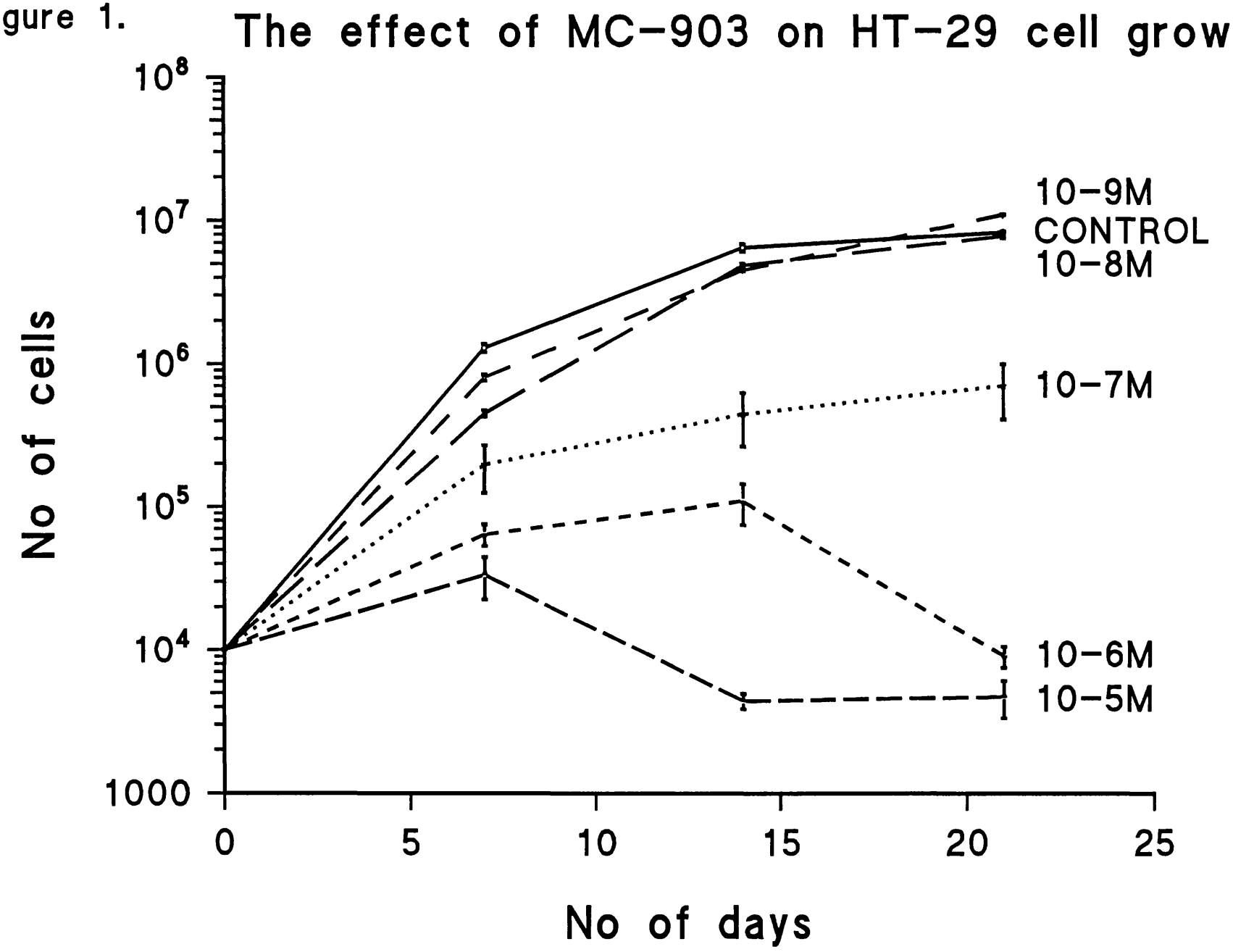
HT-29 and Caco2 cells were maintained as a monolayer of cells in Dulbecco's Modified Eagle's Medium (DMEM, Flow laboratories, High Wycombe, U.K.), with 10 % fetal calf serum, 100 u/ml penicillin and 100 $\mu$g/ml streptomycin (Serolabs, Crawley, U.K.). Cells were incubated at 37$^0$C in 95 % $O_2$ and 5 % $CO_2$. The medium was changed every 2 days. At 80-90% visual confluence, the cells were trypsinised with 0.25 % trypsin and EDTA (Flow Laboratories). After washing in phosphate buffer saline (PBS), aliquots of 1x $10^4$ cells were added to six-well tissue culture plates (Gibco). Cells were cultured in DMEM with or without the addition of the vitamin D analogue MC-903 or EB-1089 at a final concentration of 1 x$10^{-5}$ to 1x $10^{-10}$ M. All plates were set up in quadruplicate.

Following viability assessment using trypan blue exclusion, the total cell number was determined in samples at 7, 14 and 21 days by counting at least 3 samples from each concentration, using a haemocytometer. Results were analysed by means of Kruskal-Wallis one-way analysis of variance and the Mann-Whitney U-test.

**Results**

HT-29 and Caco2 cells in control media showed a rapid log-phase growth in the first 14 days of culture; thereafter the rate of cell proliferation was slower. At 7, 14 and 21 days culture, MC-903 produced a dose-dependent inhibition of the proliferation of HT-29 cells. At 21 days the total cell number was reduced by 99.9% at $10^{-5}$M, 99.8% at $10^{-6}$M, 91.5%

Figure 1.


The effect of MC-903 on HT-29 cell growth.
No of cells
$10^8$
$10^7$
$10^6$
$10^5$
$10^4$
1000
0
5
10
15
20
25
No of days
10-9M
CONTROL
10-8M
10-7M
10-6M
10-5M

at $10^{-7}$M and 5.9% at $10^{-8}$M. At $10^{-10}$M there was a slight increase in the number of cells compared to the control culture. Likewise, EB-1089 reduced total cell number by 72% at $10^{-10}$M, 64% at $10^{-9}$M and 96% at $10^{-8}$M.

The synthetic analogues had little effect on the initial rate of the proliferation of Caco2 cells. However, MC-903 produced significant inhibition of proliferation in the second week and likewise EB-1089 in the third week. A clear-cut dose-dependent inhibition of cell growth was seen after 21 days culture. MC-903 reduced the total cell number by 58% at $10^{-10}$M, 89% at $10^{-9}$M and 97% at $10^{-8}$M. Similarly, EB-1089 reduced the number of Caco2 cells by 67% at $10^{-10}$M, 82% at $10^{-9}$M and 92% at $10^{-8}$M.

**Table 1, ( Figure 1).**

**The effect of MC-903 on HT-29 cell growth.**

Values are total cell numbers (mean±s.e.m.).

| Concentration | Day 7 | Day 14 | Day 21 |
|---|---|---|---|
| Control | $1.28\pm0.88\times10^{6}$ | $6.47\pm3.97\times10^{6}$ | $8.29\pm1.45\times10^{6}$ |
| $1\times10^{-5}$M | $3.34\pm1.09\times10^{4}$** | $4.40\pm0.53\times10^{3}$** | $4.70\pm1.37\times10^{3}$** |
| $1\times10^{-6}$M | $6.40\pm1.00\times10^{4}$** | $1.09\pm3.48\times10^{5}$** | $9.00\pm1.50\times10^{3}$** |
| $1\times10^{-7}$M | $1.97\pm0.73\times10^{5}$*** | $4.44\pm1.80\times10^{5}$*** | $7.01\pm2.92\times10^{5}$*** |
| $1\times10^{-8}$M | $4.50\pm0.23\times10^{5}$* | $4.87\pm1.44\times10^{6}$* | $7.80\pm2.49\times10^{6}$ |
| $1\times10^{-9}$M | $8.00\pm0.41\times10^{5}$* | $4.50\pm0.81\times10^{6}$* | $1.10\pm0.01\times10^{7}$ |

Significance vs control (analysis of variance) $p<0.05$ *, $p<0.01$**, $p<0.001$***

Figure 2.

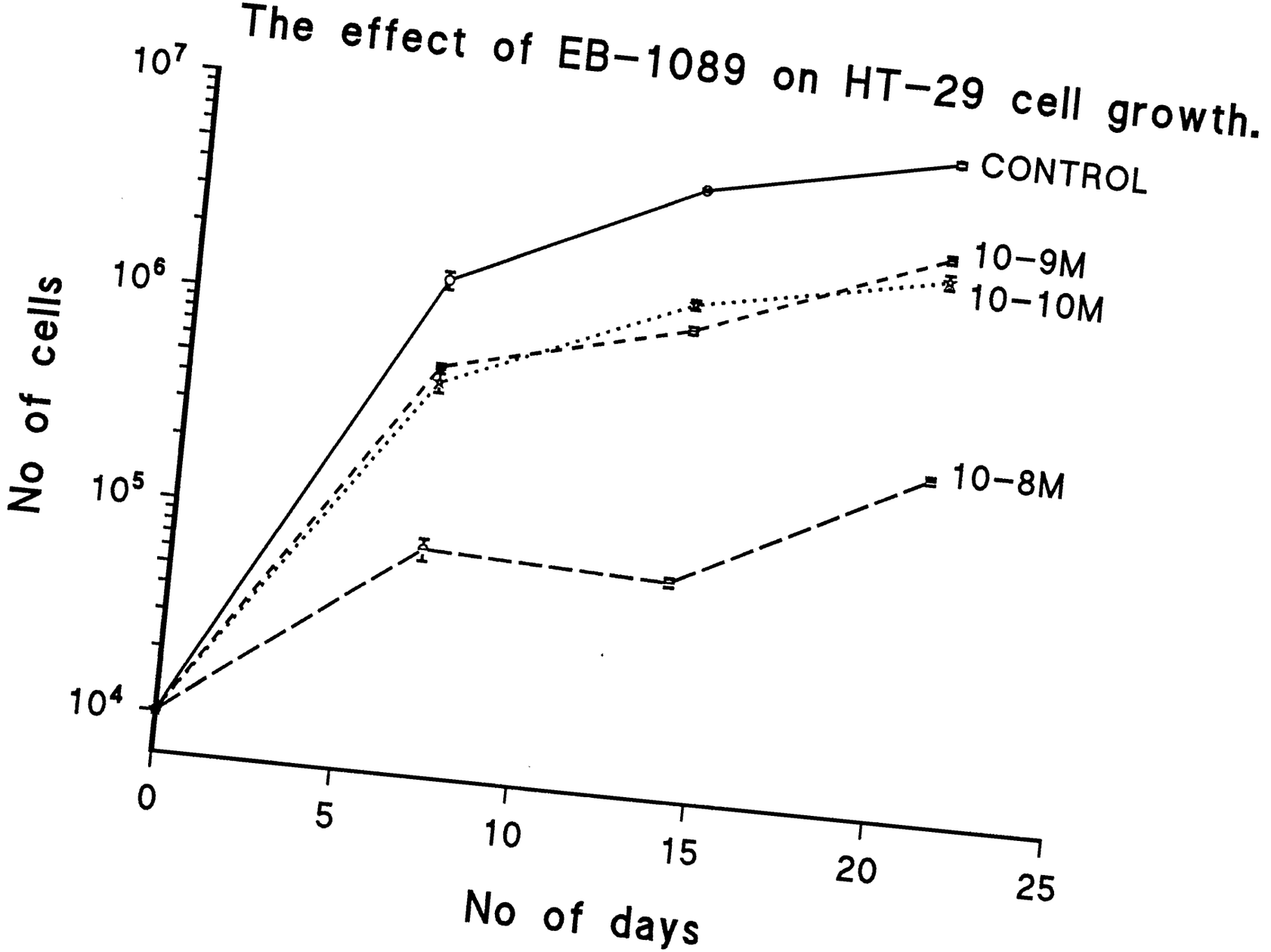

The effect of EB-1089 on HT-29 cell growth.
No of cells
$10^7$
$10^6$
$10^5$
$10^4$
CONTROL
10-9M
10-10M
10-8M
0
5
10
15
20
25
No of days

**Table 2, (Figure 2).**

**The effect of EB-1089 on HT-29 cell growth.**

Values are total cell numbers (mean±s.e.m.)

| Concentration | Day 7 | Day 14 | Day 21 |
|---|---|---|---|
| Control | $1.33\pm0.13\times10^{6}$ | $4.55\pm0.04\times10^{6}$ | $7.80\pm0.24\times10^{6}$ |
| $1\times10^{-8}$M | $7.30\pm0.88\times10^{4}$** | $6.67\pm0.32\times10^{4}$** | $2.60\pm1.00\times10^{4}$** |
| $1\times10^{-9}$M | $5.27\pm0.14\times10^{5}$* | $1.01\pm0.46\times10^{4}$** | $2.80\pm1.15\times10^{5}$** |
| $1\times10^{-10}$M | $4.43\pm0.46\pm10^{5}$* | $1.33\pm0.67\times10^{5}$** | $2.20\pm1.73\times10^{5}$** |

Significance vs control (analysis of variance) $p<0.01$*, $p<0.001$**

**Table 3, (Figure 3).**

**The effect of MC-903 on Caco2 cell growth.**

values are total cell numbers (mean±s.e.m.)

| Concentration | Day 7 | Day 14 | Day 21 |
|---|---|---|---|
| Control | $2.05\pm0.13\times10^{4}$ | $2.53\pm0.03\times10^{5}$ | $1.19\pm0.10\times10^{6}$ |
| $1\times10^{-8}$M | $7.00\pm0.50\times10^{3}$ | $3.83\pm0.33\times10^{3}$** | $3.67\pm0.60\times10^{4}$*** |
| $1\times10^{-9}$M | $1.87\pm0.67\times10^{3}$ | $7.16\pm0.60\times10^{4}$* | $1.35\pm0.30\times10^{5}$*** |
| $1\times10^{-10}$M | $1.43\pm0.23\times10^{4}$ | $7.83\pm0.93\times10^{4}$* | $4.96\pm0.69\times10^{5}$*** |

Significance vs control $p<0.05$*, $p<0.01$**, $p< 0.001$***

Figure 3.

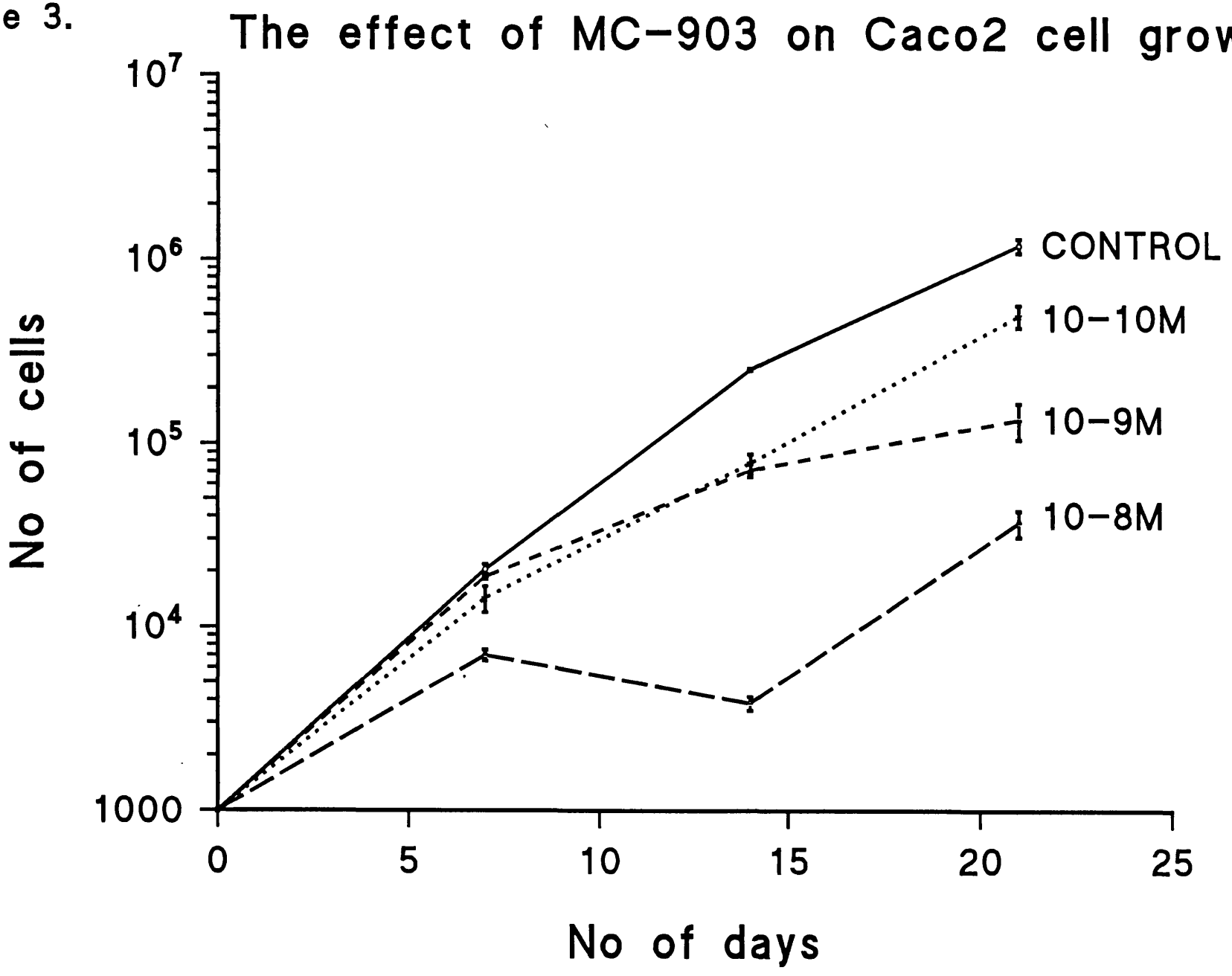

The effect of MC-903 on Caco2 cell growth.
No of cells
$10^7$
$10^6$
$10^5$
$10^4$
1000
0
5
10
15
20
25
No of days
CONTROL
10-10M
10-9M
10-8M

**Table 4, (Figure 4).**

**The effect of EB-1089 on Caco2 cell growth.**

Values are total cell numbers (mean±s.e.m.).

| Concentration | Day 7 | Day 14 | Day 21 |
|---|---|---|---|
| Control | $3.63\pm0.55\times10^{4}$ | $1.67\pm0.09\times10^{5}$ | $1.50\pm0.15\times10^{6}$ |
| $1\times10^{-8}$M | $3.00\pm0.87\times10^{3}$ | $2.17\pm0.17\times10^{4}$ | $1.20\pm0.06\times10^{5}$* |
| $1\times10^{-9}$M | $1.30\pm0.35\times10^{4}$ | $1.70\pm0.26\times10^{5}$ | $2.65\pm0.15\times10^{5}$* |
| $1\times10^{-10}$M | $1.38\pm0.20\times10^{4}$ | $1.76\pm0.12\times10^{5}$ | $4.90\pm0.15\times10^{5}$* |

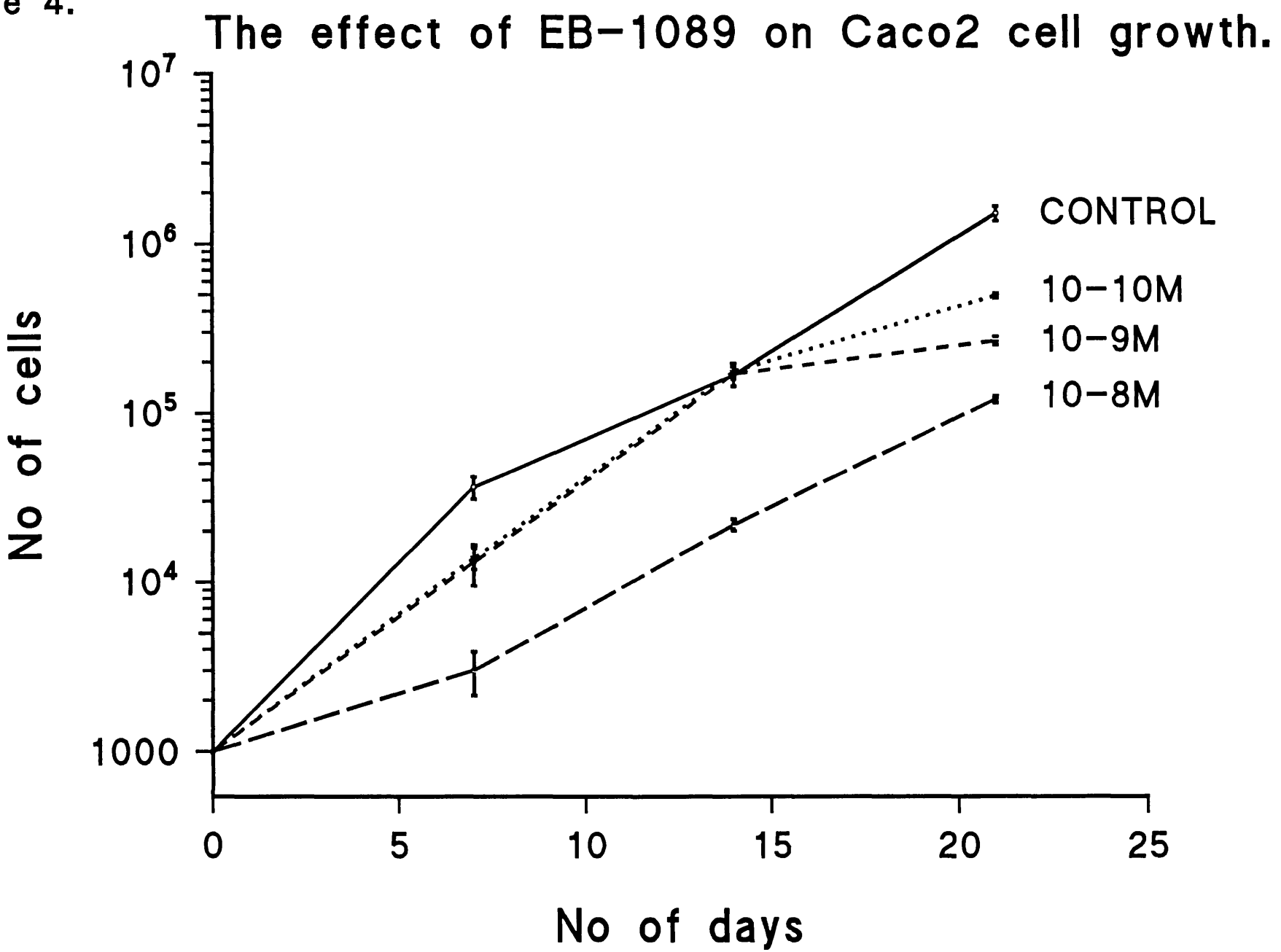
Significance vs control $p<0.001$*

**Comments**

After 21 days in cell culture synthetic vitamin D analogues produce a clear-cut dose-dependent inhibition of proliferation in both Caco2 and HT-29 cell lines. The ability of these analogues to inhibit HT-29 cell proliferation occurs within the first week, when the cells are still in the log-phase of their growth. This effect may be due to the expression of the VDR that is known to occur in undifferentiated cells. Likewise, the delay in the onset of inhibition in Caco2 cells may reflect the known paucity of receptor expression in the early or log-phase growth of these cells. As more Caco2 cells differentiate, VDR expression increases and thus the analogues will have a greater effect on the total cell number.

Caco2 cells may be similar to differentiated enterocytes, and if so colonic cells that express the VDR can respond to the steroid. The results of this experiment therefore complement the findings in Experiment 3, in which macroscopically normal rectal mucosa

Figure 4.


The effect of EB-1089 on Caco2 cell growth.
No of cells
$10^7$
$10^6$
$10^5$
$10^4$
1000
0
5
10
15
20
25
No of days
CONTROL
10-10M
10-9M
10-8M

showed a reduction in CCPR on the addition of vitamin D metabolites. Hence, safe synthetic analogues could possibly be used as therapeutic agents (perhaps in suppository form) to reduce the accelerated rate of colonic cell proliferation seen in individuals who are at a high risk of developing colorectal cancer.

Some human colorectal adenocarcinomas express the VDR, and HT-29 cells may behave in a similar fashion to the poorly differentiated cells in colorectal cancers. The ability of synthetic vitamin D analogues to reduce cell proliferation in HT-29 cells suggests that they could similarly reduce cell growth in adenocarcinoma cells expressing the VDR. There is therefore the theoretical possibility that vitamin D analogues could be used to control metastatic tissue following surgical resection of advanced colorectal cancers.

# Experiment 5.

## Epithelial cytokinetics in familial adenomatous polyposis.

**Background.**

In Experiment 3, it was seen that vitamin D and its metabolites can reduce rectal CCPR in tissue taken from patients with incidental anal conditions (i.e. controls). If a similar reduction in rectal CCPR occurred in FAP, vitamin D analogues (with limited effects on calcium metabolism) could have a potential therapeutic role in such patients to diminish the risk of colorectal cancer.

Epidermal growth factor (EGF) can accelerate epithelial proliferation in the rat colon (Goodlad et al 1987) and stimulate regeneration and ulcer healing in man (Wright Pike and Elia 1990 a, and 1990 b, Wright et al. 1990). The EGF peptide binds to the extracellular domain of its receptor EGFR (Carpenter 1983), and colorectal carcinoma cells may express this receptor (Bradly Weiss and Salem 1986). Since malignant colorectal cell lines can respond to EGF by increasing cell proliferation, this may be an EGF-stimulated autocrine effect (Huang et al. 1991, Anzano et al. 1989).

Proliferating cell nuclear antigen (PCNA) can be labelled with the PC10 antibody in formalin-fixed paraffin embedded tissue (Hall et al. 1990, Sarraf et al. 1991, Scott et al. 1991). This antibody shows a higher labelling index when compared to the exclusive S phase marker BrdU (Sarraf et al. 1991). PCNA gives estimations of the growth fraction similar to previously published figures and appears to be a reproducible and quantifiable marker of cell proliferation (Sarraf et al. 1991).

The aim of this experiment was to determine the effects of 1,25 $(OH)_2$ $D_3$, MC-903 and epidermal growth factor on the rectal CCPR in tissue from FAP patients. In addition, parameters of the cell cycle in FAP were estimated using immunohistochemistry combined with measurements of the mitotic rate and the rectal CCPR determined by organ culture.

## Method.

Clinical material.

Paired rectal biopsies were taken from 19 patients with familial adenomatous polyposis (FAP), who had previously undergone total abdominal colectomy with ileorectal anastomosis and were attending regularly for follow-up. The biopsies were taken between 9.00 and 12.00 hours during attendence at the outpatient clinic. The patients were examined sigmoidoscopically and a rectal biopsy was obtained from an area of macroscopically normal rectal mucosa. One biopsy from each patient was examined histologically to exclude the presence of microadenomas, and sections from this tissue were also used for immunohistochemical analysis. The other biopsy was divided into small pieces and these explants were used in the organ culture studies. The rectal tissue was cultured with EGF (n=8), MC-903 (n=6), and with 1,25 $(OH)_2$ $D_3$ (n=5) .

Chemicals

The active metabolite of vitamin $D_3$, 1,25 $(OH)_2$ $D_3$ in isopropyl alcohol (Roche Products, U.K.) was added to standard organ culture medium to produce a stock solution containing 100 pM 1,25 $(OH)_2$ $D_3$. Similarly, the synthetic vitamin D analogue MC-903 in isopropyl alcohol, (supplied by Leo Pharmaceutical Products, Denmark) was added to

culture medium to produce a solution containing $1x10^{-6}$M MC-903, this solution was stored in the refrigerator at $4^0$C until use. Control medium was prepared with a similar dilution of alcohol.

Mouse salivary gland EGF (Sigma chemicals, U.K.) was added to organ culture medium in produce a stock solution containg 10 ng/ml of the peptide. PC10 antibody was obtained from Professor David Lane, University of Dundee. AP12E, a Rabbit polyclonal antibody directed against a peptide from the cytoplasmic domain of the EGFR, was supplied by Dr W Gullick, R.P.M.S. London.

Organ culture.

Rectal explants were orientated upon metal grids within an organ culture dish as previously described (Experiment 3). The tissue was cultured with or without the addition of either 1,25 $(OH)_2$ $D_3$, MC-903 or EGF as paired studies, each patient's tissue acting as its own control. After 15 hours of culture, vincristine 0.6$\mu$g/ml was added to induce metaphase arrest within the rectal crypts. Explants were removed one, two and three hours later and the crypt cell production rate was determined as previously described. As each patient acted as an internal control, the CCPR values were compared using a paired Student's t-test.

Mitotic index.

Tissue from six patients was immediately fixed in Carnoys fluid and stored in 70% alcohol. Later, the tissue was rehydrated using serial dilutions of alcohol and acid hydrolysed with 1M HCl for 6 minutes at $60^0$ C. After staining with Schiff's reagent, the

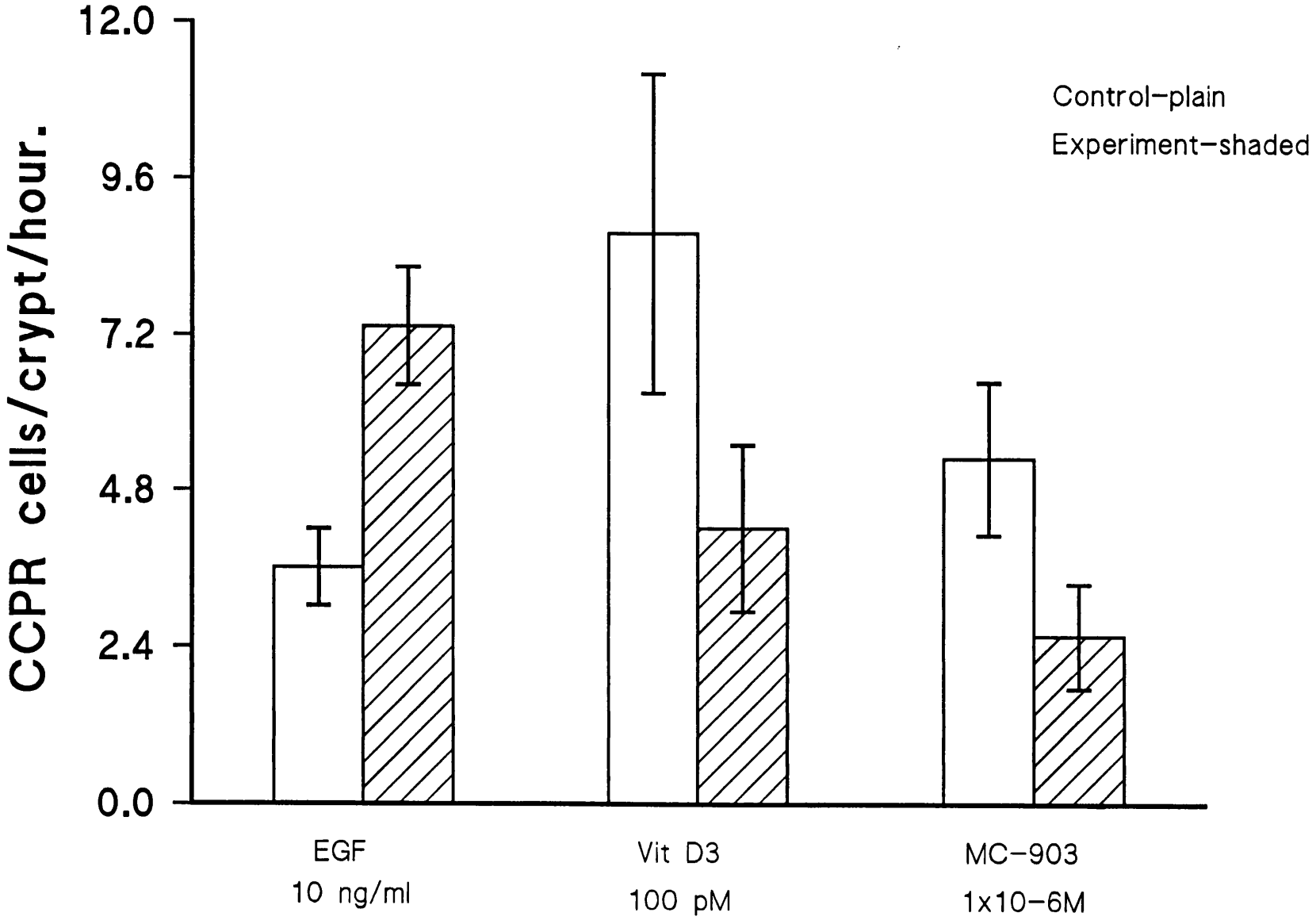
crypts were microdissected and viewed using oil-immersion microscopy. The total number of cells was counted in at least 10 crypts, and the mean number of metaphases was counted in at least 20 crypts in each specimen to determine the mean native mitotic index($I_m$), i.e number of mitoses/1,000 cells.

Immunohistochemistry.

Immunohistochemical staining was performed on 3 $\mu$m thick sections dewaxed in xylene and passed through serial dilutions of alcohol. Endogenous peroxidase activity was blocked with 0.3 % $H_2O_2$ in phosphate-buffered saline (PBS) for 30 minutes. To determine EGF receptor expression, slides were stained with AP12E rabbit polyclonal antibody using an immunoproxidase method. Three sets of slides were the prepared, one test and two controls. To the first slide anti-EGFR rabbit monoclonal antibody (AP12E) was added (170 $\mu$g/ml in a 1:40 dilution); to the second, TRIS buffer was added instead of the primary antibody, and to the third slide the antibody and the free peptide were added (4 $\mu$g/ml anti-EGFR with 10 ng/ml free peptide). The TRIS buffer and free peptide slides were used as negative controls. Slides were left for one hour and then washed three times with TRIS buffer. The second layer consisting of biotinylated swine anti-rabbit IgG (1:300 in TRIS buffer) was applied for one hour. After rinsing the slides three times in TRIS buffer, a third layer consisting of a peroxidase conjugated avidin-biotin complex, (ABC, Dakopatts U.K.) was applied for 45 minutes. The slides were again rinsed three times in TRIS buffer. Lastly, diaminobenzidine solution 250$\mu$g with 0.037% $H_2O_2$ (to demonstrate peroxidase activity) was applied for 5 minute. Slides were washed in tap water and counterstained with haematoxylin (Gullick et al. 1991, Nasim et al 1992).

Figure 1.

The effect of EGF and Vitamin D metabolites on rectal CCPR in FAP.



Tissue from six patients with FAP was routinely fixed in formalin and processed within 24 hours for optimal demonstration of PCNA with PC10 antibody. Sections (3 $\mu$m) were cut, air dried at room temperature, de-waxed and taken through serial solutions of alcohol. Endogenous peroxidase was blocked as described above, and the sections were incubated for 2 hours in a 1:20 dilution of the PC10 monoclonal antibody. The same process was employed without the addition of the primary antibody to provide negative controls and positive controls were sections of human intestine known to stain strongly with the antibody. Visualisation was performed using the three-stage peroxidase method as described above; diaminobenzidine-hydrogen peroxide was employed as the chromagen and Cole's haemotoxylin as the counterstain. The labelling index was determined by the ratio of the number of labelled cells to the total number of cells within a crypt. The mean labelling index was determined from at least ten crypts per section.

## Results

CCPR (Figure 1).

Epidermal growth factor (10 ng/ml) increased rectal CCPR by 102% from $3.62 \pm 0.59$ to $7.33 \pm 0.90$ cells/crypt/hour ($p < 0.01$). The vitamin D metabolite 1,25 $(OH)_2$ $D_3$ (100pM) reduced CCPR by 52% from $8.75 \pm 2.45$ to $4.22 \pm 1.28$ cells/crypt/hour ($p < 0.05$). Likewise, MC-903 ($1x10^{-6}$M) reduced CCPR from $5.29 \pm 1.18$ to $2.56 \pm 0.80$ cells/crypt/hour ($p < 0.05$) (Figure 1).

Mitotic Index

Counting at least ten crypts from six individuals the mean number of cells/crypt was $1034 \pm 48.75$ and the native mitotic index was $10.82 \pm 1.40$ mitoses/1,000 cells. The overall mean CCPR in 21 patients with FAP was $5.96 \pm 0.88$ cells/crypt/hour (see Experiment 2),

which gives a value for the birth rate of cells $K_b$ of 5.76/1,000 cells/hour (i.e. 5.96/1034 x 1,000).

PCNA

Strong staining with the PC10 antibody was seen in histological sections from all of the six patients studied (Figure 2). The zone of proliferation extended from the crypt base to a point just over halfway up the crypt. To calculate the labelling index and hence an approximation to the size of the proliferative compartment, only deeply stained cells were counted. The mean labelling index was $0.48 \pm 0.04$. Using this labelling index as an approximation of the growth fraction ($I_p$), then the cell cycle time ($t_c$) can be calculated from the equation $t_c = I_p/K_b$ (Wright and Alison. 1984), giving a value of 83.3 hours. In addition, the mean duration of mitosis ($t_m$) can be determined from the equation $t_m = I_p/K_b . I_m$ (Wright and Alison. 1984), giving a value for $t_m$ of 0.91 hours or 54.5 minutes.

EGFR

Expression of the EGF receptor was seen in sections from all eight patients who showed an increased CCPR with EGF in organ culture (Figure 3). EGFR expression occurred above the base of the crypt and increased in intensity further up the crypt to well above the mid-zone of the crypt. No expression was demonstrated in any of the negative controls.

## Comment.

The active metabolite of vitamin D (1,25 $(OH)_2$ $D_3$) inhibits *in-vitro* cell proliferation (CCPR) in rectal tissue from patients with FAP. Likewise, the "safer" synthetic analogue

MC-903 produces a similar reduction in rectal CCPR in FAP. If this effect can be reproduced *in-vivo*, then vitamin D analogues might have a therapuetic role in the control of mucosal proliferation in the rectal stump of FAP patients following total colectomy with ileorectal anastomosis. In other conditions that are associated with an increased risk from colorectal cancer, such as ulcerative proctocolitis, there is a well documented increase in colorectal epithelial proliferation (Bleiberg et al. 1970, Eastwood and Trier 1973, Allan Bristol and Williamson 1985, Franklin et al. 1985, Serafini Kirk and Chambers 1981) that persists even in quiescent disease (Allan Bristol and Williamson 1985 Lipkin 1988). A similar dampening of colorectal epithelial proliferation may be beneficial in this inflammatory condition: hence a suppository or enema preparation containing vitamin D analogues might help control epithelial proliferation in patients with this condition.

Epidermal growth factor has an opposite effect to vitamin D in that it increases rectal CCPR in FAP tissue that expresses the EGF receptor (EGFR). This observation correlates well with previously reported effects of EGF on rat colorectum. The effect appears to be a direct action on the tissue, but because both sides of the biopsy are bathed in the culture medium it can not be determined from this experiment whether this is a luminal or humoral action of the peptide. In addition, the dose of epidermal growth factor used was chosen in an arbitrary fashion and may be well above physiological levels.

Tissue sections from FAP patients showed strong staining with the PC10 monoclonal antibody. PCNA labelling may give an estimate for the growth fraction as the index is similar to previously reported figures for the growth fraction. (Sarraf et al. 1991). If the cell birth rate is also known, the two figures can be used to estimate the cell cycle time

(Wright and Alison 1984). Our figure of about 83 hours (3.5 days) is similar to estimates using thymidine labelling which vary from 96 hours (Lipkin Sherlock and Bell 1963) and 90 hours (Bleiberg Mainguet and Galand 1972) for the normal colon and rectum to 32 hours in patients with tubular adenomas (Bleiberg, Mainguet and Galand. 1972) and 24-48 hours in those with colorectal cancer (Lipkin 1971). Direct comparisons are not appropriate since the patients studied in this experiment had all previously undergone total abdominal colectomy. Also, PC10 may overestimate the growth fraction due to its long half life ( Hall et al. 1990, Scott et al. 1991, Sarraf et al. 1991). Nevertheless, it is clear that the combination of immunohistochemistry with static mitotic and dynamic metaphase counting can give useful kinetic information about the gastrointestinal epithelium in disease states.

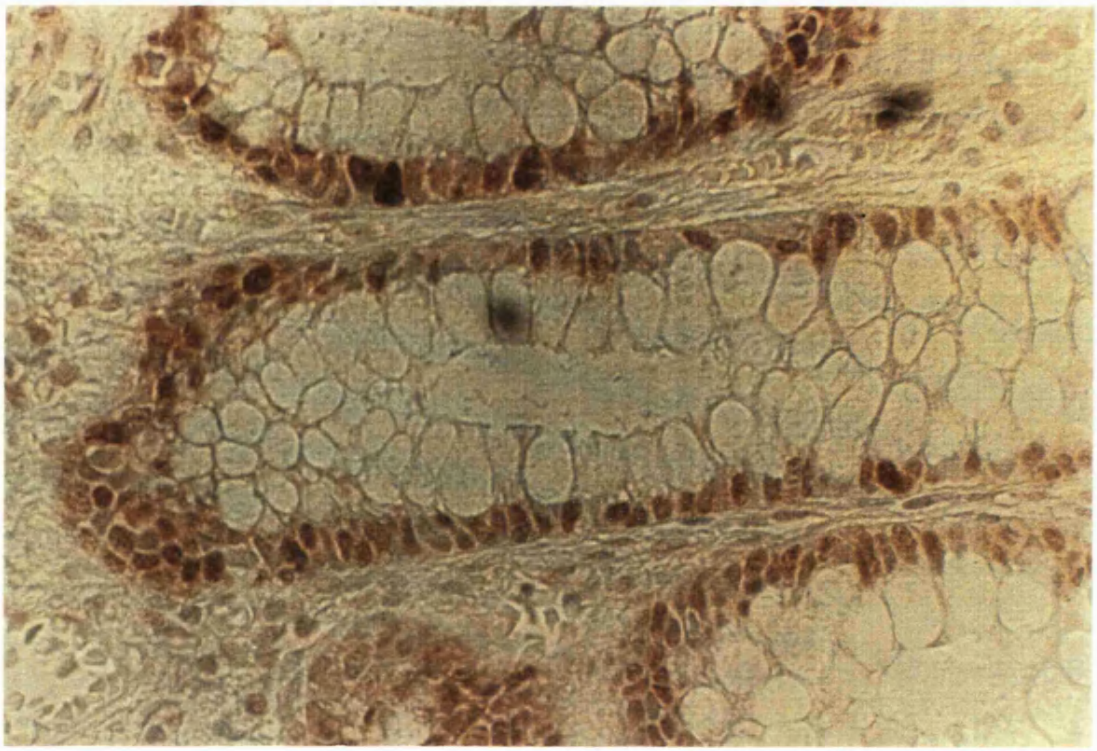


Figure 2. Immunohistochemical localisation of proliferating cells in FAP tissue using PCNA.

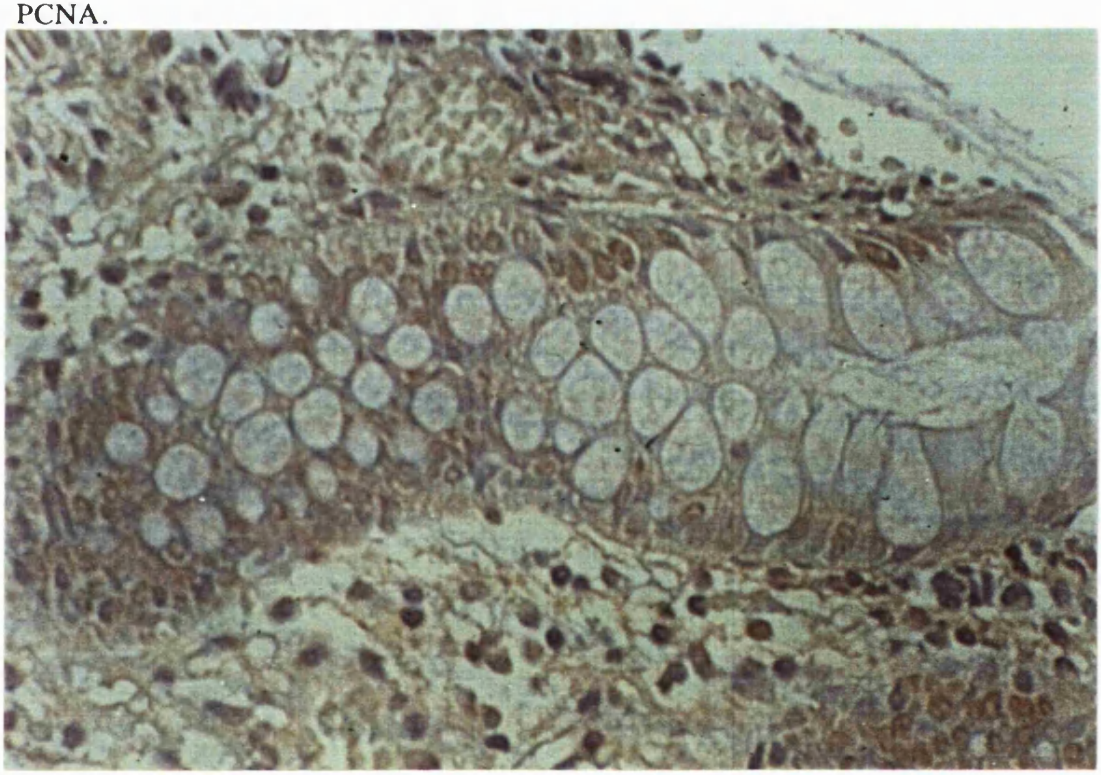


Figure 3. EGFR expression in a rectal crypt in tissue from a patient with FAP.

# Chapter 6.

## General discussion.

Colorectal cancer affects both in individuals recognised as having a high risk of developing the disease and sporadically among those with an apparently "average" chance of developing the disease. Allelic deletion at the FAP locus on the long arm of chromosome 5 appears to cause the phenotypic expression of FAP (Herrera et al. 1986, Bodmer et al. 1987, Leppart et al. 1987). This region may represent a recessive suppressor gene (the apc gene) that requires gene deletion or mutation of both alleles to produce colon cancer (Vogelstein et al. 1988). Adenomatous tissue from patients with FAP however, appears to be a monoclonal proliferation of cells that are not associated with allelic deletions at the FAP focus. It has been suggested that the FAP locus may be responsible for the epithelial hyperplasia that precedes the development of adenomas in patients with polyposis (Vogelstein et al. 1988). The second genetic event responsible for transition from abnormal proliferation to adenoma in FAP does not seem to be the loss of the remaining wild-type FAP gene. Indeed, it may not be one specific event but a series of three or more genetic alterations that accounts for this transition, for example *ras*-gene mutations or allelic losses on chromosome 17 and 18 (Vogelstein et al. 1988). In addition, approximately 20% of patients with non-polyposis colorectal cancer have allelic losses at chromosome 5 (Solomon et al. 1987), and pedigree analysis suggests that an inherited susceptibility to colonic adenomatous polyps and colorectal cancer is responsible for the majority of colonic neoplasms (Cannon-Albright et al. 1988). It therefore seems likely that multiple genetic steps are responsible for the development of colorectal cancer (Vogelstein et al. 1988, Bodmer et al. 1989, Ravikumar et al. 1989), and the pathogenesis is thus

different from the Knudson Two-Hit model of tumorigenesis (Knudson. 1985), which occurs in the development of retinblastoma (Fung et al. 1987, Lee et al. 1987). In addition to genetic factors, environmental factors, in particular diet, have an important role in the development of colorectal cancer (Kune Kune and Watson 1986, 1988, 1989). Genetic factors could be responsible for a person's relative susceptibility to colorectal cancer, and the environmental factors determine which of these susceptible people are affected (Cannon-Albright et al. 1988).

Epidemiology, together with animal and human experimental evidence, suggests that the powerful promoters of colorectal cancers also induce colorectal epithelial hyperplasia. As a corollary, there is good evidence in animals that operative procedures and dietary intervention that can cause a reduction in the large bowel mucosal proliferation rate may protect against chemical carcinogenesis. Epidemiological evidence suggests that the same may hold true in man (Chapter 3).

The aim of this thesis has been to examine the effect of some of these dietary and humoral influences on colorectal epithelial cell proliferation. I have used as my standard cytokinetic parameter the crypt cell production rate (CCPR) as this is generally considered to be a very reliable measurement of cellular proliferation (Chapter 3and 4). The use of an organ culture system to measure CCPR is both an accurate and reproducible method with which to obtain *in-vitro* kinetic information from colorectal tissue (Allan Bristol and Williamson 1985, Appleton et al. 1991). The consistent figures for the CCPR in normal human rectal mucosa together with the low standard errors seen in Experiment 3 tend to support this view. I have extended this technique in Experiment 5, by combining static and

dynamic mitotic counts with immunohistochemistry to increase the amount of kinetic information that can be obtained from FAP tissue. Using this combination I have been able to estimate both the duration of mitosis and the cell cycle time. A similar approach could be used to further investigate the effects of calcium, vitamin D and epidermal growth factor (EGF) on the different phases of the cell cycle and might help to explain their actions.

In Experiment 1, the type of fibre present in the lumen was shown to have a profound effect on colonic mucosal proliferation. The use of a soy-fibre diet, which caused an elevation in total faecal bile acids and secondary bile acids, ameliorated the hypoplasia induced by enteral feeding. The neutral steroid analysis suggested that this effect might be due to an increase in bacterial degradation of bile acids. The consequent colonic acidification would reduce luminal pH, and this could limit further bile acid production by inhibiting 7-alpha dehydroxylation (Thornton 1981). Low pH may have a direct effect on the colorectal mucosa, causing an increase in epithelial cell proliferation (Lupton Coder and Jacobs 1988). Fermentable fibres also increase short chain fatty acid production and these may act as promoters of chemical carcinogenesis (Chapter 3). An unresolved question is whether luminal pH plays a pivotal role in the induction of hyperplasia or whether it is merely a secondary phenomenon.

Calcium has been shown to have both direct and possibly indirect effects on colonic CCPR (Appleton et al. 1991). Calcium lactate might reduce the luminal pH, which could similarly reduce secondary bile acid production, thus dampening proliferation. The more direct effect of low pH postulated by Lupton would be expected to increase CCPR, however (Lupton Coder and Jacobs 1988). In Experiment 2 dietary supplementation with calcium carbonate reduced the rectal CCPR in patients with FAP. This double-blind trial

has confirmed the preliminary findings reported by Lipkin (Lipkin and Newmark 1985), and also supports a recent study of oral supplementation in patients with solitary adenomatous polyps (Barsoum et al. 1992). The mechanism by which calcium reduces colonic CCPR remains unresolved. Calcium may act directly by binding to free bile acids and fatty acids (Newmark Wargovich and Bruce 1984) or by lowering intraluminal pH and thereby reducing secondary bile acid production. Alternatively it may act via the calcium-phosphate complex (Experiment 2). It has also been suggested that calcium has a direct action on the large bowel mucosa (Appleton et al. 1991). To further investigate the actions of calcium would probably require the recruitment of more patients with prolonged ingestion of calcium supplements. A combination of an accurate dietary assessment with frequent sigmoidoscopic examination could help determine if oral calcium supplementation can prevent microadenoma formation in FAP. In addition, faecal steroid analysis similar to that employed in experiment 1, could also aid the interpretation of dietary supplementation trials in man. In 21 patients with FAP the mean rectal CCPR (5.96 cells/crypt/hour, Experiment 2) is slightly higher than the value seen in 17 normal individuals (4.74 cells/crypt/hour Experiment 3). These figures can not be directly compared because all the FAP patients had previously undergone total colectomy with ileorectal anastomosis. Even so, if these figures are compared using non-parametric analysis (the Mann-Whitney U-test) there is no significant difference between them. This failure to show an elevated rectal CCPR in FAP patients may reflect the wide range of values (1.14-19.80 cells/crypt/hour) or alternatively may suggest that a field change does not occur in this disease, however, the growth fraction estimated with PC10 labelling in Experiment 5 suggests that there is an expansion of the growth fraction in FAP. The cell cycle time calculated in Experiment 5

would also tend to support a slight elevation in the proliferation rate of colorectal epithelial cells in FAP, though perhaps not as much as previously suggested (Lipkin 1988).

It is clear from Experiments 3, 4 and 5 that vitamin D metabolites have a reproducible anti-proliferative effect on normal, premalignant and malignant colorectal epithelial cells maintained in culture. The exact mechanism of this effect is not known, and clearly more studies are required in this area. The likely modes of action are an intranuclear receptor-mediated genomic action, or a more rapid non-genomic effect possibly related to calcium transport across the cell membrane. The inhibition of rectal epithelial cell proliferation by the "non-toxic" synthetic vitamin $D_3$ analogues has not previously been reported. If these compounds have the same effect *in-vivo*, then their future use as therapeutic agents may offer an alternative treatment to control epithelial proliferation in the rectal stump of individuals with FAP, or to dampen down cell turnover in ulcerative proctocolitis. In the rat, the effect of novel synthetic vitamin $D_3$ analogues on the development of colorectal cancer could be tested using azoxymethane combined with small bowel resection to induce colonic hyperplasia and promote chemical carcinogenesis in the large intestine.

I have also shown that EGF can increase rectal CCPR in FAP tissue that expresses the EGF receptor. It seems increasingly likely that the rate of cell proliferation in the large intestine of man is dependent on many different humoral and intraluminal factors. The relative contributions of factors that either increase or decrease cellular proliferation may be responsible for the well documented environmental influences on the incidence of colorectal cancer. More detailed analysis of the effect of dietary and therapeutic manipulation may in the future, help to decrease the incidence of colorectal cancer and improve the dismal prognosis currently associated with this disease.

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**Publications related to this thesis.**

1. The inhibitory effect of calcium on colorectal cytokinetics and carcinogenesis. M G Thomas, G V N Appleton, R C N Williamson. Chapter 9 in; Calcium, Vitamin D and Prevention of Colon Cancer. CRC press inc. 1991. Eds: Lipkin, Boon, Kelloff, Malone and Steel.

2. Vitamin D and its metabolites inhibit cell proliferation in human rectal mucosa and a colon cancer cell line. M G Thomas, S Tebbutt, R C N Williamson. Gut. 1992; 33: 1660-1663.

3. Oral calcium inhibits rectal epithelial proliferation in familial adenomatous polyposis. M G Thomas, J P S Thomson, R C N Williamson. Br. J. Surg. 1993; 80: 499-501.

4.The effect of enteral feeding on intestinal cell proliferation and faecal bile acid profiles in the rat. M G Thomas, B Alexander, R Owen, R C N Williamson. J.P.E.N. 1993; in press.

**Abstracts**

1. Vitamin D inhibits cell proliferation in human colorectal mucosa. M G Thomas, R I S Swift, R C N Williamson. Gut. 1990; 31: A1163.

2. The assessment of rectal epithelial proliferation in familial polyposis coli. M G Thomas, R I S Swift Gut. 1990; 31: A1170.

3. Effect of vitamin D *in vitro* on rectal epithelial proliferation in familial polyposis coli. M G Thomas, B Alexander, J P S Thomson, R C N Willliamson. Br. J. Surg. 1991; 78: 757.

4. 1,25 $(OH)_2$ $D_3$ inhibits crypt cell proliferation in human colorectal mucosa. M G Thomas, S Tebbutt, R C N Williamson. In vitamin D, Gene Regulation, Structure-Function, Analysis and Clinical Application. Eds; Norman, Bouillon Thomasset. Walter de Gruyter. Berlin 1991. p453-454.

5. Epidermal growth factor stimulates rectal proliferation in familial adenomatous polyposis. M G Thomas, W J Gullick, S Tebbutt, R C N Williamson. Gut. 1991; 32: A1219.

6.Vitamin D and its analogue MC-903 inhibit cell proliferation in human colorectal tissue. M G Thomas, L Binderup, S Tebbutt, R C N Williamson. Gut. 1991; 32: A1219.

7. Oral calcium inhibits rectal epithelial proliferation in familial adenomatous polyposis. M G Thomas, J P S Thomson, R C N Williamson. Br. J. Surg. 1992; 79: 461.

8. Inhibition of colorectal cell proliferation by vitamin D analogues. M G Thomas, R C N Williamson. Br. J. Surg. 1992; 79: 1233.

**Oral Presentations**

1. Vitamin D inhibits cell proliferation in human colorectal mucosa.
British Society of Gastroenterology, Southampton, September 1990.

2. Vitamin D inhibits epithelial proliferation in Familial polyposis coli.
Surgical Research Society, London, January 1991.

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**Submitted papers**

Divergent effects of epidermal growth factor and calcipotriol on human rectal cell proliferation. M. G. Thomas, G Brown, M R Alison, R C N Williamson. Gut.