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EXPERIMENTAL COLONIC CARCINOGENESIS.

THE INTERACTION OF DIET, BILE ACIDS, COLONIC KALLIKREIN ACTIVITY AND COLONIC PROSTAGLANDIN CONTENT.

A THESIS PRESENTED IN
FULFILMENT OF THE REQUIREMENTS FOR THE
DEGREE OF DOCTOR OF MEDICINE.

BY

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S U M M A R Y

1. Colorectal cancer is currently the fourth most common killing malignancy. There is good epidemiological and experimental evidence that the incidence of this tumour reflects the nature of the diet consumed. Principally, this evidence suggests that a diet which is relatively high in fat or low in dietary fibre content will increase the likelihood of the development of colorectal cancer.

2. Epidemiological and experimental evidence have also demonstrated that the development of colorectal neoplasia is related to the presence of high concentrations of bile acids and their metabolites in the faeces. The concentration of the faecal constituents are dependent on the diet.

3. At the cellular level there are a large number of substances which may control growth and differentation - the autacoids. The kinins and prostaglandins are autacoids which have been shown to have significant effects on the structure and metabolism of the colonic epithelium.

4. There is an established animal model for colonic carcinogenesis whichgives a fair representation of the human condition.

5. This study has examined such an animal model for colorectal carcinogenesis to establish if there is any detectable relationship between the diet, the faecal constituents and the levels of autacoids in the colonic tissue.

6. Furthermore, this study has examined if there are changes in the concentration of autacoids with the development of tumours in the animal model and if the tumour incidence can be altered by pharmacological manipulation of the autacoid activity.

7. 575 male albino Swiss rats were entered into the study and 555 survived until completion. These animals were fed one of three diets which were either a) high fat, low fibre; b) low fat, high fibre or c) their standard laboratory diet from entry into the study until completion.

8. In addition, some animals received the carcinogen azoxymethane by weekly, subcutaneous injection over a period of twelve weeks. Selected animals also received daily injections of aprotinin which is a protease inhibitor blocking the formation of kinins. Other animals received indomethacin continuously in their drinking water to inhibit prostaglandin synthesis. Appropriate control treatments were also administered to other animals.

9. Animals were sacrificed at 4, 16 and 24 weeks after entry into the experiment. Samples of colonic tissue were taken to measure:
a) the tissue kallikrein-like amidase activity which indicates the kinin-forming activity of the tissue.
b) the tissue prostaglandin E_2 content since this is the most abundant prostanoid in the colon.
c) the crypt cell production rate.

10. Samples of all tumours were assessed histologically and samples of the faeces were taken for bile acid analysis.

11. The animals grew and developed normally on all three diets but those receiving the low fat, high fibre diet gained least weight over the experimental period. Administration of the carcinogen was associated with a reduction in overall weight gain but none of the other drug treatments significantly affected the growth of the animals.

12. Tissue kallikrein-like amidase activity was demonstrated to be present in the rat colon in a distinct pattern with the levels in the caecum being three to five times higher than those elsewhere in the colon.

13. The colonic tissue kallikrein-like amidase activity was unaffected by the diet which the animals consumed and by the use of carcinogen.

14. Regular injections of aprotinin resulted in a significant decrease in the colonic tissue kallikrein-like amidase activity but none of the other treatments had a significant effect on this activity.

15. Prostaglandin E_2 was demonstrated to be present in the rat colon in a distinct pattern with the levels in the distal areas of the colon being higher than those in the caecum.

16. The colonic prostaglandin E_2 content was unaffected by the diet which the animals consumed but animals receiving the carcinogen had increased levels of prostaglandin E_2 in the colon.

17. The regular administration of indomethacin resulted in a significant reduction in the colonic content of prostaglandin E_2 but none of the other treatments had a significant effect on the Prostaglandin E_2 content.

18. Only animals which received the carcinogen developed tumours of the colorectum. No relationship was seen between the development of tumours and the levels of either tissue kallikrein-like amidase activity or prostaglandin E_2.

19. Analysis of the crypt cell production rates by a stathmokinetic method failed to demonstrate any effect of diet on the crypt cell turnover. Aprotinin and indomethacin were both associated with reductions in the crypt cell production rate but use of the carcinogen and tumour development was without effect.

20. The faecal bile acids, neutral sterols and free fatty acids were seen to be significantly related to the diet which the animals consumed.

21. Animals on the high fat, low fibre diet had raised levels of secondary bile acids in the faeces, with elevated levels of all other faecal constituents also. Animals on the their standard

laboratory diet were noted to have a relative excess of faecal unsaturated fatty acids.

22. The tumour incidence was higher in the the animals receiving the high fat, low fibre diet compared to those on the low fat, high fibre diet. The tumour incidence of the animals receiving their standard diet was as great as that of animals on the high fat, low fibre diet.

23. Regular injection of aprotinin was found to have a slight protective effect against tumorigenesis in animals on the low fat, high fibre diet. This was not seen in the other dietary groups.

24. Indomethacin was not shown to be protective against tumour development. The animals receiving ethanol as a control medium did not develop any tumours.

25. These results are taken to confirm the central role of the diet in determining the risk for colorectal cancer.

26. The role of the two autacoids examined in this study has not been clearly defined but the evidence obtained could be taken as suggestive of a promotional role for both the kinins and prostanoids in colorectal carcinogenesis since pharmacological manipulation altered tumour incidence in some instances. The use of carcinogen increased colonic prostaglandin E_2 content.

27. Whether the autacoids form a further link in the pathway between diet, bile acids and the colonic cell has not been established.

28. The lack of a consistent response in the crypt cell production rate is consistent with recent work.

29. The most impressive determinant of colorectal cancer risk was the diet. However it is clear from this study that simple consideration of one or two dietary elements is inadequate to explain the alterations in risk.

30. It is proposed that the relationship between risk, dietary fat content and dietary fibre content is non-linear and modified by other factors both endogenous and exogenous.

INTRODUCTION

EPIDEMIOLOGY

Each year throughout the World there are around 5.9 million neoplasms diagnosed. (Muir & Parkin, 1985) About 0.5 million of these are cancers of the colorectum making this, on a world-wide basis, the fourth most common killing malignancy (after carcinoma of the stomach, bronchus and female breast). It would seem that in the aetiology of human cancers, environmental factors play the most important role. This is certainly true for colorectal cancer as epidemiological studies have shown.

In 1971, Burkitt presented work which highlighted the familiar facts that there was both a great range in the incidence of colorectal cancer throughout the world and that it was common in the same geographic areas as certain non-infective bowel diseases such as diverticular disease and haemorrhoids and as certain non-bowel diseases such as ischaemic heart disease and diabetes. Burkitt further noted that the Western diet was high in refined carbohydrate and low in unabsorbable fibre and that those who adopted such a diet had an increased risk of developing colorectal cancer. He postulated that the deficiency in fibre was important as it led to a slow transit time for the faecal stream and allowed more time for the degradation and activation of carcinogens (Burkitt, Walker & Painter 1974)

Contemporaneously Haenszel and Correa (1971) undertook an epidemiological review and demonstrated that the incidence of colorectal cancer in the population of Colombia depended on the social class there being a startling sevenfold variation between

those in the high risk upper class and the low risk lower class. They also demonstrated that the disease behaved differently in the two groups; colonic cancer being more frequent than rectal when the risk was high and the converse true when the risk was low. Additionally, in the high risk group a preponderance of left-sided cancers were seen. In a further paper they (Haenszel, Correa & Cuello, 1975) indicated that those at excess risk were consuming a more "affluent" diet with more meat, eggs and milk.

Since these early studies, repeated observations have re-asserted the role of dietary fat, fibre and protein and other "indicators of affluence" in determining the ultimate risk for colrectal cancer. The precise contribution of each component was unclear and its importance depended to some extent on the standpoint of the writer. (Drasar & Irving, 1973; Berg and Howell 1974; Burkitt, 1975; Hegsted, 1975)) By virtue of large corrlation studies the roles of dietary fat and protein as positive risk factors for colonic cancer became dominant (Armstrong & Doll, 1975) and despite the enthusiasm with which the protective role of dietary fibre was promulgated, the evidence for it was the least rigorous of all. (Stubbs, 1983) Not all the data were consistent. (Enstrom, 1975; Graham, Dayal, Swanson, et al., 1978) Zaridze (1983) expanded on this observing that while geographic pathology had given rise to the theories on large bowel cancer aetiology the subsequent epidemiological studies did not uniformly support the theories. Other components of the diet came in for scrutiny also. The role of alcohol was

questioned when beer drinking and rectal cancer were found to be associated (Breslow & Enstrom, 1974; Enstrom 1977). Jansson (1982) described an apparently protective effect of potassium salts in the diet and the possible protective powers of the antioxidant selenium have been discussed on several occasions. (Shamberger, 1970; Willett & MacMahon, 1984; Salonen, Salonen, Lappetelainen et al., 1985)

Despite the varied opinions, it seems clear from the combined body of work that the risk to an individual of developing colorectal cancer is largely determined by his or her diet and by two components in particular. These observations on dietary fat and dietary fibre have laid the foundations of current thinking on the aetiological mechanisms of this disease.

THE COLONIC MICROENVIRONMENT

Wynder (1975) had come to the conclusion from his earlier epidemiological study that the colonic contents contained tumorigenic components and other factors, probably related to dietary fat, that directly and indirectly affected the composition of the faeces and consequently the tumorigenic components. He suggested that the diet determined the intracolonic contents and the colonic microflora produced metabolites which may have carcinogenic or cocarcinogenic activity (Wynder & Reddy, 1975). This idea was not new.

The carcinogenic potential of metabolites of bile acids and sterols was known. (Lacassagne, Buu-Hoi & Zajdela, 1966) In 1968 Hill and Drasar showed experimentally that, *in vitro*, bacteria could deconjugate bile salts and could perform the dehydroxylation of the bile acid nucleus which converted a primary bile acid into a secondary bile acid. Thus, cholic acid was metabolised to deoxycholate acid and chenodeoxycholic acid to lithocholic acid. Many anaerobes had the capacity to perform this reaction but it became clear that *Bacteroides* and *Clostridia* were the most active species. Some organisms could metabolise bile acids without prior deconjugation. (Aries, Crowther, Drasar et al., 1969a; Aries & Hill, 1969a & b) In short, in the intestinal metabolism of bile salts it was the strict anaerobes which were most important. Examination of the faecal flora of two populations at greatly differing risks of developing colorectal cancer - the English and Ugandans - found that the dominant type

of bacteria in both instances was the non-sporing anaerobe. This organism was present in the English population in thirty times the numbers seen in the Ugandans. Both sets of bacteria were equally active in degrading bile salts but the quantities of bacteria in the English faeces resulted in greater degradation occurring. (Aries, et al., 1969b)

From these observations it was proposed that the intestinal flora and the diet were inter-related and that bacteria could form carcinogens from dietary substrates. Further studies consistently showed that groups at a high risk of developing colorectal cancer had more bacteria capable of metabolizing bile acids. The faeces of these groups contained raised amounts of bile acid and cholesterol metabolites, principally from nuclear-dehydrogenating clostridia. (Hill & Aries, 1971; Hill, Crowther, Drasar et al., 1971; Goddard, Fernandez, West et al., 1975; Reddy & Wynder, 1973; Wynder & Reddy, 1974) Similarly other studies showed that diet had profound effects on the faecal composition of bile acid metabolites. (Mastromarino, Reddy & Wynder, 1976; Nair, Turjman, Goodman et al., 1984; Turjman, Goodman, Jaegar et al., 1984)

While it was generally agreed that diet was a major factor in the aetiology of colonic cancer there was still considerable debate as to which component was responsible - protein, fat, fibre or refined carbohydrate. There was little evidence to suggest a dietary carcinogen but it was feasible for one or more components of the diet to form carcinogens *in vivo*.

(Hill, 1974) Thus, proteins could form phenols, N-nitrosamines or ethionine. Fat metabolism could produce similar compounds. More attractive was the concept that it was the bile acids which were converted into the carcinogen since full aromatization of the bile acid nucleus would form a cyclopentanophenanthrene with carcinogenic potential. This would require only four types of nuclear dehydrogenation which could all be carried out by gut bacteria - almost exclusively *Cl. paraputrificum.* It was further suggested that the gut bacteria may affect the enterohepatic circulation of carcinogens, hepatic detoxification or immune defence mechanisms. Thus the dietary fat could control both the colonic flora and the flow of substrate to it. An examination of the bile acids and flora in patients with large bowel cancer showed them to have the expected high levels of faecal bile acids and also to have a high proportion of *Cl. paraputrificum* present capable of performing the nuclear dehydrogenation required. (Hill, Drasar, Williams et al., 1975)

Several other experimental studies gave further support to the relationship of dietary fat, bile acids, colonic bacteria and colonic cancer. (Antonis & Bersohn, 1962; Aries, Crowther, Drasar et al., 1971; Crowther, Drasar, Hill et al., 1976) These studies demonstrated in various ways that consuming higher amounts of dietary fat resulted in higher amounts of secondary bile acids in the faeces due to altered microflora activity. Faecal fatty acids were also increased (Cummings, Wiggins, Jenkins et al., 1978) but interestingly the faecal fatty acids

were not those of the diet. Meat protein was not a major determinant of faecal bile acid excretion. (Cummings, Hill, Jivraj et al., 1979; Cummings, Hill, Bone et al., 1979)

Not all evidence was clear cut. A study in the West of Scotland - an area of exceptionally high incidence of colonic cancer - showed patients with colorectal cancer to have a lower concentration of faecal bile acids but a higher concentration of nuclear-dehydrogenating clostridia. (Murray, Blackwood, Trotter, et al., 1980) It was suggested that the very high levels of indigenous bacterial activity may have led to very extensive metabolism of the bile acids to a paradoxically low level. A similar study at the same time (Mudd, McKelvey, Norwood, et al., 1980) could find no difference in faecal bile acid concentrations between patients with large bowel cancer and those with a variety of conditions which exposed them to an increased risk of carcinoma. A more recent study from Japan (Hikasa, Tanida, Ohno et al., 1984) compared the faecal bile acid profiles in 14 patients with colorectal cancer and 14 age-sex matched controls. They could not demonstrate any significant alteration in the faecal bile acid profiles between patients and control. However they did demonstrate the excretion of a unique product - 5B-chol-3-en-24-oic acid - by a high proportion of the patients in amounts. The significance of this was not clear.

While the inter-relationship of dietary fat and bile acids with colonic cancer was becoming clearer, the interaction of dietary fibre remained ambiguous. Bran could apparently alter

the composition of bile acids in bile by influencing the bacterial metabolism or colonic environment. (Pomare and Heaton, 1973) However, dietary fibre did not appear to modify the faecal microflora to any detectable extent. (Drasar & Jenkins, 1976; Drasar, Jenkins & Cummings, 1976; Fuchs, Dorfman & Floch, 1976)

The addition of bran to the diet had a variable effect on the daily excretion of sterols and bile acids but their concentration in the faeces seemed to be universally reduced. (Stanley, Paul, Gacke et al., 1973; Hill, 1975; Cummings, Hill, Jenkins et al., 1976 & Cummings, Branch, Jenkins et al., 1978) These effects were dependent on the type of fibre - bran reduced the faecal concentrations of acid and neutral sterols but bagasse increased the excretion of acid sterols only without affecting the concentrations of acid or neutral sterols. (Walters, Baird, Davies et al., 1975) Large concentrations of bran may bind steroids leading to an increased faecal concentration and increased excretion. This is unusual but is seen with agents such as pectin and guar where the diluting effect of increased bulk is offset by a 'washout' effect leading to increased faecal excretion and concentration. A direct study of the binding capacity of bile acids and bile salts by several types of fibre showed that there were considerable variations between the different fibre types and bran had only a third of the binding capacity of lignin. (Story & Kritchevsky, 1976). The mechanism of binding was seen to be complex and varied depending on the type of fibre. The transit time increases with the increased

faecal bulk but this does not affect the amount of bacterial metabolite formed. (Hill, 1978) The precise effect of fibre depends on each of its components and the amounts present. Fibre may influence bacterial enzyme activity by modifying the flora itself or by altering the milieu e.g. lowering the pH of the faeces. (Hill, 1982).

The bulk of the evidence supports the hypothesis laid down by Wynder and by Hill. Thompson (1982) summarised the situation : there is a positive correlation between the incidence of colon cancer and the consumption of animal protein and fat and an inverse relationship with dietary fibre. There is also a positive correlation with faecal bile acid concentration and the concentration of nuclear dehydrogenating clostridia in the faeces. Increasing the consumption of fat and protein leads to an increase in the faecal bile acids; fibre has a variable effect depending on the source. All of the dietary components are interlinked - altering one invariably alters another.

Using this evidence Hill, Morson and Bussey (1978) proposed that "Adenomas are initially caused by an environmental agent which acts on the colorectal cells of adenoma prone persons, a further agent causes small adenomas to grow and a carcinogen causes malignancy in a high proportion of large adenomas". It was well- recognised that the malignant potential of large adenomas was greater than small ones but was equally great whether the individual was at high or low risk of developing colorectal cancer. The incidence and subsite

distribution of large adenomas follows that of carcinoma and it followed that the factor determining carcinoma incidence also determined growth of adenomas. They proposed that this factor was a bacterial metabolite of bile acids. A study of the intracolonic environment and the presence of colonic adenomas in man (Van der Werf, Nagengast, Van Berge Henegouwen et al. 1983) has shown that patients with adenomas have increased bile acid turnover and increased absorption of deoxycholic acid indicating increased exposure of the colonic mucosa to secondary bile acids. Additionally these patients have a higher proportion of anaerobic bacteria present and the combination of these two observations favours a link between a colonic environment well-suited to the growth of anaerobic bacteria and exposure of the mucosa to tumour-promoting bile acids.

EXPERIMENTAL COLORECTAL CARCINOGENESIS

Within the rodent kingdom the spontaneous development of tumours is uncommon and especially so for colonic lesions. Miwa, Takenaka, Ito et al.(1976) describe a spontaneous occurrence of colonic tumours (not necessarily carcinomas) of less than 1%. The discovery and use of the hydrazine derivatives of cycasin to induce tumour formation has passed into laboratory folklore and been adequately reviewed elsewhere. (LaMont and O'Gorman, 1978) The animal models for colonic carcinogenesis based on these agents have been utilised extensively in the laboratory and have confirmed much of the epidemiological studies described above.

Studies using other carcinogens have also been described with interesting results. Early experiments demonstrated the similarity of induced colon tumours in rats to human lesions. (Spjut & Noall, 1971) The selectivity of some carcinogens for the colon was demonstrated in transposition experiments (Gennaro, Villanueva, Sukonthaman et al., 1973) and the necessity of a faecal stream for tumours to develop was also rapidly appreciated. (Spjut & Noall, 1971) Direct instillation of the powerful mutagen N-Methyl-N'-Nitro-N-Nitrosoguanidine (MNNG) into the rectum also resulted in tumour formation wwhich was augmented by the concurrent use of a secondary bile acid whether free or conjugated. (So, Magadia & Wynder, 1973; Narisawa, Magadia, Weisburger et al., 1974)

While assessing the experimental potential of dimethylhydrazine and azoxymethane, Nigro, Bhadrachari & Chomchai

(1973) found that administering cholestyramine resulted in an increased yield of colonic tumours presumably due to the increased local concentration of bile acids. They gave their work further support in a later study (Chomchai, Bhadrachari & Nigro, 1974) in which they demonstrated that reimplanting the bile duct to the mid small bowel also resulted in a greatly increased number of colonic tumours developing for the same reason.

Dietary fat and bile acids

Reddy, Weisburger & Wynder (1974) used a rat model with 1,2-dimethylhydrazine (DMH) as the carcinogen to examine the effects of dietary fat on faecal sterol excretion and carcinogenesis. They found that the faecal excretion of neutral sterols and bile acids increased when the animals were given a high (20%) fat diet. Tumour induction was greatest in animals on the high fat diet. DMH caused a rise in the excretion of bile acids and neutral sterols in all groups regardless of diet. Paradoxically, animals on their standard laboratory diet (Purina lab chow) had the highest levels of neutral sterol and bile acid excretion but a level of tumour induction which was the lowest of any the groups. There was no obvious explanation for this. Subsequently, dietary fat from various sources has been universally shown to increase the yield of tumours regardless of the carcinogen used. (Nigro, Singh, Campbell et al., 1975; Reddy, Narisawa & Weisburger, 1976; Reddy, Narisawa, Vukusich et al., 1976; Reddy, Watanabe & Weisburger, 1977)

Reddy, Narisawa, Weisburger and Wynder (1976) used MNNG

in germ-free rats to examine the role of deoxycholic acid in tumour formation. While the total number of tumours induced was about the same, the animals which received deoxycholic acid after MNNG administration developed more adenocarcinomas than the group receiving MNNG alone. Those receiving deoxycholic acid alone did not develop tumours and it was deduced that deoxycholic acid was behaving as a promoter of carcinogenesis in this model. The primary bile acids cholic and chenodeoxycholic acid, and another secondary bile acid, lithocholic acid, were similarly shown to promote MNNG-carcinogenesis. (Reddy, Watanabe, Weisburger et al., 1977; Reddy and Watanabe, 1979) Another group (Deschner, Cohen & Raicht, 1981) examined the effect of giving cholic acid with the diet on N-methyl-N-Nitrosourea (MNNU)-induced rectal carcinogenesis. This also increased the number of tumours developing.

In an involved series of experiments the possibility that dietary fat acted as a promoter was suggested. (Bull, Soullier, Wilson, et al., 1979). Their studies using the azoxymethane (AOM)-rat model allowed the animals to have either a high or low fat diet for varying times before, during and after induction of carcinogenesis. Animals on the high fat diet developed more tumours than those on the low fat diet. The highest rate of tumour induction was seen when the high fat diet was given for four or more weeks after the carcinogen administration was complete. Thus the high fat diet exhibited most of the features of a promoter - it was not carcinogenic by itself; it increased

tumour incidence after AOM administration; delaying administration produced the same effect. As well as confirming the role of the high fat diet this study gave some substance to the notion that colonic carcinogenesis was at least a two-step process. A later study by Reddy and Maruyama (1986) supported the role of dietary fat as a promoting substance.

Several studies have consistently shown that unsaturated fats in the diet are more efficatious as tumour promoters. Reddy and Maeura (1984) found that colonic tumorigenesis depended crucially on the type of fat and that polyunsaturated fats increased faecal bile acid concentrations. Broitman and coworkers (Broitman, Vitale, Vavrousek-Jakuba et al., 1977) linked their observations on tumour development to studies on the immune system and showed depressed immune reactivity associated with the ingestion of unsaturated fats. Sakaguchi and coworkers (Sakaguchi, Hiramatsu, Takata et al., 1984) described clear changes in cell membrane phospholipids associated with unsaturated fat consumption as well as increased tumour formation. They later confirmed that unsaturated fats increased faecal bile concentrations more than saturated fats (Sakaguchi, Minoura, Hiramatsu, et al., 1986).

Not all studies concurred. An exacting study of the effect of alterations in the quality and quantity of dietary fat (Nauss, Locniskar & Newberne, 1983) could find no difference between the number, site or invasiveness of the tumours developed in animals on either a 5% mixed fat diet, 24% beef fat diet, a

24% corn oil diet or a 24% Crisco diet (partially hydrogenated fat). These workers expressed reservations about previous studies which had all been based on unbalanced diets. Interestingly, they administered the carcinogen (DMH) by gavage whereas it was injected in all other studies, a point which they subsequently examined and showed to be of no importance. (Locniskar, Nauss, Kaufmann et al., 1985)

Dietary fibre

While the effects of dietary fat on experimental colonic carcinogenesis seemed to be largely consistent with the epidemiological hypotheses the effect of fibre was somewhat less predictable. Two studies on DMH-induced colon cancer (Fleiszer, MacFarlane, Murray et al., 1978; Freeman, Spiller & Kim, 1978) suggested that fibre protected against colonic cancer development. Indeed there seemed to be a "graded" response with the tumour incidence increasing as the fibre content decreased. A similar protective effect of wheat bran was seen by Wilson, Hutcheson and Wideman (1977) who found that neither the type nor quantity of dietary fat influenced tumour development. The protective effect of dietary fibre was confirmed by Reddy, Mori & Nicolas (1981) who observed a significant decrease in the number of animals developing tumours when diets with wheat bran and citrus fibre supplements were used. There was no difference between the two fibre groups.

In contrast, one study (Bauer, Asp, Dahlqvist, et al., 1981) did not demonstrate any protective effect of pectin, guar

gum or bran in DMH-induced carcinogenesis. Jacobs (1983 & 1984) found that the effect of fibre depended on the time of administration. His experiments, although small in numbers, suggested that feeding a 20% wheatbran supplement to rats during carcinogen (DMH) exposure resulted in a marked increase in the number of tumours developed. In contrast, feeding bran after carcinogen exposure led to a significant reduction in the yield of tumours. It was suggested that there was an increase in the proliferative activity of the colon with the bran diet which had an additive effect with the hyperplasia of carcinogen administration.

Barnes, Clapp, Scott et al (1983) showed that the effect of bran depended on the source with wheat bran being protective against DMH-induced colonic tumours but rice and soybean bran being ineffective. Corn bran appeared to potentiate the effect of the carcinogen. The protective effect of wheat bran was only apparent if given before or shortly after tumour initiation.

Trudel, Senterman & Brown (1983) examined the effect of fat and fibre simultaneously using equicaloric pair-feeding of animals. The usual lab diet was supplemented with cellulose, safflower oil or lard. The fats increased the tumour incidence (the fat type was immaterial) and cellulose "..counteracted the deleterious effect of fat."

The protective effect of wheat bran was shown to be independent of faecal bile acid concentration by Calvert, Klurfield, Subramaniam et al., (1987). These authors argued that

fibre did not work by "dilution" of bile acids but perhaps by another mechanism such as altered transit time, altered mucins or a bulking effect. Agents causing faecal dilution had already been shown not to be protective. (Karlin, O'Donnell & Jensen, 1980)

Some studies have examined the effects of fibre on the colonic microenvironment. Dietary fibre (bran) does not lead to any change in the total faecal bile acids (Brydon, Tadesse & Eastwood, 1980) but animals on a low fibre diet have higher levels of cholic, chenodeoxycholic and muricholic acid. The total bile acid pool does not alter and the main effect of bran seems to be to alter the physical distribution of bile acids along the intestine since the levels of bile acids in the small intestine rise. (ibid). Shiau and Chang (1983) showed that a variety of fibres - pectin, guar gum, carrageenan and cellulose - would reduce the activity of colonic mucinase and B-glucuronidase which may explain their protective effect. (Goldin & Gorbach, 1976)

Galloway and coworkers (Galloway, Owen, Jarrett et al., 1986) showed that tumour induction with azoxymethane was profoundly influenced by the dietary content of fat and fibre. Most tumours developed when the animals received a diet high in fat and low in fibre. The least number of tumours were seen when the diet was low in fat and high in fibre. Altering this to a diet which was high in fat and high in fibre did not alter tumour incidence. This, taken with the faecal bile acid profiles for each of the dietary groups, suggested that the fibre content of

the diet was of primary importance.

Biliary secretion

The role of biliary secretion has been examined by diversion of the biliary secretions to the mid small bowel via a transposed Vaterian segment of duodenum. (Williamson, Bauer, Ross, et al., 1979) This resulted in an increased yield of colonic tumours no matter whether the diversion was done before or after the administration of carcinogen. Additionally, after diversion, most tumours occurred in the caecum and metastases were more frequent. Oddly, there was no significant change in the level of faecal bile acids. The diversion led to colonic hyperplasia which may have been responsible for the increase in tumour induction but similar hyperplasia occurred in the ileum which did not have an increased tumour incidence.

The role of bile in carcinogenesis has been examined also by establishing a segment of the colon as an isolated loop (Thiry-Vella fistula) in animals receiving a carcinogen. (Rainey, Davies, Bristol et al., 1983) Tumour induction in such a fistula was generally reduced but was increased when the fistula was irrigated with a solution of faeces. Irrigation with saline or sodium deoxycholate solution was without effect. In this instance deoxycholic acid was not acting as a cocarcinogen but there is little doubt that the TVF microflora must be altered as there is no faecal stream present.

Williamson and Rainey (1984) also showed that proximal and distal enterectomy would increase colonic tumorigenesis with

increased cell turnover. Partial colectomy did not greatly affect tumour development but tumours did cluster at sites of anastamosis. Defunctioned colon (as in the Thiry-Vella Fistula) had a reduced tumour development.

The effect of small bowel transection, 50% jejunoileal resection or bypass was assessed by Scudamore and Freeman (1983) on small and large bowel tumours. They found that the total number of tumours increased in the operated animals as did the number of tumours per animal. This was most apparent in the group undergoing 50% jejunoileal bypass. Colonic tumours tended to occur more distally. The observed effects may relate to hyperplasia or increased exposure to pancreaticobiliary secretions. Other workers also found that resection of the terminal ileum enhanced DMH tumorigenesis and increased the total faecal bile acids. They also noted that tumours occurred mostly in the distal colon. (Koga, Kaibara & Takeda, 1982)

This has been contradicted by a more recent study which showed that small bowel resection resulted in a fall in the faecal bile acids with promotion of tumour development. (Savage, Sian, Matthews, et al., 1988)

The bile acid pattern in a strain of rat with a high incidence of spontaneous colonic cancer has been desribed. (Hayashi, Amuro, Endo et al., 1986) Animals with colonic tumours were found to have a significantly raised faecal concentration of deoxycholic acid when compared to animals without tumours. This was accompanied by a generalised reduction in the other bile

acids measured such that the total faecal bile acids were no different between the tumour-bearing and tumour-free groups.

Microscopic Detail of Experimental Tumour Development.

One particularly useful facet of the animal model is that it allows the study of the development of tumours and has made possible the study of the mucosal changes associated with carcinogenesis.

The gross and microscopic features of human colorectal carcinoma are well recognised and are extensively described in standard textbooks. The relationship between the colonic adenoma and carcinoma has presented an enigma which is of some importance clinically and is central to consideration of the role of bile acids and bacteria in colonic carcinogenesis. (page 9)

It is established that colonic adenomas have a relationship in time and distribution to colonic carcinomas. (Grundmann, 1985) There are also certain polyposis syndromes which are associated with the premature development of cancer. (Neel, 1971; Bulow, 1984) In an extensive review Enterline (1976) submitted that, if the true villous adenoma is ignored since it has never been part of the issue, then there is good evidence that adenomas mirror the subsite distribution of colonic carcinoma and that they exhibit a range of changes from focal atypia to frank carcinoma. The minute carcinoma is rarely, if ever, discovered independent of an adenoma. In addition the peak incidence of adenoma predates that of carcinoma by about five years which would be a feasible "latent" period for the

development of clinical carcinoma. Morson (1984) and Day (1984) also support this view.

The changes in the mucosa around a neoplasm have received some attention since they may give some insight into the precursor changes in the development of carcinoma. Histochemical studies have clearly shown changes in the type of mucin production in areas which are neoplastic. Essentially this comprises a relative increase in the amount of sialomucin produced. Moreover, this type of change can be demonstrated in what is histologically normal colonic mucosa. (Filipe & Branfoot, 1974 & 1976; Jass, Strudley & Faludy, 1984) Ultrastructural examination of the mucosa adjacent to the carcinoma has shown that marked abnormalities of structure and organisation exist in areas of apparently normal epithelium. (Riddel & Levin, 1977; Traynor, Costa, Blumgart, et al. 1981)

In all studies which have examined the pathological anatomy and histology of experimental tumours it seems clear that the animal tumours closely reflect the human situation. (e.g. Ward, Yamamoto & Brown, 1973; Nigro, Sardesai, Chomchai et al., 1973; Reddy, Narisawa, Weisburger et al., 1976). A detailed comparison between human tumours and those induced experimentally in animals using DMH, AOM and methylazoxymethanol (MAM) has shown similarities in the general morphology, patterns of invasion, association with adenomas and the greater invasiveness of mucinous tumours. Distinct differences also exist in the experimental situation such as the occurrence of multiple

tumours, an association with Peyer's patches, a lesser invasiveness of non-mucinous tumours and the development of extra-colonic tumours. Nonetheless "..the comparison is dominated by a striking overall histological resemblance between the two groups." (Ross, 1982)

Cell Kinetics and Neoplastic Change.

The organised structure of the colonic epithelium and the rapid turnover of the cells has lent itself to extensive studies of the cell kinetics. It is well established that the colonic epithelium derives from the proliferative zone occupying the lower one third of the colonic crypt in man (Lipkin, 1971) and rodents (Wright, 1978). From here the cells migrate and differentiate to become mature, non-dividing enterocytes on the epithelial surface. Cell kinetic studies using thymidine labelling and stathmokinetic arrest has shown that the cell turnover exhibits a diurnal rhythm and varies along the length of the colon. Studies on the proliferation of normal and neoplastic cells in human colon (Lipkin, 1971) have suggested that carcinoma cells proliferate at rates equal to or slower than normal cells. The histological changes occurring in the animal mucosal structure have been examined by several authors. (Deschner, 1974; Lipkin, 1974; Maskens & Deschner, 1977; Pozharisski, 1975; Barkla & Tutton, 1977; Shamsuddin & Trump, 1981; Shamsuddin, 1982; Kikkawa & Sasai, 1982; Pan, Hamilton, Hyland et al., 1985) These detailed studies have demonstrated in animals (mice and rats) and in some cases, in man, a clear set of events occurring in the

colonic mucosa prior to tumour development. Essentially there is a shift of the DNA synthetic cells to occupy the whole colonic crypt and the repopulation of the crypt and mucosa by actively dividing cells. Various categories of light and electron microscopic changes have been depicted which all appear to be related to abnormal epithelial maturation and division. Such changes have also been seen adjacent to human carcinomas. However, none of the studies in experimental animals have demonstrated an adenoma-carcinoma sequence.

In contrast, a study using the scanning electron microscope (Cooke, Kirkham, Stainthorp, et al., 1984) has shown the development of lesions around the mouth of the colonic crypts which the authors refer to as "microadenomas" and were found as early as five weeks after starting carcinogen treatment. They take this as being supportive evidence for an adenoma-carcinoma sequence.

In an experimental study with a literature review Maskens and Dujardin-Loits (1981) concluded that experimental tumours in rats arose de novo without a prior adenoma and that adenomas behaved as distinct entities. However they did note that the mouse model was more akin to the human situation since a range of lesions are seen. The changes in the epithelium of the mouse colon have been described extensively by Chang (1984) who found lesions to arise both de novo and from within adenomas.

The effect of carcinogen administration on the colonic epithelial cell kinetics has been described. (Wright, 1983)

Initially, there was inhibition of DNA synthesis with necrosis of the crypt cells in the proliferative region. A compensatory proliferative response followed leading to crypt hyperplasia and by one week normality returned. Chronic administration of a carcinogen led to a variety of hyperplastic changes. The labelling index increased associated with an expansion of the proliferative zone to the top of the crypt. The growth fraction in the hyperplastic crypts fell as did the colonic cell cycle time. The overall kinetics remained about the same. The cell birth rate in the proliferative zone fell but the cell cycle time tended to remain constant in this zone and the resultant fall in the growth fraction was due to a falling growth fraction within the proliferative compartment.

Deschner (1982) described a similar series of changes and divided them into three stages. She and Wright agree that such hyperplastic crypts have a selective advantage over others in allowing them to express neoplastic transformation and Wright proposes that a slowly cycling transformed cell evolves which gives rise to subsequent tumours.

Tutton and Barkla (1976, 1980a, 1981, 1982) have examined the rat - DMH model kinetics extensively by stathmokinetic methodology. They showed that treating rats with DMH led to marked enlargement of the crypt cell circumference and elongation of the proliferative zone at the base of the crypt. (1976) They went on to show that there was evidence of autonomic control over the proliferation. (1980a) In 1981 they demonstrated that

adrenalectomy almost abolished cell division in tumours and that it was restored by hydrocortisone but normal cell division seemed to be unaffected. They demonstrated a similar dependency of tumour division on androgens (1982) and other workers had shown the presence of androgen receptors in DMH-induced tumours. (Mehta, Fricks & Moon, 1980)

Deschner, Cohen and Raicht (1981) showed that feeding cholic acid to rats in their diet significantly increased the thymidine labelling of the crypts and subsequently to enlargement of the proliferative zone and elongation of the crypt. These effects were are due to deoxycholic acid since cholic acid is metabolised to deoxycholic acid in the colon.

The evidence to date reinforces the concept that the experimental animal model of tumour development is broadly similar to the human situation and is under similar control. The question of the adenoma-carcinoma sequence has not been fully resolved but there is a striking lack of supportive evidence for it in the animal model.

<u>Other features of the experimental model.</u>

The rat model has been explored in more diverse ways. Two observations are of interest.

In 1983 Summerton, Flynn, Cooke & Taylor demonstrated the presence of specific deoxycholic acid receptors in a high percentage of human colonic tumours. This was subsequently demonstrated to be true in the animal model (Summerton, Goeting, Trotter, et al., 1985) and it was shown at the same time that

tumour incidence increased by administering rectal deoxycholic acid with parenteral carcinogen. The authors speculated that the receptor had a role in promotion - in particular the change from adenoma to carcinoma.

Barthold and Jonas (1977) exploited the susceptibility of the mouse to a specific variant of *Citrobacter freundi* which causes severe, transient hyperplasia of the distal colon. Administration of this agent led to a decrease in the latent period to the development of tumours. There was no effect on established lesions.

As regards the relationship of fat, fibre, bile acids and tumour development an underlying problem has always been the mechanism involved. If the dietary contents or bile acids do not directly act on the colonic mucosal cell then by what means do they influence it? In partial answer to this it is worthwhile considering two systems - the kallikrein-kinin system and the prostaglandins. Both of these systems have been shown to have a role in cellular communication and to be involved with cellular proliferation and the development of the neoplastic state.

THE KALLIKREIN-KININ SYSTEM

The kallikreins (EC 3.4.21.8) can be defined as those serine proteases which liberate kinins from kininogen by limited proteolysis and which have little or no proteolytic activity on other proteins. (Pisano, 1975) All mammals studied to date have two kallikreins - glandular (or tissue) and plasma - which differ in physicochemical properties, rates of reaction with kininogens and synthetic substrates, the kinins produced and the response to a variety of natural and synthetic inhibitors.

The discovery and characterisation of kallikrein and its product bradykinin have become part of pharmacological history and need not be reviewed here as they are covered comprehensively elsewhere. (Werle, 1970; Elliot, 1970; Schachter,1980; Fritz, 1983)

The serine proteases are a broad group of enzymes which are active at neutral pH and require no cofactors (Barrett, 1980). The group includes enzymes such as trypsin, chymotrypsin, elastase, thrombin and plasmin. (Schachter, 1980) Not all serine proteases are capable of cleaving kininogen - the substrate of kallikrein - to form a kinin but kallikrein, trypsin and plasmin may do so. Kallikreins are unique in that they show virtually no proteolytic activity on any other protein.

Glandular or tissue kallikrein is an heterogeneous group of acidic glycoproteins of MW 24000 - 43600. (Pisano, 1975) It acts on two substrates - High MW Kininogen and Low MW Kininogen - to form kallidin (lys-bradykinin). Plasma kallikrein is a basic

glycoprotein of MW 100000 acting only on High MW kininogen to form bradykinin. Plasma kallikrein exists as an inactive precursor which is activated by limited proteolysis by activated Hageman factor or by trypsin. (Fritz, 1983)

Bradykinin is a nonapeptide of short half-life which is rapidly inactivated by kininases which are widely distributed. (Erdos and Yan, 1970) It has been shown to have many properties;

- vasodilatation (Elliot, 1970 , Haddy, Emerson, Scott, et al., 1970)
- contraction of the longitudinal muscle of the jejunum, ileum and taenia coli; relaxation of the circular muscle of the ileum and colon (Fishlock, 1968)
- vasodilatation of the stomach and gut with an increase in gastric secretion due transudation of extracellular fluid. (Jacobsson, 1970)
- renal arteriolar dilatation and natriuresis (Jacobsson, 1970)
- increased salivary secretion and bronchoconstriction. (Pisano, 1975)
- stimulation of cell proliferation in bone marrow, epithelial basal cells of tongue and in the gastrointestinal tract (Pisano, 1975).

Much work has been done on the distribution and localisation of kallikrein within the tissues. Kinin-forming activity was shown in all areas of the gastrointestinal tract by

Amundsen and Nustad (1965) although this was not specifically kallikrein activity. Interestingly they demonstrated the presence of kininase activity in all the tissues they examined which included ones without kinin-forming activity. The kinin-forming activity was thought to reside in cellular granules and was further demonstrated in salivary glands, pancreas and urine (Webster, 1970). Orstavik (1978 & 1983) showed the presence of kallikrein in rat submandibular and sublingual glands and pancreas. He subsequently demonstrated that it resided in the secretory granules of the tubular cells and the pancreatic acinar cells. This was subsequently shown to be true in the pig and in human pancreas. (Amouric, De La Porte, Colomb et al., 1983). In the rat kidney glandular kallikrein was found in the distal tubular cells (Nustad, Orstavik, Gautvik, et al., 1978). Immunofluorescent localisation of glandular kallikrein (Fink, Dietl, Seifert, et al., 1979) failed to show any definite localisation in the kidney although kallikrein was present in blood and excreted by the kidney. Human tissue kallikreins have been isolated from duodenal juice, urine, large intestine, blood plasma and seminal plasma and have been shown to be identical with each other and similar to porcine pancreatic kallikrein. (Geiger, Hofman, Franke, et al., 1983)

Following the work of Amundsen & Nustad, Zeitlin (1971) investigated the nature of the kinin-forming activity of rat intestinal mucosa and showed that the kinin-forming enzyme was distinct from trypsin, plasmin, plasma kallikrein and rat

pancreatic kallikrein. He also showed that the activity was almost completely inhibited by the proteinase inhibitor aprotinin, slightly inhibited by soybean trypsin inhibitor (unlike plasma kallikrein which was completely inhibited) and was unaffected by a specific trypsin inhibitor. Other workers showed that normal human colon tissue and that from colon adenocarcinoma contained kininogenase activity which existed at least in part as a pre-kallikrein. (Seki, Nakajima & Erdos, 1972).

The plasma and tissue enzymes were later shown to be distinct entities with the tissue enzyme having a molecular weight (MW) of 33000 and the plasma enzyme a MW of 61500 and 125000. (Zeitlin, Singh, Lembeck, et al., 1976)

Studies in the human (Zeitlin & Smith, 1973) showed that in the normal human colon kallikrein activity resided in the mucosa while the kininogen resided in the muscular layers of the bowel wall. Colonic tissue involved by diverticular disease was indistinguishable from normal but tissue from patients with ulcerative colitis had high levels of kallikrein activity in the the colonic muscle while the mucosal levels were relatively unchanged. Intervening, unaffected tissue was normal and the change was thought to be due to an increase in permeability of the colonic wall due to the inflammatory process which may, in part, be related to the production of kinins locally.

Frankish and Zeitlin (1977a) demonstrated that bile salts (deoxycholic acid) stimulated the release of kallikrein from strips of duodenum *in vitro*. This was not a simple detergent

effect. The levels of kallikrein activity, as determined by bioassay, in the duodenum could be increased by fasting the animal (Frankish & Zeitlin, 1977b & 1980). Similarly the level of kinin-forming activity in the duodenum was increased by giving the animal (rat) only water for 24 hours but the levels of activity in the caecum fell while those in the distal colon rose. Giving glucose only for 60 hours resulted in duodenal levels slightly greater than normal, caecal levels which were considerably lower than normal and distal colon levels which were relatively high in comparison to normally fed animals.

Using a glandular kallikrein-selective chromogenic substrate, Al-Dhahir & Zeitlin (1982 & 1983) showed that direct instillation of bile salts in physiological concentrations into the colonic lumen *in vitro*, led to a large increase in the kallikrein activity of the instillate associated with a fall in the tissue glandular kallikrein-like activity. They also showed that the macroscopic changes of bile instillation - oedematous congested mucosa with haemorrhagic discharge - were inhibited by the concurrent administration of aprotinin which totally inhibited tissue glandular kallikrein activity.

Aprotinin, Protease Inhibitors and Tumour Formation.

Aprotinin is a broad-spectrum protease inhibitor which was originally identified in the pancreas of the cow by Kunitz and Northrop in 1935 . It has been described in several species and in a variety of organs; an aprotinin-like inhibitor has also been isolated from human serum. Its structure, inhibitory

characteristics, spectrum of activity and the molecular mechanisms have been extensively reviewed (Fritz & Wunderer, 1983). Aprotinin exhibits activity against several enzymes including trypsin, chymotrypsin, plasmin, plasma kallikrein and tissue kallikrein. In biochemical research aprotinin has been useful as an aid to protease purification, molecular weight estimation and in the estimation of kallikrein activity. It has also some other properties of interest in cellular systems - in particular it can inhibit the growth of malignant transformed cells and may depress the growth and invasiveness of tumours. It may also improve immunity by acting on lymphocytes.

In cell culture normal cells grow to a monolayer and stop. Resting cells can be triggered into mitosis by hormones, lectins or proteases. (Verloes, Atassi, Dumont, et al., 1978) Tumour cells in culture do not stop growing and it has been proposed that they carry or can secrete their own mitotic protease. The field of protease inhibitors and malignant cell growth has been a fruitful one for researchers and will be briefly reviewed with respect to aprotinin.

The interest in the effects of protease inhibitors on carcinogenesis arose from work on skin carcinogenesis in the mouse. In the two-stage model of skin carcinogenesis, the application of the tumour promoter 12-O-tetradecanoyl-phorbol-13 acetate resulted in the appearance of a trypsin-like protease. A variety of protease inhibitors were effective anti-tumour agents. The development of this research has been reviewed by Rossman and

Troll (1980).

In 1970, Burger reported that adding small amounts of trypsin to mouse fibroblast cells in culture lead to their escape from contact inhibition with renewed division and cell overgrowth. Adding a trypsin inhibitor (ovomucoid) prevented the escape phenomenon. He observed that the changes at the cell surface resembled those occurring in chemical or viral transformation of normal cells to malignant ones. A similar phenomenon was seen in embryonic chick cell culture (Sefton & Rubin, 1970).

Schnebli and Burger (1972) subsequently examined a range of compounds whose only common feature was their proteinase-inhibitory activity. These were

1. Tosyl-arginine methyl ester (TAME)
2. Tosyl-phenylalanyl-chloromethyl ketone (TPCK)
3. Tosyl-lysyl-chloromethylketone (TLCK)
4. Soybean trypsin inhibitor (SBTI)
5. Ovomucoid
6. Aprotinin.

Using transformed mouse fibroblast and baby hamster kidney cells it became clear that the growth of transformed cells was inhibited in a dose-dependent fashion by all the inhibitors except SBTI. Untransformed cells were unaffected by the inhibitors. They concluded that the transformed cells required a protease-like activity to exhibit unrestrained growth. Another group using the same inhibitors with transformed hamster cells in

culture made the same observations (Goetz, Weinstein & Roberts, 1972).

SBTI was shown to promote the adhesion of Ehrlich ascites cells to the host peritoneum in a way not usually encountered (Whur, Robson & Payne 1973). This was considered to be due to inhibition of intrinsic proteolytic activity and that the abnormal growth rates and adhesive properties of tumour cells are related to modifications of the cell surface binding sites by the activity of intrinsic proteases.

In contrast to the above, McIlhinney & Hogan (1974) found variable and contradictory effects of TPCK and aprotinin on the growth of normal and transformed baby hamster kidney cells *in vivo*, such that they could not support the hypothesis that transformed cells maintain their higher growth rate by increased proteolytic activity.

Latner, Longstaff & Pradhan, (1973) took a more direct approach to show that aprotinin could inhibit invasion of subsequent kidney explants by transformed cells. They further showed (Latner, Longstaff & Turner, 1974) that aprotinin could decrease the invasiveness of tumours implanted into test animals but the effect depended on the route of administration with the intraperitoneal route apparently stopping invasion while the local administration led to reduced invasion and increased necrosis.

Latner and Turner (1976) demonstrated that aprotinin slowed the development of a second implanted tumour in hamsters

and decreased the number of metastases. Interestingly, prior inoculation with cortisone abolished the effectiveness of aprotinin. A role for the immune system was proposed.

The other side of the problem was addressed by Bosmann & Hall (1974) when they examined the enzymic activity of malignant human breast and colon tumours compared with control tissue. Malignant tumours had increased activity of several enzymes including proteases. The increased enzymic activity represented increased degradative capacity which could be responsible for tumour characteristics.

LeBlanc & Back (1975a & b) made an extensive study of proteases during the growth of Ehrlich ascites tumour. They confirmed the production of plasminogen activators by tumour cells and went on to demonstrate the presence of prekallikrein in ascitic fluid from the tumours. Kininogen was also present in levels which increased with tumour growth. The ascitic fluid had kinin-forming activity which was eightfold that of plasma and this activity was inhibited by aprotinin, SBTI and heparin. Kininase activity was initially high, fell off and then rose again suggesting that kinin activity acted only at certain phases of tumour growth - perhaps altering capillary permeability. Other workers showed that SBTI inhibited the growth of the ascites tumour in a dose dependent fashion. (Verloes & Kanarek, 1976)

With the knowledge that specific kinin-forming enzymes could be isolated from solid rodent tumours, ascites tumours and rodent fibroblasts Back & Steger (1976) examined the effects of

three protease inhibitors - aprotinin, e-aminocaproic acid (EACA) and heparin - on tumour growth. Aprotinin and heparin were effective in reducing tumour growth. Changes in the kinin-forming system were documented which suggested that the tumour-inhibitory activity of aprotinin was related to " effects on the kinin-forming enzyme system. ... aprotinin apparently interfered with the ability of the tumour to utilise prekallikrein and kininogen"

The effects of aprotinin *in vivo* were not always so striking. Thomson and coworkers (Thomson, Pugh-Humphreys, Horne et al., 1977) injected aprotinin into the peritoneal cavity of rats inoculated with tumour cells from a variety of routes and found no increase in survival but a partial attenuation of the effects of the tumour. Turner and Weiss (1981) reported that aprotinin could enhance the metastatic capacity of a lung tumour.

Koppelman, Moore and Porter (1978) assessed the effect of direct injection of bradykinin into a hamster sarcoma on the plasma kallikrein activity. Bradykinin reduced the tumour growth and produced a marked mononuclear infiltrate into the tumour. Animals which were inoculated with the tumour but which did not develop lesions had higher levels of plasma kallikrein than those which did develop the tumour. Contrariwise the level of kallikrein inhibitor in those not developing tumours was lower than those which did. Injection of bradykinin produced an increase in the plasma kallikrein activity. Thus the plasma

kallikrein was reduced by tumour progression and growth of established tumours was slowed by bradykinin.

Human and experimental colonic tumours have been partly examined. An early study (Goldberg, McAllister & Roy, 1969) found decreased proteolytic activity in human colonic tumours. The capacity of colonic carcinoma tissue to produce plasminogen activators has been found to be both increased (Corasanti, Celik, Camiolo, et al., 1980; Tissot, Hauert & Bachmann, 1984) and decreased (Gelister, Mahmoud, Lewin, et al., 1986) when compared to normal colonic tissue. The latter group found the carcinomas had a raised level of urokinase. Plasminogen activators are of interest since plasmin is a prekallikrein activator and therefore activation of plasminogen may ultimately reflect activation of kallikrein and kinin formation. Corasanti, Hobika & Markus (1982) reported that giving e-aminocaproic acid (EACA) to mice in drinking water conferred significant protection for colonic carcinoma induced by dimethyl-hydrazine (DMH).

In summary it may be said that there is a considerable body of evidence to implicate protease enzymes in tumour development and metastasis and kallikrein may be involved in this process either directly as a protease or through its specific product bradykinin.

PROSTAGLANDINS

The prostaglandins are an extensive family of compounds derived from 20-carbon polyunsaturated fatty acids - principally arachidonic acid. They are present in some form in every tissue of the body examined to date. The history of the discovery and understanding of this diverse and important group of substances has been reviewed. (Moncada & Vane, 1983; Crawford, 1983). The initial descriptions of these substances came from von Euler and Goldblatt in the 1930's but it was to be over three decades before the relationship between the prostaglandins and the essential fatty acids from which they were derived was established. Of all the attributed effects of prostaglandins three particular areas have come to be particularly well-studied: the role of the prostanoids in mediating the inflammatory process, the role of the prostaglandin endoperoxides in platelet aggregation and vessel wall protection, and the description of the lipoxygenase products and their role in chronic inflammation and asthma.

In vivo and *in vitro* the prostanoids (= eicosanoids = cyclo-oxygenase products (COP)) are synthesised and catabolised within the same tissue and probably within the same group of cells and as such are correctly classified as local hormones (Bakhle, 1983). The pivotal point in the metabolic cascade of the eicosanoids is the enzyme cyclo-oxygenase which mediates the conversion of the fatty acid into the cyclic endoperoxide precursors of the common prostaglandins. The overall scheme of

the reactions is shown as Fig 1. In essence arachidonic acid is liberated from cell membranes by the action of phospholipase and converted by cyclo-oxygenase into its respective products. A proportion is converted by the lipoxygenase pathway into a separate group of products - the leukotrienes.

Cyclo-oxygenase activity is widely distributed but the mixture of products formed shows marked specificity for cell, tissue and species. In the gastrointestinal tract E and F prostaglandins have been found in all tissues examined. (Bennett & Fleshler, 1970) Little is known about the distribution within the gut wall nor the cellular location. The main effects of prostaglandins are to contract the longitudinal muscle and relax the circular muscle coats although the effects were not entirely constant. In the human it was shown that PGE_2 was the main type of prostaglandin present in the stomach and colon and that mucosal levels were lower than those in muscle of the colon but the converse was true in the stomach. (Bennett, Stamford & Stockley, 1977). Exposing gastrointestinal homogenates to a hypertonic environment results in an increase in prostanoid production from stomach, jejunum or colon (Knapp, Oelz, Sweetman et al., 1978). Again, PGE_2 was the main prostanoid recovered although PGF_{2a} and PGD_2 were also detectable.

In a remarkable and comprehensive series of experiments Craven and DeRubertis examined the relationship between colonic mucosal cell turnover, cyclic monophosphates and the prostaglandins. They showed (DeRubertis, Craven & Saito, 1980)

that administering the colonic carcinogen MNNG to isolated colonic epithelial strips produced an increase in the production of cGMP but did not affect cAMP. The bile salt, sodium deoxycholate, could stimulate cAMP production by the epithelial strips when present in high concentration but in low concentration only potentiated the effect of MNNG on cGMP. In the colonic epithelium neoplastic change was associated with a fall in the levels of cAMP and raised or normal levels of cGMP. (Craven & DeRubertis, 1981) They examined the cyclic nucleotide metabolism of rat colonic epithelial cells by sequentially scraping the mucosa. Incorporation of tritiated thymidine was greatest in the cells from the scrapings from the deeper regions of the pits. cAMP and cGMP levels were lower in the deep scrapings when compared with superficial scrapings; basal adenylate cyclase activity was higher than the superficial cells and could be stimulated by sodium fluoride, PGE_2 and VIP. Guanylate cyclase activity was highest in the superficial scrapings. cAMP, PGE_2 and VIP produced a reduction in the uptake of tritiated thymidine by colonic segments; cGMP was without effect. The authors noted that the lower cyclic AMP levels of rapidly proliferating epithelial cells was ".. analogous to the change observed in human and experimental colonic tumours" and that the reduction in cell proliferation induced by dibutyryl cyclic AMP and prostaglandin E_2 was consistent with an inhibitory influence of cAMP on proliferation."

In further experiments on the colon they examined the

prostanoid production by the intact colon, by colon devoid of epithelium and of the epithelial cells alone. (Craven & DeRubertis, 1983). This showed that while PGE_2 was the most abundant prostanoid formed in total it was virtually all formed in the submucosa. In contrast, the mucosal epithelial cells contained 71% of the total degradative capacity and the superficial cells were sixfold more active in degrading PGE_2 than the deep cells. Interestingly, adding exogenous arachidonic acid led to increased PGF_{2a} in the epithelial cells and adding deoxycholate - which stimulates prostanoid synthesis by enhancing arachidonate release - produced a similar effect. This latter result was followed up in 1984 (DeRubertis, Craven and Saito, 1984) when they examined the effect of bile salt stimulation of colonic proliferation and the role of arachidonic acid metabolites. Their results showed that bile salts (deoxycholic acid, chenodeoxycholic acid and cholic acid) increased the release of arachidonic acid by the colon *in vitro*. Instillation of deoxycholic acid into the rat colon produced an increased uptake of tritiated thymidine *in vivo* and when the colon was incubated thereafter *in vitro* prostanoid production was elevated as was the production of 12-HETE, a lipoxygenase product (fig 1). Indomethacin reduced the prostanoid production and enhanced the the thymidine uptake increase produced by the bile acid. Thus it seems possible that the lipoxygenase products have a role to play in modulating the turnover of the colonic epithelium.

An examination of the human colon (Boughton-Smith, Hawkey

& Whittle, 1983) showed that it could synthesise cyclo-oxygenase products from added radiolabelled arachidonic acid with PGE_2 being the principal product. In addition lipoxygenase products were also formed and inflammed mucosa from colitic patients produced more of all products. The chemotactic and chemokinetic properties of the lipoxygenase products could well be responsible for the inflammatory manifestations of the disease and could explain why inhibitors of cyclo-oxygenase are ineffective in the disease in spite of the raised levels of prostaglandin production. (Sharon, Ligumsky, Rachmilewitz et al., 1978; Harris, Smith & Swan, 1978; Rampton & Sladen, 1981) Indeed, inhibitors of cyclo-oxygenase may aggravate the disease. (Hawkey and Truelove, 1983)

Prostaglandins and Tumours.

The evidence linking the prostaglandins and tumours was initially sketchy. Jaffe reviewed the knowledge in 1974. The essence was that neoplastic cells were known to synthesise prostaglandins *in vivo* and *in vitro* and malignant cells produced more prostaglandin than normal cells, the dominant prostanoid being PGE_2. Effects of prostanoids on cell proliferation were recognised (but were not to be elucidated for some years as detailed above) and Jaffe's own work had shown that colonic tumours in organ culture produced eight times as much PGE_2 as normal cells. Additionally, indomethacin, an inhibitor of prostaglandin production delayed the establishment of inoculated tumours in mice.

The increased formation of prostanoid material by tumours was also seen in human breast cancers (Bennet, Simpson, McDonald et al., 1975) and it was also noted that those with bony metastases had particularly high levels. (Bennett, Charlier & McDonald, 1976) Similarly, elevated levels of prostanoid were seen in some human colonic tumours when compared with normal mucosa from the same individual (Bennett & DelTacca, 1975; Bennett, DelTacca, Stamford, et al., 1977) but the pattern was not consistent. Rolland and coworkers (Rolland, Martin, Jacquemier, et al., 1980) showed that there was an inverse relationship between tumour size and the levels of prostanoid produced; metastatic lesions appeared to produce more prostanoid than the primary tumour. A relationship with the oestrogen and progesterone receptor status of the tumour was suggested. (Karmali, Welt, Thaler et al., 1983)

Prostanoid production was not just the preserve of breast and colon tumours: it was also demonstrated in thyroid cancer, neuroblastoma, phaeochromocytoma, islet cell tumours, bronchial carcinoma and renal cell carcinoma and several experimental tumours. (Levine, 1981; Smith, Wills & Savory, 1983) Moreover, in the familiar tetraphorbol acetate(TPA)-croton oil mouse skin cancer model it was recognised that TPA could stimulate several cell types to produce prostaglandins by stimulating the release of arachidonic acid from membrane phospholipids. Additionally, indomethacin could block the promoting effect of TPA and this could be over come by adding PGE_2. (Levine, 1981; Honn, Bockmann

& Marnett, 1981) Increased production of prostaglandins has been shown for other tumour promoters. (Snoek & Levine, 1983) The inhibition of tumour growth by indomethacin (and other inhibitors of cyclo-oxygenase) has been demonstrated on several occasions. In 1973 aspirin and indomethacin were shown to inhibit the development of osteolytic tumour deposits and the development of hypercalcaemia in rats inoculated with Walker tumour. (Powles, Clark, Easty et al., 1973). Humes, Cupo and Strausser (1974) showed that indomethacin delayed the onset of Moloney Sarcoma virus-induced tumours inoculated into mice and also suppressed the tumour growth. This was correlated with tumour prostaglandin production. Transplantable fibrosarcoma growth was inhibited by aspirin and indomethacin (Lynch, Castens, Astoin et al., 1978; Lynch & Salomon 1979). It was suggested that the role of indomethacin may be to unmask the tumour and allow full expression of "anaphylactic reactions". Pollard and Luckart (1981a & b) demonstrated a general reduction in DMH-induced colonic tumorigenesis in rats by the coadministration of indomethacin. Bennett, Berstock & Carroll (1982) showed that the survival time after excision of a transplantable mammary tumour in mice was prolonged by both indomethacin and flurbiprofen. The tumour prostaglandins were also reduced by the treatment.

An antitumour effect of indomethacin on DMH and MAM - induced colonic tumours in rats was shown by Pollard & Luckert (1983). They showed that the inhibitory effect of indomethacin was demonstrable even if its use was initiated long after the

inoculation of carcinogen. Indomethacin was shown to block the promotion of rat colonic tumours induced by N-nitrosomethylurea but curiously the addition of PGE_2 to the regime by injection did not reverse this effect. (Narisawa, Hermanek, Habs et al., 1984) Indomethacin inhibited the growth of transplanted experimental breast tumour in rats and completely abrogated the promotional effect of increased dietary fat. (Kollmorgan, King, Kosanke et al., 1983) A high fat diet increased the incidence and size of 7,12-Dimethylbenz(a)anthracene induced mammary tumours in mice. This effect of fat was blocked by the coadministration of indomethacin. (Carter, Milholland, Shea et al., 1983) Serum cortisone levels in the rats receiving indomethacin and a normal fat diet were elevated but were normal in the animals on the high fat group. Cortisone would block the action of phospholipase and prevent the release of arachidonic acid from the cell membrane. This would lead to depressed levels of prostaglandin. High levels of prostaglandin are known to inhibit cell-mediated immunity and would be the result of the high fat diet. Prostaglandins have been shown to be capable of an inhibitory influence on natural killer cells (Brunda, Heberman & Holden 1980) and this effect may be mediated by their stimulating the accumulation of cAMP which is associated with depression of lymphocyte stimulation and production, cell-mediated immunity and antibody formation. (Lewis, 1983). Immunosuppression in cancer patients is well recognised and has been documented in those with colorectal cancer. (Wanebo, Baskar, Attiyeh et al. 1980) The

immunosuppression may be mediated by prostaglandins (PGE_2) but not necessarily derived from the tumour cells but from the monocyte response. (Balch, Dougherty, Cloud et al., 1984; Attallah, Lee, Ambrus et al. 1984) Not surprisingly, a possible role for the potent lipoxygenase products has been proposed in immunoregulation. (Valone, 1983)

As regards tumorigenesis, it has been repeatedly suggested that the metabolic activation of the primary carcinogen may be linked to prostaglandin formation. This has been best demonstrated for benz(a)pyrene (Sivarajah, Anderson & Eling, 1978; Sivarajah, Lasker & Eling, 1981; Craven, DeRubertis & Fox, 1983; Krauss & Eling, 1984). The colonic mucosa microsomes have been shown to possess an inducible mixed function oxidase system capable of hydroxylation of carcinogens to mutagenic products. This system can be stimulated by PGE_2. (Strobel, Fang & Oshinsky, 1980)

It would not be unreasonable to presuppose a relationship between the kallikrein-kinin system and the prostanoids since both are involved in inflammatory processes. (Williams, 1983) It has been shown that protease treatment of peritoneal macrophages will cause release of PGE_2 but not in a fashion related to proteolytic strength (Chang, Wigley & Newcombe, 1980). This prostanoid release can be inhibited by proteinase inhibitors (including aprotinin). Interestingly, aprotinin will also inhibit the prostanoid release stimulated by tetradecanoyl phorbol acetate (TPA) - a tumour promoter.

A relationship between the kinins and prostaglandin synthesis has been demonstrated in the kidney (Vio, Bednal & McGiff, 1983) and it has been proposed that bradykinin may control the activity of phospholipase C which is essential to the release of arachidonic acid from the cell membrane. Studies *in vitro* have shown that bradykinin can release arachidonic acid from cells in a dose-dependent fashion with accompanying increases in prostaglandin formation. (Juan & Sametz, 1983) Some of the most important interactions of the prostaglandins and kinins are involved in regulating the micro-circulation to the metabolic demands of the tissue.

AIMS

Existing evidence suggests that the development of experimental colonic cancer may depend upon a number of variables. Important among these are the diet, the faecal bile acids, the colonic crypt cell proliferation rate and the presence of a carcinogen. There is ample evidence to implicate also other agents which may interact with these variables. In particular the kallikrein-kinin system and the prostaglandins have established roles in cellular growth and in some experimental tumour systems. In addition, dietary constituents, bile acids and carcinogens may influence the activity of these two endogenous mediators - the kinins and the prostaglandins. Therefore it may be proposed that these substances provide an important link in the overall relationship between diet and the development of experimental colorectal cancer.

The principle aim of this study is to examinethe effect of diet on experimental colorectal carcinogenesis and to attempt to simultaneously examine the mechanisms whereby diet may produce its effects. The hypothesis outlined above indicates that there are six variables to be studied:

1. the diet
2. the kallikrein - kinin system
3. the prostanoids
4. the faecal bile acids
5. the crypt cell proliferation rates
6. the presence of carcinogen.

The aims of the study can be formulated as a series of questions as follows:

1. What is the effect of high and low risk diets for colorectal cancer on the following factors in normal experimental animals: the colonic kallikrein activity, the colonic prostanoid content, the faecal bile acids and the colonic crypt cell production rates?

2. In animals receiving high and low risk diets what is the effect of pharmacological inhibition of kallikrein activity on the colonic kallikrein activity, the colonic prostanoid content and the colonic crypt cell production rates?

3. In animals receiving high and low risk diets what is the effect of pharmacological inhibition of prostaglandin synthesis on the colonic kallikrein activity, the colonic prostanoid content and the colonic crypt cell production rates?

Once these "baseline" measurements are established then the same questions can be addressed for animals receiving a carcinogen:

4. What is the effect of high and low risk diets for colorectal cancer on the following factors in animals exposed to the carcinogen: the colonic kallikrein activity, the colonic prostanoid content, the faecal bile acids, the colonic crypt cell production rates and the tumour induction?

5. In animals receiving high and low risk diets and the carcinogen what is the effect of pharmacological inhibition of kallikrein activity on the colonic kallikrein activity, the

colonic prostanoid content,the colonic crypt cell production rates and tumour induction?

6. In animals receiving high and low risk diets and the carcinogen what is the effect of pharmacological inhibition of prostaglandin synthesis on the colonic kallikrein activity, the colonic prostanoid content,the colonic crypt cell production rates and tumour induction?

In addition to these central questions one other aspect needs evaluation:

7. In what way do animals receiving defined high and low risk diets differ from those on their usual laboratory diet in terms of the the colonic kallikrein activity, the colonic prostanoid content, the faecal bile acids, the colonic crypt cell production rates and ultimately the tumour induction?

The study falls therefore into two phases - firstly studying animals which have not received carcinogen and secondly studying those which have had carcinogen treatment to induce tumours. While the data will be presented for each variable individually, this basic division will always be observed.

MATERIALS AND METHODS

(GRAPHS 1-3)

ANIMALS

The animals used in this study were male Swiss albino rats bred from a closed colony in the animal unit of the Department of Surgery, Western Infirmary, Glasgow. They were recruited into the experiment at about 250gm body weight (10 weeks of age). They lived in controlled quarters with a 12 hour light-dark cycle in cages of up to 5 animals. Diet and water were supplied ad libitum and the animals were weighed weekly during the course of the experiment.

DIETS

Three diets were given during the course of the experiment. All animals were raised on Oxoid breeding diet. When recruited into the experiment some animals continued on this; others received one of two special diets designed to be either high in fat and low in fibre content or low in fat and high in fibre content. These were prepared by a commercial firm to bulk order (Special Diet Services, Witham, Essex). The composition of the diets is shown in Appendix 1. The special diets differ only in their fat and fibre content and consequently their caloric value. Details of the fatty acid composition are presented in the appendix for the high fat, low fibre diet. The main saturated fatty acids are palmitic acid (5.2%) and stearic acid (3.6%). The main unsaturated fatty acids are oleic acid (7.5%) and linoleic acid (2.6%). The fatty acid composition of the low fat, high fibre diet is a similar mix of essential and non-essential fatty

acids. The fibre present in the diet (crude fibre) is essentially cellulose and lignin. The Oxoid diet has a fat and fibre content between the two special diets but also differs in caloric value, vitamin and micronutrient content. The precise fatty acid and fibre composition is not available.

The consumption of diet and water during the experiment was assessed by weighing the amount of diet consumed by the animals every week and by measuring the volume of water consumed over two 24 hour periods (09.00 Monday - 09.00 Tuesday and 09.00 Thursday - 09.00 Friday). When calculated, the diet and water consumption was expressed per animal day. Whilst this method provides no data on the individual variability of the consumption per animal it was considered justifiable since all animals in a cage belonged to one experimental group.

During the course of the experimental period the animals received some combination of diet and drug treatment with or without a carcinogen for one of three time periods. The various combinations used are shown in Table 1 with the numbers of the animals and the code numbers for each group. The experimental plan is illustrated in figure 2.

AZOXYMETHANE

Approximately half of the animals received azoxymethane as a tumour-inducing agent. This was given by weekly subcutaneous injections over twelve weeks beginning on the fourth week of the experiment. The dose was 10mg/kg or a total dose of 120mg/kg for

each animal. Administration of the carcinogen required several precautions to be taken which are detailed in Appendix 2.

APROTININ

This drug was given by daily subcutaneous injection in a dose of 20000 KIU/kg every day during the course of the experiment to animals due to receive it. Control animals received an injection of 0.9% saline which was the vehicle. This drug was gifted by Bayer UK Ltd.

INDOMETHACIN

Indomethacin was given to the animals in their drinking water in a dose of 2mg/kg. As indomethacin is insoluble in water ethanol was added to a concentration of 0.25%. A stock solution of indomethacin in ethanol was made at a concentration of 8mg/ml and this was added to the drinking water of the animals using 0.25ml in 99.75ml of water. The method of administration does not provide an exact dosing regime but on the assumption that all animals drink about the same (which was established prior to the experiment) and that this method has been successfully used elsewhere then it was considered reasonable so to do. Control animals for this group received 0.25% ethanol in their drinking water.

SACRIFICE

Animals were sacrificed at either 4, 16 or 24 weeks after entering the experiment. Immediately prior to sacrifice some animals received vincristine (0.25mg) as a stathmokinetic agent by a single intraperitoneal injection.

Animals were killed by ether anaesthesia and cervical dislocation. Where stathmokinetic studies were to be undertaken the animals were sacrificed at intervals of 15 minutes.

On opening the body cavity a small plastic cannula was placed in the thoracic aorta and the inferior vena cava divided. The lower body viscera including the colon were then perfused free of blood using heparinized Kreb's solution initially and then plain Kreb's solution. Simple injection with a small volume syringe performed this adequately and an animal of 300g would be adequately perfused with about 75ml of solution.

Following this the colon was removed from rectum to caecum, the terminal ileum being cut off flush with the caecum. The entire colon and rectum was opened longitudinally and faecal material collected for analysis of faecal bile acids. The entire faecal content of the colon was collected and samples from any one group pooled for analysis.

The colon was then washed with Kreb's solution and pinned out under minimal tension and the length measured. It was cut into five equal segments labelled A,B,C,D & E. These corresponded to the caecum, proximal colon, mid colon, distal colon and rectum respectively. Each segment was split longitudinally and samples

taken for histological and stathmokinetic analysis. Tumours were recorded as occurring at a particular site by length from the caecum and were removed entire for histological study. The remaining tissue was snap frozen in liquid nitrogen and stored deep frozen (-40°C) until analysis

MEASUREMENT OF TISSUE KALLIKREIN-LIKE ACTIVITY IN COLON.

Tissue kallikrein-like activity was estimated by a semi-micro, quantitative spectrophotometric assay which essentially follows the descriptions of Amundsen, Putter, Friberger et al., (1979) and Al-Dhahir and Zeitlin (1982).

Preparation of the tissue

Colonic tissue was stored at -40°C until used. It was thawed at room temperature and washed briefly with fresh distilled water, blotted dry and weighed. To the tissue was added fresh distilled water in a volume 40 times the weight of the tissue in grams. [Thus 0.1g of tissue had 4ml of water added]. The tissue was homogenised in an Ultraturrax blender at the temperature of melting ice for three minutes. This homogenate was then allowed to stand at room temperature under toluene vacuum overnight to allow activation of the cellular debris. After this the homogenate was centrifuged at 2000g and 4°C for 30minutes and the clear supernatant containing the kallikrein activity aspirated. This was then stored deep frozen in aliquots until used. Once thawed no sample was refrozen (Zeitlin et al.,1976)

Assay

At the time of the assay each aliquot of homogenate supernatant was thawed at 37°C in a water bath. A sample from each aliquot was incubated with synthetic chromogenic kallikrein-selective substrate S-2266 (KabiVitrium, Stockholm, Sweden). Appendix 3 shows the individual steps of the assay and the calculation of the kallikrein-like amidase activity is shown

in Appendix 4. All results of the kallikrein studies are expressed as micromoles of substrate cleaved. g wet weight of tissue^{-1}.min^{-1}. The principle of the assay and data on the substrate, S-2266, are given in Appendix 5.

Calibration of spectrophotometer

The cleavage of S-2266 produces the coloured product p-nitroaniline. This shows maximal absorbance at 405nm. The spectrophotometer was calibrated at this wavelength for solutions of differing concentrations of p-nitroaniline when read against distilled water. This allows the extinction coefficient for the assay to be calculated. Table 2 (Graph 1) shows the results of the calibration curve and the calculation of the extinction coefficient for these experiments.

Characterisation of the assay.

It was necessary to understand the behaviour of the assay prior to undertaking any definitive analyses. To this end several experiments were carried out to assess:

1. the linearity of the assay with concentration of homogenate extract
2. the course of the reaction with time
3. the optimal pH for the reaction
4. the coefficient of variation for the experimenter
5. that the activity being measured was tissue kallikrein-like amidase

1. Linearity of the assay with concentration of extract.

Tissue extract of rat colon was diluted initially to 1/10

and from this stock further dilutions were made to 1/20, 1/40, 1/50 and 1/100. These samples were assayed in standard fashion with replicates. The results are shown in Table 3. These data show that there is a linear relationship between the concentration of the extract and the activity produced.

2. Course of the reaction with time.

Rat colon extract was prepared in the usual fashion and diluted to 1/10 and 1/20 strength. The samples were assayed in standard fashion with replicates and the reaction stopped at 5, 15, 30, 60 and 120 minutes after the addition of the substrate by acidification.

The results are shown in Table 4 and Graph 2. This shows that the reaction is linear with time for the 1/20 dilution up to 120 minutes and for the 1/10 dilution up to 60 minutes. In the latter case the reaction then goes onto a plateau due to exhaustion of the substrate.

3. Optimal pH for the reaction.

The buffer for the incubation was made up to various pH values in the range of pH 7 to pH 11. Colonic tissue was assayed in standard fashion. The results are shown in Table 5 and Graph 3.

It proved impossible to establish an optimal pH for the reaction despite several repetitions of the experiment. This is in contradistinction to other workers who were able to demonstrate an optimal pH for their reaction. While this was unexpected the reaction occurred in a predictable fashion and

appeared to be reproducible (see coefficient of variation, below). In view of this it was decided to carry out the assay at a pH of 8.2 as was used by Amundsen et al. (1979). This will be discussed again. (page 97)

4. Coefficient of variation.

A single sample of tissue extract was assayed in standard fashion on 9 separate occasions and the results recorded. The coefficient of variation was calculated to be 2.8%. (Table 6)

5. Specificity of the assay.

While the substrate S-2266 is selective for tissue kallikrein it does give some reaction with similar proteases, namely plasma kallikrein, trypsin and plasmin. Soybean trypsin inhibitor is known to inhibit all three of these proteases while leaving tissue kallikrein unaffected. Aprotinin is a broad-spectrum protease inhibitor which will block the activity of tissue kallikrein as well as other 'interfering' proteases. By using these inhibitors in the assay system it is possible to deduce if all the activity present is due to tissue kallikrein.

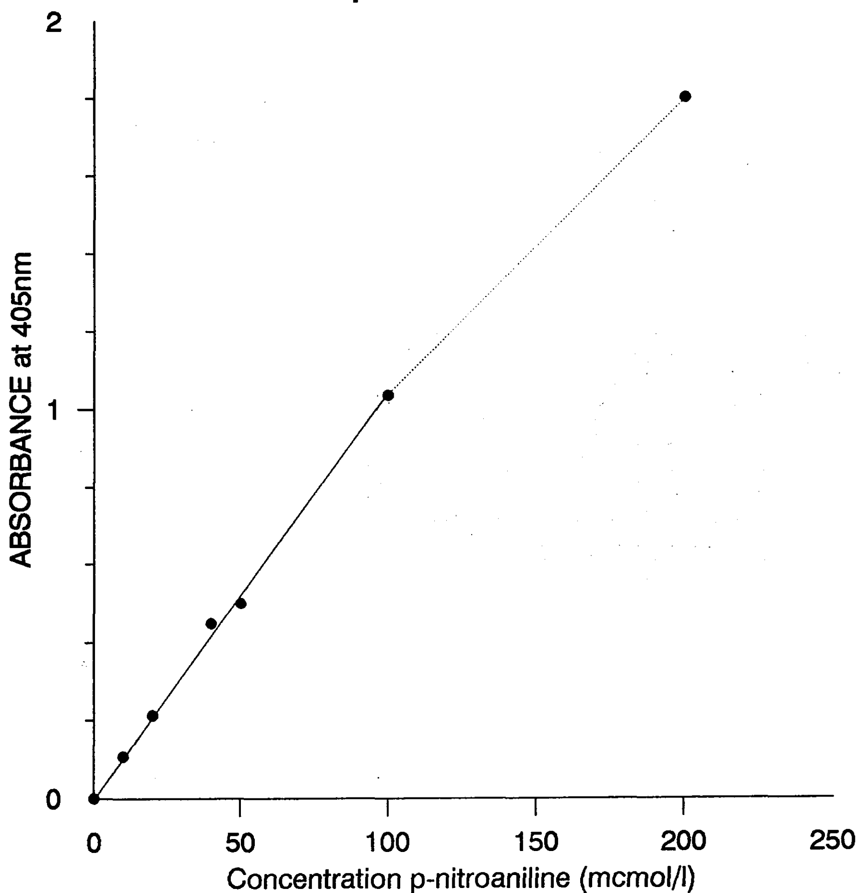
A sample of colon extract was incubated in standard fashion with buffer which contained soybean trypsin inhibitor in three concentrations and without inhibitor. In a further experiment aprotinin was substituted for soybean trypsin inhibitor. The results are shown in Table 7.

Addition of soybean trypsin inhibitor produced a small, significant fall in the activity of the extract indicating the presence of some other protease activity. Aprotinin completely

abolished all activity in the assay system.

In view of this all future assays were undertaken using soybean trypsin inhibitor in the buffer solution at a concentration of 250mcg/ml.

GRAPH 1



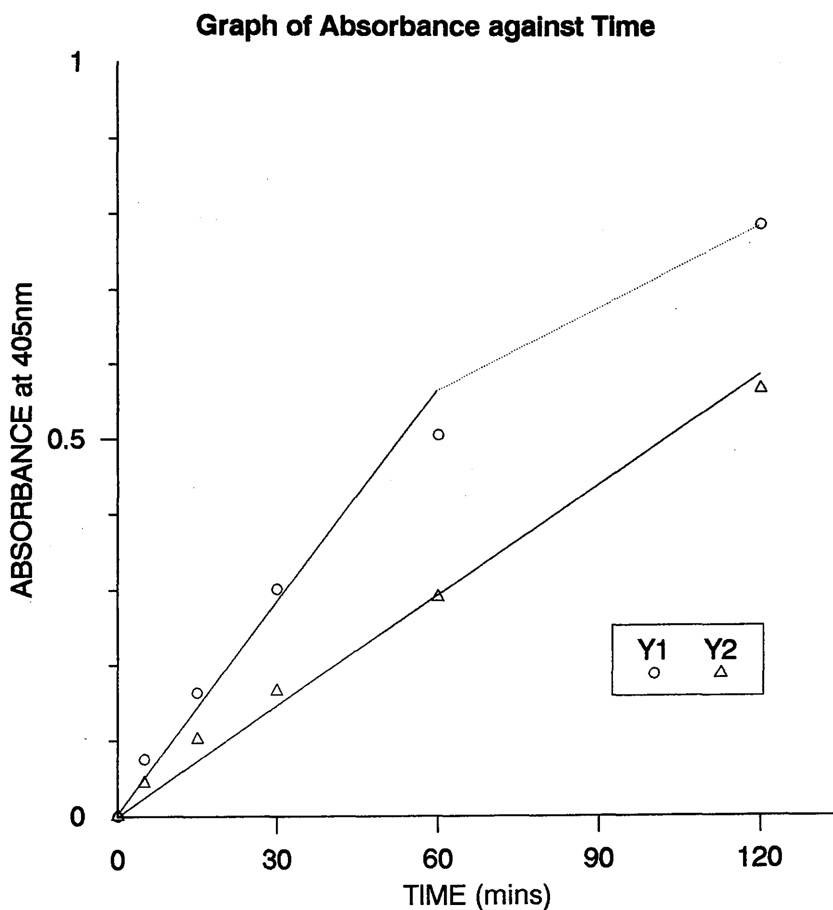
Solid line has r = 0.999

Broken line indicates loss of linearity with concentrations of pNA greater than 100 micromol/l and absorbance greater than 1.

Extinction coefficient = 10343.367

Data: Table 2

GRAPH 2



Standard incubation conditions.

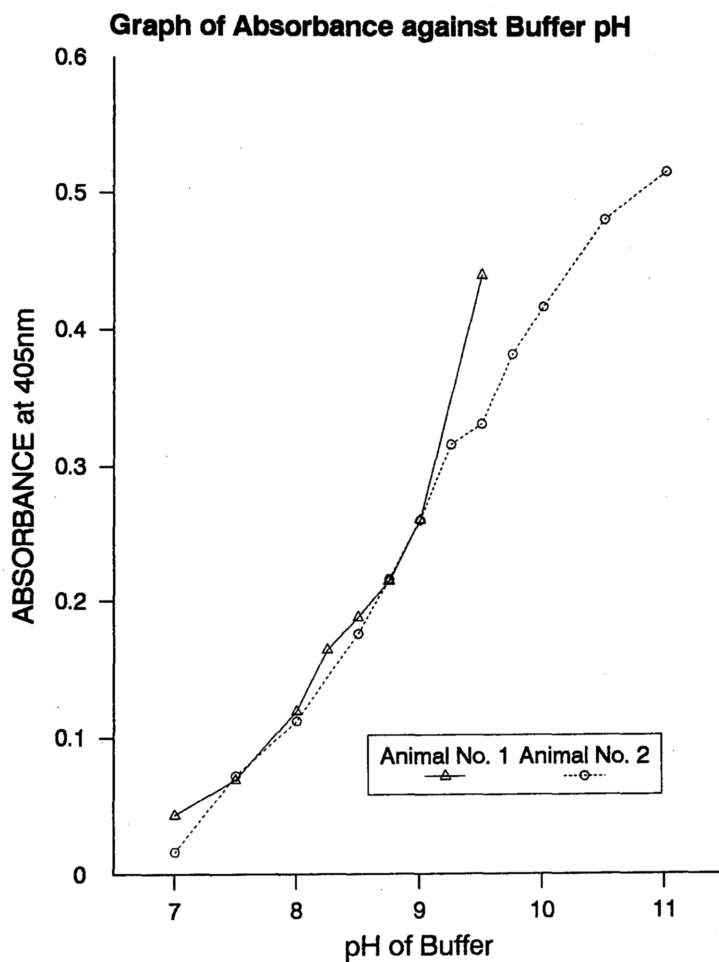
y1 = colonic extract at 1/10 dilution.
y2 = colonic extract at 1/20 dilution.

The 1/10 dilution extract shows linearity up to 60 minutes. After this time point the rate of reaction decreases and linearity fails. (broken line).

The 1/20 dilution extract shows linearity up to 120minutes.

Data: Table 4

GRAPH 3



Colonic extracts for 2 animals assayed.

Standard incubation conditions.

Data: Table 5

MEASUREMENT OF PROSTAGLANDIN E_2 IN COLONIC TISSUE.

The content of PGE_2 in colonic tissue was estimated by performing radioimmunoassay on an extract of colon tissue.

Preparation of the Tissue

Tissue was stored deep frozen until used. It was then thawed at room temperature and weighed. To it was added a volume of phosphosaline buffer (PSB) containing indomethacin (10mcg/ml). The volume added was 25 times the weight of the tissue in grams. [Thus 0.1g of tissue had 2.5ml of buffer added.] The mixture was homogenised in an Ultraturrax blender for 3 minutes at the temperature of melting ice. The homogenate was then centrifuged for 20 minutes at 2000g and 4^0C and the supernatant decanted into a volume of PSB to provide a 1:10 dilution. This diluted extract was then stored at -40^0C until assay.

Assay of PGE_2

Samples of the diluted colonic extract were thawed at the time of assay. A suitable aliquot was dispensed into glass tubes in duplicate and an assay for PGE_2 carried out using a method already established and validated in this laboratory. (Coker, Clark and Zeitlin, 1982). Details of the methodology and the source and characteristics of the reagents are given separately as appendices 6 & 7.

Since tissue damage will activate prostanoid production the effect of homogenisation on the measured levels of PGE_2 was assessed. Samples of colonic tissue were homogenised using the Ultraturrax blender as described. Samples of the homogenate were

taken after 3, 5 and 10 minutes of homogenising and assayed for PGE_2 using the radioimmunoassay as described. The results are shown in Table 8. The levels of PGE_2 measured did not differ between the time periods and did not increase with prolonged homogenising. (823$\pm$36pg for 3 minute values vs 824$\pm$112pg and 904$\pm$ 72 for 5 and 10 minute values respectively)

To ensure that the concentration of indomethacin used in the phosphosaline buffer (PSB) produced maximal inhibition of prostanoid production during homogenisation the effect of increasing the concentration was examined. Samples of colonic tissue from the same area of the colon were homogenised in PSB containing indomethacin in concentrations of 10, 20 and 50mcg/ml and the homogenates assayed for PGE_2. The results are presented in Table 9. Increasing the concentration of indomethacin in the buffer did not affect the levels of PGE_2 measured. (732$\pm$25pg.mg^{-1} for 10mcg.ml^{-1}, 714$\pm$81pg.ml^{-1} for 20mcg.ml^{-1}, 688$\pm$65pg.ml^{-1} for 50mcg.ml^{-1}, NSD)

The effect of delaying separation of the homogenate was assessed by dividing samples of colonic homogenate into three separate specimens which were then either:

a) separated immediately by centrifugation

b) left at 4°C overnight in a refrigerator prior to separation

or c) left at room temperature under toluene overnight prior to separation

These samples were then assayed for PGE_2 content in

standard fashion. The results are shown in Table 10. Delaying separation of the homogenate resulted in some increase in the measured prostaglandin content (392 ± 82pg vs 537 ± 108pg, $p<0.05$)

For this study, all colon samples were homogenised in phosphosaline buffer containing indomethacin in a concentration of $10 mcg.ml^{-1}$ for three minutes and were separated by centrifugation immediately after homogenisation.

The coefficient of variation of the prostanoid assay was estimated by analysis of a sample of colonic homogenate which was aliquoted into several separate vials and kept at $-40^{0}C$. One vial (sufficient for a replicate assay) was assayed periodically with the main assay. The results of the individual analysis are shown in Table 11. The interassay coefficient of variation is calculated to be 24%. The intra-assay coefficient of variation was estimated by the multiple analysis of one specimen in the course of one assay. This was shown to be 9.5%

It was impossible to assay all samples simultaneously. In view of the high interassay variation and to avoid "batch" effects on the results of the prostaglandin assay, the samples were assayed in a random fashion. This ensured that any differences observed between groups was not simply a reflection of different assay conditions. Each batch of samples which was assayed had a set of known prostaglandin standards included. This set of standards provided the necessary data to calculate the standard curve for the assay and, by derivation, the absolute prostaglandin content of the sample. The prostaglandin standards

were all made from a single standard solution of prostaglandin E_2. This ensured that all measured levels of prostaglandin related to a single, known standard. All samples were assayed in duplicate. (Appendix 6)

STATHMOKINETIC ANALYSIS

The preparation of specimens for stathmokinetic analysis followed established methods for crypt microdissection, staining and evaluation of crypt cell production rates. (Ferguson, Sutherland, MacDonald et al., 1977; Cooke et al., 1984)

Specimens from each of the five areas of the colon were taken from the animals which had received vincristine (Eli Lilly & Co., Basingstoke) prior to sacrifice. These were fixed in Carnoy's solution and dehydrated through alcohols for storage. Following rehydration they were stained with Schiff reagent for DNA (Feulgen reaction). (Appendix 8.) After staining the intact specimen the mucosa was dissected from the muscularis and the crypts teased from this using fine knife, needles and forceps. A squash preparation of the crypts was made onto a microscope slide and in a modification to the method a coverslip was bonded to this under pressure with an epoxy resin (Araldite).

By light microscopy at x100 or x400 magnification, crypts were assessed and the distinct metaphase figures counted for each crypt. (Figure 3) For each specimen a minimum of 10 crypts were counted. The subsequent crypt cell metaphase counts were converted into the crypt cell production rate (CCPR) by plotting the regression line through the metaphase count versus time. This provides a measure of the efflux of cells from the proliferative compartment to the functional compartment of the crypt. The CCPR was measured for each animal at each segment of colon studied.

MEASUREMENT OF FAECAL CONSTITUENTS.

Samples of faeces were taken from the colons of animals at sacrifice. These samples comprised the entire colonic content. The samples were then freeze-dried to constant weight and stored at -40oC until assay. The faecal specimens from one group of animals were pooled and the subsequent measurements made on this sample.

These samples were then assayed by the method of Owen, Thompson and Hill (1984) as described in Appendix 9.

HISTOLOGICAL EXAMINATION

All specimens for histological assessment were fixed in formol saline solution and processed through routine histological methods using alcohol dehydration, chloroform and xylene clearing prior to paraffin wax embedding. Sections (4mcm) were stained with haematoxylin and eosin for examination. All specimens were examined by one individual.

MEASUREMENT OF PLASMA INDOMETHACIN

The plasma indomethacin was assayed in a number of animals receiving the drug at the time of sacrifice. Blood was obtained by cardiac puncture and placed in heparinised tubes. The samples were centrifuged at $4^{o}C$ to separate the plasma which was then stored at $-40^{o}C$ until assay.

At the time of assay the plasma samples were thawed and the indomethacin concentration measured by the method of Hucker, Zacchei, Cox et al. (1966) as described in Appendix 10.

STATISTICAL METHODS.

Several statistical methods were used in the analysis of the different sets of data.

1. Tissue kallikrein-like amidase activity and tissue prostaglandin E_2 content.

The design of the study was such that data were obtained under a total of 300 different experimental conditions. [2 diets (high fat, low fibre & low fat, high fibre) by 5 treatments (no drug, saline control, ethanol control, aprotinin, indomethacin) by 3 times (4, 16 & 24 weeks) by 2 levels of carcinogen (present or absent) by 5 colon segments (A, B, C, D & E)]. This gives rise to 38850 (300x299/2) possible pairwise comparisons. Clearly it is neither feasible nor sensible to compare the findings over each pair of experimental conditions. Either it would be necessary to ignore the multiple comparisons and thereby generate a large number of false positive findings (Type I error), or by correcting for the multiple comparisons, so reduce the statistical power of the study such that a large number of false negatives would result. (Type II error).

Fortunately statistical methods for the analysis of data from such complex but carefully structured experiments are well-developed and are based upon the analysis of variance. (Scheffe, 1959) In essence a very general statistical model is fitted to the data and then terms which do not contribute significantly to the variability of the data (hence "analysis of variance") are successively removed by dropping variables and/or

pooling categories. The end result should be a simple model which adequately describes the observed variability of the data and implicitly, by pooling over irrelevant factors, greatly increases the precision with which the key comparisons may be made. The crux of the approach is that one regards the data as a coherent whole which has arisen from a single, carefully designed and structured experiment rather than an unstructured mass of 300 sets of data.

The data for the kallikrein activity and prostaglandin content were analysed using repeated measures analysis of variance, taking colon segment as the within-animal factor and taking carcinogen, diet, drug and time as grouping factors. A square root transformation was used throughout the formal analysis to stabilise the variability which tended to increase with increasing levels of kallikrein activity and prostaglandin content. Wherever possible, and as set out in detail in the results section, categories were pooled and/or factors were dropped from the analysis to give clear answers to the primary questions of interest.

The calculations were performed on an ICL 3980 mainframe computer, using the BMDP program P2V. (Dixon, Brown, Engelman et al., 1985)

2. Crypt cell production rates.

These data were initially plotted so that clear outliers could be rejected visually. Points were rejected if they lay more than 50% from a visually plotted line on the graphs. In practice

this only involved a total of less than 50 points in all the metaphase counts made and such points were very obviously at variance with the trend of the plotted line.

The crypt cell production rates were then estimated by fitting linear regression lines to the data. This was done using weighted least squares, with the weighting according to the variability of the 10 replicated counts. The calculations were performed using the MINITAB statistical package running on an ICL 3980 mainframe computer. (MINITAB Inc., 3081 Enterprise Drive, State College, PA 16801, USA.)

The crypt cell production rates were simply tabulated along with their standard errors, as the sparse structure of the data precluded any more formal analysis of the effects of the factors such as colon segment or diet.

<u>3. Animal growth, diet consumption, faecal bile acids and tumour induction.</u>

Where numbers permitted, mean values were compared using Student's t-test. Where numbers were small (<10) the data were analysed by using the Mann-Whitney U-test. Analysis of tumour induction was done using the Chi-square test.

TABLES 1 - 11

TABLE 1

DESCRIPTION OF THE GROUPS OF ANIMALS.

GROUP NO.	ANIMAL NUMBERS	DIET	DURATION	DRUGS
9	83/42 - 83/51	normal	4	none
10	83/52 - 83/61	normal	16	none
11	83/62 - 83/71	normal	24	none
12	84/01 - 84/10	normal	4	aprotinin
13	84/11 - 84/20	normal	16	aprotinin
14	84/21 - 84/30	normal	24	aprotinin
15	84/31 - 84/40	normal	4	indomethacin
16	84/41 - 84/50	normal	16	indomethacin
17	84/51 - 84/60	normal	24	indomethacin
18	84/61 - 84/64			
	85/100 - 85/104	normal	4	vehicle
19	84/65 - 84/68	normal	24	vehicle
20	84/69 - 84/72	normal	4	ethanol
21	84/73 - 84/76	normal	24	ethanol
22	84/77 - 84/80	high fat, low fibre	4	none
23	84/81 - 84/84			
	84/921 - 84/924	high fat, low fibre	16	none
24	84/85 - 84/88			
	84/801 - 84/804	high fat, low fibre	24	none
25	84/90 - 84/99	high fat, low fibre	4	aprotinin
26	84/100 - 84/109	high fat, low fibre	16	aprotinin
27	84/110 - 84/119	high fat, low fibre	24	aprotinin
28	84/120 - 84/129	high fat, low fibre	4	indomethacin
29	84/130 - 84/139	high fat, low fibre	16	indomethacin
30	84/140 - 84/149	high fat, low fibre	24	indomethacin
31	84/150 - 84/153	high fat, low fibre	4	vehicle
32	84/154 - 84/157	high fat, low fibre	24	vehicle
33	84/158 - 84/161	high fat, low fibre	4	ethanol
34	84/162 - 84/165	high fat, low fibre	24	ethanol
35	84/166 - 84/169	low fat, high fibre	4	none
36	84/170 - 84/173			
	85/21 - 85/24	low fat, high fibre	16	none
37	84/174 - 84/177	low fat, high fibre	24	none
38	84/178 - 84/187	low fat, high fibre	4	aprotinin
39	84/188 - 84/197	low fat, high fibre	16	aprotinin
40	84/198 - 84/207	low fat, high fibre	24	aprotinin
41	84/208 - 84/217	low fat, high fibre	4	indomethacin
42	84/218 - 84/227	low fat, high fibre	16	indomethacin
43	84/228 - 84/237	low fat, high fibre	24	indomethacin
44	84/238 - 84/241	low fat, high fibre	4	vehicle
45	84/242 - 84/245	low fat, high fibre	24	vehicle
46	84/246 - 84/249	low fat, high fibre	4	ethanol
47	84/250 - 84/253	low fat, high fibre	24	ethanol

TABLE 1 (continued)

DESCRIPTION OF THE GROUPS OF ANIMALS.

GROUP NO.	ANIMAL NUMBERS	DIET	DURATION	DRUG
48 *		normal	4	none
49 *	84/264 - 84/273	normal	16	none
50 *	84/274 - 84/285	normal	24	none
51 *		normal	4	aprotinin
52 *	84/296 - 84/305	normal	16	aprotinin
53 *	84/306 - 84/317	normal	24	aprotinin
54 *		normal	4	indomethacin
55 *	84/328 - 84/337	normal	16	indomethacin
56 *	84/338 - 84/349	normal	24	indomethacin
57 *		normal	4	vehicle
58 *	84/354 - 84/358	normal	24	vehicle
59 *		normal	4	ethanol
60 *	84/363 - 84/367	normal	24	ethanol
61 *		high fat, low fibre	4	none
62 *	84/372 - 84/376 85/01	high fat, low fibre	16	none
63 *	84/377 - 84/382 84/901 - 84/904	high fat, low fibre	24	none
64 *		high fat, low fibre	4	aprotinin
65 *	84/393 - 84/402	high fat, low fibre	16	aprotinin
66 *	84/403 - 84/414	high fat, low fibre	24	aprotinin
67 *		high fat, low fibre	4	indomethacin
68 *	84/425 - 84/434	high fat, low fibre	16	indomethacin
69 *	84/435 - 84/446	high fat, low fibre	24	indomethacin
70 *		high fat, low fibre	4	vehicle
71 *	84/451 - 84/455	high fat, low fibre	24	vehicle
72 *		high fat, low fibre	4	ethanol
73 *	84/460 - 84/464	high fat, low fibre	24	ethanol
74 *		low fat, high fibre	4	none
75 *	84/469 - 84/473 85/11	low fat, high fibre	16	none
76 *	84/474 - 84/479 84/911 - 84/914	low fat, high fibre	24	none
77 *		low fat, high fibre	4	aprotinin
78 *	84/490 - 84/499	low fat, high fibre	16	aprotinin
79 *	84/500 - 84/511	low fat, high fibre	24	aprotinin
80 *		low fat, high fibre	4	indomethacin
81 *	84/522 - 84/531	low fat, high fibre	16	indomethacin
82 *	84/532 - 84/543	low fat, high fibre	24	indomethacin
83		low fat, high fibre	4	vehicle
84 *	84/548 - 84/552	low fat, high fibre	24	vehicle
85 *		low fat, high fibre	4	ethanol
86 *	84/557 - 84/561	low fat, high fibre	24	ethanol

* = carcinogen given

TABLE 1 (continued)

DESCRIPTION OF THE GROUPS OF ANIMALS.

GROUP NO.	ANIMAL NUMBERS	DIET	DURATION	DRUG
87	85/201 - 85/205	normal	16	vehicle
88	85/105 - 85/109	normal	16	ethanol
89	85/110 - 85/114	high fat, low fibre	16	vehicle
90	85/115 - 85/119	high fat, low fibre	16	ethanol
91	85/120 - 85/124	low fat, high fibre	16	vehicle
92	85/125 - 85/129	low fat, high fibre	16	ethanol
93 *	85/130 - 85/134	normal	16	vehicle
94 *	85/135 - 85/139	normal	16	ethanol
95 *	85/140 - 85/144	high fat, low fibre	16	vehicle
96 *	85/145 - 85/149	high fat, low fibre	16	ethanol
97 *	85/150 - 85/154	low fat, high fibre	16	vehicle
98 *	85/155 - 85/159	low fat, high fibre	16	ethanol

* = carcinogen given

GROUP NO.	ANIMAL NUMBERS	DIET	DURATION	DRUGS
1	83/10 - 83/13	normal	2	none
2	83/14 - 83/17	normal	2	aprotinin
3	83/18 - 83/21	normal	2	aprotinin
4	83/22 - 83/25	normal	2	aprotinin
5	83/26 - 83/29	normal	2	indomethacin
6	83/30 - 83/33	normal	2	indomethacin
7	83/34 - 83/37	normal	2	indomethacin
8	83/38 - 83/41	normal	2	indomethacin

These animals were used for a 2 week pilot study and are included for completeness for the group numbering.

TABLE 2

CALIBRATION OF SPECTROPHOTOMETER FOR p-NITROANILINE (p-NA)

p-NA (uM)	ABSORBANCE I	II	III	Mean +/- SEM
10	0.106	0.107	0.108	0.107 $\pm$ 0.000
20	0.211	0.213	0.213	0.212 $\pm$ 0.001
40	0.448	0.449	0.448	0.448 $\pm$ 0.000
50	0.500	0.500	0.501	0.500 $\pm$ 0.000
100	1.036	1.039	1.042	1.039 $\pm$ 0.001
200	1.800	1.812	1.818	1.810 $\pm$ 0.004

By linear regression, the slope of the line for values from 0 - 100mcM p-NA is 10343.367 . This is equivalent to the extinction coefficient.

$r = 0.999$

The graph of these values (Graph 1) shows that beyond 100 mcM the relationship ceases to be linear.

TABLE 3

LINEARITY OF ASSAY FOR TISSUE KALLIKREIN

WITH EXTRACT CONCENTRATION.

Concentration of extract	Absorbance (450nm)
1/10	0.910 ± 0.047
1/20	0.528 ± 0.036
1/40	0.298 ± 0.007
1/50	0.200 ± 0.026
1/100	0.155 ± 0.020

By linear regression : r = 0.996

slope = 8.557

TABLE 4

LINEARITY OF THE ASSAY FOR TKLA WITH TIME.

Time of Reaction (mins)	Absorbance 1/10 dil'n	1/20 dil'n
5	0.075 ± 0.003	0.044 ± 0.001
15	0.163 ± 0.001	0.102 ± 0.003
30	0.301 ± 0.002	0.165 ± 0.002
60	0.506 ± 0.007	0.290 ± 0.001
120	0.787 ± 0.005	0.567 ± 0.009

For 1/20 dilution : r = 0.998

For 1/10 dilution : r = 0.994 for values up to 60 mins

r = 0.986 for values up to 120 mins.

These results are shown in Graph 2.

TABLE 5

EFFECT OF pH ON THE ASSAY FOR TISSUE KALLIKREIN

pH (TRIS buffer)	ABSORBANCE Colon 1	Colon 2	Small Intestine
7.0	0.043	0.016	-----
7.5	0.069	0.072	-----
8.0	0.119	0.112	0.379
8.25	0.164	0.156	-----
8.5	0.188	0.176	0.408
8.75	0.215	0.216	-----
9.0	0.264	0.260	0.485
9.25	-----	0.316	-----
9.5	0.440	0.331	0.513
9.75	-----	0.382	-----
10.0	-----	0.417	0.638
10.5	-----	0.481	-----
11.0	-----	0.516	0.683

Notes:
1. The colon samples came from two animals.
2. The small intestine sample was included since the activity from it is higher and it has also been shown to have an optimum pH.
3. None of the samples examined here by the standard assay method showed a distinct optimal pH for the reaction (although the reaction was pH-dependent). The results are shown graphically in Graph 3.

TABLE 6

COEFFICIENT OF VARIATION OF THE ASSAY FOR TISSUE KALLIKREIN.

A single sample of colon extract was aliquoted and the activity assessed on several separate occasions. The results of each individual assay are shown below.

Aliquot	Absorbance
1	0.167
2	0.160
3	0.154
4	0.156
5	0.165
6	0.159
7	0.155
8	0.160
9	0.161
Mean	0.160
S.D.	0.004
Coefficient of variation	2.8%

TABLE 7

SPECIFICITY OF THE ASSAY FOR TISSUE KALLIKREIN-LIKE AMIDASE ACTIVITY

Assay Conditions	Absorbance (Mean +/- S.D.)
No SBTI	0.303 ± 0.009
SBTI 250ug/ml	0.276 ± 0.001
SBTI 500ug/ml	0.278 ± 0.001
SBTI 1000ug/ml	0.273 ± 0.002
No APROTININ	0.129 ± 0.002
APROTININ 3000KIU/ml	0.000

Notes : 1. SBTI = Soybean trypsin inhibitor.

2. SBTI and Aprotinin experiments carried out on different samples.

3. SBTI causes a reduction in measured TKLA by 8.9% which is significant at the 5% level

4. Aprotinin abolishes all activity in the sample.

TABLE 8

EFFECT OF HOMOGENISATION ON TISSUE PGE_2 MEASURED

Time of homogenising (mins)		3	5	10
Sample	1	880	906	854
	2	762	868	1018
	3	830	865	942
	4	824	658	801
	MEAN$\pm$SD	823$\pm$36	824$\pm$112	904$\pm$72

Values are ng $PGE_2.g^{-1}$ tissue

Samples were made from a pool of colonic tissue homogenate.

TABLE 9

EFFECT OF INCREASING INDOMETHACIN CONCENTRATION OF BUFFER IN ASSAY OF PGE_2.

Indomethacin concentration (mcg/ml)		10	20	50
Sample	1	614	542	618
	2	606	693	690
	3	818	727	706
	4	801	759	692
	5	820	837	600
	6	737	730	821
	MEAN$\pm$SD	732$\pm$25	714$\pm$81	688$\pm$65

Values are ng $PGE_2.g^{-1}$ tissue

Samples were made from a pool of colonic tissue homogenate.

TABLE 10

EFFECT OF DELAYING SEPARATION OF HOMOGENATE. ON LEVELS OF PGE_2 MEASURED.

SEPARATION:	Immediate	$4^{o}C$ overnight	Room temp. overnight
Sample 1	614	918	919
2	606	801	737
3	542	730	759
4	693	727	937
5	334	706	456
6	390	693	439
MEAN±SD	530±116	762±71	708±181

Values are ng $PGE_2.g^{-1}$ tissue

'Immediate' group less than other two groups by U-test ($p<0.05$)

Samples were made from a pool of colonic tissue homogenate.

TABLE 11

INTERASSAY COEFFICIENT OF VARIATION FOR ASSAY OF PGE_2.

SAMPLE	PGE_2 (pg)
1	107
2	84
3	138
4	67
5	67
6	73
7	112
8	102
9	87
MEAN	93
SD	22
CV	24%

The sample was made from a pooled sample of homogenate which was individually aliquoted and assayed in duplicate in the course of the routine assay.

RESULTS

GROWTH AND DEVELOPMENT OF THE ANIMALS

In total 575 animals were introduced into the study and all but 20 survived until sacrifice. The 20 premature deaths occurred in a variety of groups with no particular pattern except that three of the deaths occurred in the group receiving standard diet and indomethacin but no carcinogen. There was no obvious cause for any of the deaths and in particular none of the deaths could be attributed to carcinomatosis.

The growth of the animals is shown in summarised form in Tables 12a & b. This table shows the weights of the animals at the start of the experiment and at two later time periods - 16 weeks and 24 weeks the latter being the endpoint for the experiment. Groups of animals were sacrificed at 4, 16 and 24 weeks after the start of the experiment.

There were no significant differences in the weights of the animals at the start of the experimental period. The mean starting weight was 250g with a range for the means of 231g to 279g for each of the groups. The animals receiving the high fat, low fibre diet gained most weight over the experimental period (129g in 24 weeks) and those on the low fat, high fibre diet gained least. (77g in 24 weeks) Administration of the carcinogen significantly reduced the weight gain in all groups of animals (73g in high fat, low fibre group, 56g in low fat, high fibre group) and was characterised by a sharp fall in the weight of the animals after the start of carcinogen treatment followed by a slow recovery period. The animals receiving their standard diet

gained 118g during the 24 week experimental period and 105g when the carcinogen was administered.

The daily injections of aprotinin were well-tolerated by the animals but those on the low fat, high fibre diet did not gain as much weight as their contemporaries on other treatment. (non-carcinogen group: 66g vs 77g; carcinogen-treated group: 39g vs 56g) It was found from a pilot experiment that injection of aprotinin in a dose higher than that used in this study (50000 and 100000 $KIU.kg^{-1}$ vs 20000 $KIU.kg^{-1}$) was poorly tolerated. On the higher doses animals rapidly lost weight and failed to thrive. None of the other drugs affected the growth of the animals.

The diet which the animals consumed was measured for each of the groups as described. (Table 13) Over a 24 week period the animals on the high fat diet consumed an average of 13.6g of diet per day while those on the low fat diet consumed 29.2g per day. With the knowledge of the energy content of the diet this means that the animals on the high fat diet consumed 218KJ per day and those on the low fat diet consumed 216KJ per day. Those on the standard laboratory diet ate 19.6g of their diet per day (= 235KJ). Administering the experimental drugs did not significantly alter the dietary intake although there was an apparent significant increase in the consumption of the high fat diet by the animals receiving the control injection of saline and ethanol in drinking water. In the animals receiving the carcinogen there was a bipartite effect. Those on the high fat

diet showed a significant increase in the dietary intake (+9.2g/day) while those on the low fat diet showed a reduction in the intake (-9.0g/day). This was also seen in the animals receiving aprotinin and indomethacin. There was an apparent decrease in the dietary intake in the groups receiving a high fat, low fibre diet and saline or ethanol when given carcinogen compared with the untreated controls but the values in the carcinogen group do not differ from those on the other treatments.

These changes necessarily produce a change in caloric intake which is increased to 367KJ/day for the animals on the high fat, low fibre diet and decreased to 149KJ/day for the animals on the low fat, high fibre diet when the carcinogen was administered.

The animals on their standard diet showed both increases and decreases between the carcinogen and non-carcinogen group. The untreated controls showed a net increase in dietary consumption (+3.7g/day) when given carcinogen leading to an increased caloric intake of 279KJ/day.

The water intake was broadly similar across all the groups but did tend to be higher in the low fat, high fibre group. In the animals receiving indomethacin there were no significant differences between any of the groups and the animals drank an average of 25.3 ml of water/indomethacin solution per day. This provided a dose of 0.51mg of indomethacin per rat.day (=2.02mg/kg for a 250 g rat; target dose = 2mg/kg for 250 gram

rat.) This implies that at the end of the 24 week period the rats were receiving a dose of about 1.3 - 1.6mg/kg.day . It was noted that the animals consumed more diet and water at a younger age than at later stages.

DISTRIBUTION OF KALLIKREIN ACTIVITY IN THE COLON

The tissue kallikrein-like amidase activity (TKLA) was measured by the method described at five levels in the colon corresponding to the caecum, proximal colon, mid colon, distal colon and rectum. These are referred to as segments A, B, C, D, and E respectively. The results are shown in Tables 14a - c which illustrate the mean values of the colonic TKLA for each colon segment at each time period for the different drug treatments. Each diet is considered separately. The results from the specialised diets will be presented first and then comment on the animals receiving the standard diet will be made.

In the animals receiving the two specialised diets - namely the high fat, low fibre and low fat, high fibre diet - it is clear that the tissue kallikrein-like amidase activity is distributed in a unique pattern along the colon with the level of activity in the caecum being significantly higher than elsewhere in the colon. (tables 14a & b) Values in the caecum are in the order of three to five times greater than anywhere else in the colon. The values tend to decrease from the proximal colon to rectum but there is no consistent pattern present and no significant differences are present between any of the segments B to E. The pattern described above is also seen in animals receiving their standard laboratory diet. (table 14c)

Analysis of the multiple subgroups and comparison with their respective controls and with each other suggests that differences may exist between the groups but these do not occur

in any regular, predictable fashion. For example, in the animals on the high fat diet, if the group receiving aprotinin is compared with the saline control group then it is possible to demonstrate in colon segments A, B, D & E that there is an apparent reduction in the kallikrein activity at 4 weeks. However, this is not readily demonstrated at the 16 week period but there is a suggestion that it is present again at the 24 week period in segments C, D & E. A similar set of results is possible for the low fat diet group.

In an attempt to bring some form to these results it was necessary to reduce the number of comparisons to the ones which were of greatest importance. These comparisons examined:

1. If there were any systematic differences between the "control" groups?

2. If there were any systematic effects of diet on the levels of kallikrein activity measured?

3. If there were any effects of drug treatment on the levels of kallikrein activity measured.

4. If there were any other interactions which affected the levels of kallikrein activity measured?

Since it was apparent that there were no constant differences between the colonic segments B, C, D & E then comparisons were made between segment A and a pooled figure for segments B - E which was average of all the B - E values. Additionally since there were no clear demonstrable differences between the time periods examined then the values at 4 and 16

weeks were merged and compared with the 24 week values. This is of some use in examining the group receiving carcinogen treatment since this covers the period during tumour induction or initiation and then the period of tumour growth or promotion. The values generated by this are shown in Table 15.

1. THE CONTROL GROUPS. A comparison of the saline and ethanol control groups with the group of animals receiving no drug treatment at all has shown that they do not differ significantly from each other in their levels of kallikrein activity. The probability values for each of the comparisons is shown in the Table 16. These values have been derived by the statistical methods described. The only significant effect demonstrated is that of segment (segment A higher than the rest) as has been previously noted. With this knowledge the control groups were merged into one large group for further comparisons. Each of these placebo groups remained distinct for the time period, use of carcinogen and colon segment under study.

2. THE EFFECT OF DIET. These results are shown in Table 17. Direct comparison of the TKLA between the animals receiving the high fat, low fibre diet and the TKLA of the animals receiving the low fat, high fibre diet has shown that for any given group (controls, aprotinin, indomethacin or carcinogen) there is no appreciable effect of diet on any differences observed. ($p = 0.1250$) In other words, segment for segment and treatment for treatment there is no systematic effect

of diet on the levels of TKLA measured. This is apparent from the figures in table 17 which shows a broad similarity of the results in each of the two specialised dietary groups. A single discrepancy is seen in the case of the low fat, high fibre diet receiving indomethacin at 4 & 16 weeks where the caecal TKLA is lower in the group receiving carcinogen than in either the non-carcinogen control or the levels in the high fat, low fibre group. (34 ± 20 vs 106 ± 66 & 189 ± 59 nmolpNA.g^{-1}.min^{-1} respectively; $p < 0.005$).

The animals receiving their standard diet have not been demonstrated as being significantly different from those on the specialised diets.

It would therefore appear that there is little if any effect of the diet on the colonic TKLA and therefore for further analysis the two specialised dietary groups will be considered as one but the animals on their standard diet will be considered separately.

3.THE EFFECT OF DRUG TREATMENT. The results for this analysis are shown in table 18. The animals receiving the drug treatment (aprotinin or indomethacin) are compared against the pooled control groups for each of the two time periods (4 & 16 weeks versus 24 weeks).

In the group which did not receive carcinogen the mean TKLA in the caecum was higher than the other segments at both time periods and in all groups. ($p < 0.0001$) In the group receiving aprotinin the mean TKLA was significantly lower than

the placebo in all segments of the colon at 4 & 16 weeks (116 ± 58 vs 152 ± 38 nmolpNA.g^{-1}.min^{-1} for caecum; 25 ± 6 vs 39 ± 20 nmolpNA.g^{-1}.min^{-1} for distal colon; $p < 0.0001$) and in the distal colon segments at 24 weeks. (30 ± 5 vs 58 ± 21 nmol.pNA.g^{-1}.min^{-1}; $p < 0.0001$) The TKLA in the animals receiving indomethacin did not differ significantly from their controls. ($p > 0.05$) In the group which received the carcinogen treatment as well as the drugs there were no differences between the treated (aprotinin or indomethacin) and control groups. The significant reduction ($p <0.005$) in the TKLA of the A (caecal) segment in the low fat, high fibre group receiving indomethacin for 4 or 16 weeks was noted above. This was not seen at 24 weeks and in the pooled analysis there was no significant effect of indomethacin on the TKLA (table 18) The carcinogen treated placebo group did not differ from the one which did not receive carcinogen treatment.

In the animals receiving their standard diet (table 17) the TKLA was reduced in the groups receiving aprotinin at both time periods when compared with the placebo group. (Caecum: 136 ± 27 vs 161 ± 64 & 119 ± 21 vs 150 ± 57; Distal segments: 26 ± 17 vs 37 ± 22 & 24 ± 12 vs 33 ± 18; $p<0.05$ for each pairing) The carcinogen groups also showed a reduced TKLA in comparison with the placebo.

Administering indomethacin also resulted in a reduced TKLA in the 4 & 16 week group on standard diet (caecum: 122 ± 50 vs 161 ± 64; distal colon 27 ± 15 vs 37 ± 22, $p<0.05$) but this was not seen at the 24 weeks stage or in the carcinogen treated animals.

4. OTHER EFFECTS The analysis showed that there was a significant interaction between the segment of colon and the time ($p < 0.01$). This emphasises that the effect of aprotinin is not seen at 24 weeks in the caecum but is seen prior to this and is seen in the distal colon at both time intervals. This has been described above. None of the other variables - diet, drug or carcinogen treatment - interact nor do any permutations of these with colon segment or time.

In summary, it has been shown that the regular, daily injection of aprotinin significantly reduces the detectable tissue kallikrein-like amidase activity of the rat colon at all the levels examined. With the possible exception of one group of animals receiving indomethacin for 4 to 16 weeks no other agent altered the levels of tissue kallikrein-like amidase activity to a significant degree. This effect was more marked in the earlier stages of the experiment. Dietary change was without effect. The animals on their standard diet demonstrated the same changes.

DISTRIBUTION OF PROSTAGLANDIN IN THE COLON

The tissue levels of prostaglandin were measured as prostaglandin E_2 (PGE_2) by the methods described at the same five levels of the colon - A to E - as for the tissue kallikrein-like amidase activity. (The segment of colon was split longitudinally and one half used for tissue kallikrein-like amidase activity estimation, the other for PGE_2 estimation). The results are shown in Tables 19a - c which illustrate the mean values of the colonic PGE_2 concentration for each colon segment at each time period for the different drug treatments. Each diet is considered separately. As before, the results for the specialised diets will be presented first and then the results from the standard diet.

In the animals receiving the two specialised diets - the high fat, low fibre and low fat, high fibre diet - the colonic content of PGE_2 varies along the colon such that the lowest levels are found in the caecum (segment A) and the highest levels in the more distal segments (B to E). (tables 19a & b) The PGE_2 content of the distal segments does not vary in any predictable fashion and none of these segments differ significantly from each other. While there are values which appear to be different from each other the spread of results is very great encompassing a tenfold difference within one group. On average the values for the distal colon are twice those of the caecum. Animals receiving their standard diet demonstrated a similar gradient of PGE_2 content along the colon with the highest levels being in the distal colon. (table 19c)

As with the results of the tissue kallikrein-like amidase activity, analysis of the multiple subgroups and comparison with their respective controls for the tissue prostaglandin content produces a set of statistics suggestive of differences which may be present but not in any readily identifiable fashion. For example, in the animals receiving the high fat, low fibre diet, those receiving indomethacin may have reduced levels of PGE_2 after 24 weeks treatment but not at 4 or 16 weeks and not necessarily in every segment examined. In the same way as for the results of the analysis of the tissue kallikrein-like amidase activity it was necessary to reduce the comparisons made to those which were of greatest importance and which corresponded to those made for the tissue kallikrein-like amidase activity analysis.

These comparisons examined:

1. If there were any systematic differences between the "control" groups?

2. If there were any systematic effects of diet on the levels of PGE_2 measured?

3. If there were any effects of drug treatment on the levels of PGE_2 measured.

4. If there were any other interactions which affected the levels of PGE_2 measured?

Since it was again apparent that there were no detectable differences between the colonic segments B - E then comparisons were made between segment A and a pooled figure for segments B - E which was the average of all the B - E values. As before the

values for the 4 and 16 week periods were pooled and compared against the 24 week value. These values are shown in Table 20

1. THE CONTROL GROUPS. A comparison of the ethanol and saline control groups with the group of animals receiving no drug treatment has shown that they do not differ from each other as far as the treatments or diets are concerned but differ between the time periods examined and the use of carcinogen. The probability values for these comparisons are shown in table 21 and are derived by the statistical methods described. The animals treated with carcinogen have significantly higher levels of prostaglandin than do the non-carcinogen control animals ($p < 0.0001$). There is a significant effect of segment also as noted above (segment A lower than the rest). While these differences do exist it is still possible to pool the control groups as before to form one control group for each time stage and for the carcinogen treatment as was done for the analysis of TKLA.

2. THE EFFECT OF DIET Comparison between the PGE_2 content of the colon in the animals on the high fat, low fibre diet and the PGE_2 content of the colon of the animals on the low fat, high fibre diet shows that there is no no appreciable effect of diet on any differences observed for any given group (control, indomethacin, aprotinin or carcinogen; table 22, $p = 0.1575$) In other words, segment for segment and treatment for treatment there is no systematic effect of diet on the levels of PGE_2 measured. This is apparent from the figures in

table 22 which shows a broad similarity of the results in each of the two specialised dietary groups. For example, in the placebo groups at 4 & 16 weeks the caecal colonic PGE_2 is $93 \pm 35 ng.g^{-1}$ in the high fat, low fibre group and is $93 \pm 26 ng.g^{-1}$ in the low fat, high fibre group; the distal segments have corresponding values of 179 ± 119 and $170 \pm 90 ng.g^{-1}$. This reflects the relatively higher levels in the distal colon. The range of values obtained for some of the colonic PGE_2 analysis was wide and is reflected in the large standard deviations for some groups. Consequently, the range of mean values is great but it is impossible to separate the groups statistically. The animals receiving their standard diet have not been shown to be significantly different from those on the specialised diets.

For further analysis the two specialised dietary groups will be considered together but the animals on their standard diet will be considered separately.

3. THE EFFECT OF DRUG TREATMENT. The results for this analysis are shown in table 23. The animals receiving the drug treatment (indomethacin or aprotinin) are compared against the pooled control groups for each of the two time periods (4 & 16 weeks and 24 weeks).

In the group which did not receive the carcinogen the mean prostaglandin content expressed as PGE_2 was lower in the caecum than in the other segments at both time periods and in all groups. ($p < 0.0001$) In the group receiving indomethacin a significant ($p < 0.005$) reduction was seen in the colonic PGE_2 in

the distal colonic segments but not in the caecum. (83 ± 36 vs 94 ± 32 ng.g^{-1} for caecum; 141 ± 57 vs 176 ± 88 ng.g^{-1} for distal colon at 4 & 16 week stage; 56 ± 23 vs 52 ± 20 ng.g^{-1} for caecum; 87 ± 36 vs 121 ± 30 ng.g^{-1} for distal colon at 24 weeks) In the group receiving aprotinin there was no difference in the measured levels of PGE_2 from those in the control group.

In the group receiving the carcinogen as well as indomethacin a significant reduction in the content of PGE_2 was seen when compared with the control groups receiving carcinogen at the combined 4 & 16 week stage (caecum: 95 ± 31 vs 191 ± 125 ng.g^{-1}; distal colon 145 ± 31 vs 259 ± 117ng.g^{-1}, $p < 0.025$). This was also evident at 24 weeks in the distal colon (144 ± 46 vs 191 ± 66 ng.g^{-1}, $p < 0.025$) but was not detectable in the caecum. (87 ± 59 vs 105 ± 44ng.g^{-1}, N.S.D.) In the group receiving aprotinin and carcinogen treatment there was a significant difference between the treated group and the placebo at the 4 & 16 week stage with the aprotinin group having a significantly lower PGE_2 content. ($p < 0.05$).

Comparison between the two drug-treated groups overall showed that the levels of PGE_2 were significantly lower in the indomethacin group ($p < 0.0006$) although in individual drug-segment-time combinations they may not appear so: e.g. aprotinin + carcinogen at 4 and 16 weeks compared to indomethacin + carcinogen at the same stage. (Caecum: 92 ± 24 vs 95 ± 31; distal colon: 113 ± 19 vs 145 ± 31ng.g-1; aprotinin vs indomethacin.)

4. OTHER EFFECTS Analysis of the interactions between the

various experimental components shows that the other major variable to determine the PGE_2 levels is the use of carcinogen. (table 23) The use of carcinogen was associated with higher levels of PGE_2 in the colon than in the untreated (non-carcinogen) groups. (e.g. placebo group, caecum: 191 ± 125 vs $94 \pm 32 ng.g^{-1}$ (4 & 16 wks); 105 ± 44 vs $52 \pm 20 ng.g^{-1}$ (24 wks); distal colon: 259 ± 117 vs $176 \pm 88 ng.g^{-1}$ (4 & 16 wks); 191 ± 66 vs $121 \pm 30 ng.g^{-1}$ (24 wks); carcinogen vs non-carcinogen, $p < 0.0005$)

There was also a significant interactive effect between the use of drug and the length of time it was used but this effect was rather more obscure than it appeared statistically. If animals not receiving carcinogen are considered then prolonged drug treatment (aprotinin & indomethacin) seemed to result in relatively lower levels of PGE_2 at 24 weeks than at 4 & 16 weeks. However, a similar fall in the PGE_2 levels was also seen in the placebo groups. This was not evident in the animals receiving carcinogen treatment.

In summary, it has been shown that the levels of colonic PGE_2 were significantly reduced by the use of indomethacin when compared with untreated controls. The weekly injection of a carcinogen produced a rise in the colonic PGE_2 when compared with animals which did not receive carcinogen treatment. The absolute levels of PGE_2 seemed to fall with increasing age of the animals - an effect which was abolished by carcinogen treatment.

CRYPT CELL PRODUCTION RATES

The crypt cell production rates for the various groups were calculated by the stathmokinetic method as described. From the individual metaphase counts the regression line has been calculated and this is presented in tables 24a - c for each of the time periods and drug combinations. Each diet has been presented separately.

It became clear during the counting of the crypt cell dissections that the preparations made from the region of the colon labelled B (proximal colon) never produced satisfactory specimens for counting despite repeated attempts. The reason for this is not clear but has been recognised by other workers and presumably relates to the structure of the mucosa which does not lend itself to microdissection. Consequently it has been omitted from all further analysis.

The results have been examined both individually and in the groupings previously described for the kallikrein and prostaglandin studies.

Unlike the pattern seen in the autacoid studies no significant differences were seen between the segments (segment A versus segments C, D & E ; $p = 0.87$). There was no discernible effect of diet on the crypt cell production rate (high fat, low fibre vs low fat, high fibre; $p = 0.30$) Similarly no differences between the groups was seen with time of the experiment (4 & 16 weeks vs 24 weeks; $p = 0.76$) Administration of the carcinogen did not affect the crypt cell turnover ($p = 0.36$). However the

administration of aprotinin and indomethacin did appear to reduce the crypt cell turnover when compared with the control ($p < 0.05$) but both drugs seemed to produce the effect to the same extent ($p = 0.43$).

Thus it has proved impossible to demonstrate any effect of diet on the crypt cell production rates (CCPR) in the colon nor to demonstrate any variation in the CCPR along the colon. However the administration of the drugs is associated with a significant reduction in the CCPR.

FAECAL CONSTITUENTS.

The results of the analysis of the faecal bile acids, faecal neutral sterols and fatty acids are presented in tables 25a and 25b. The data are presented under three broad headings - neutral steroids, long chain fatty acids and free bile acids. Each value is the mean of several samples of faeces taken from the colon of the rats at the time of sacrifice. The samples were only distinguished by the diet the animals consumed and whether the animal received the carcinogen. This analysis takes no account of any other drug treatment they may have received. Figure 4 illustrates diagramatically the relative proportions of the various components for each of the dietary groups. The data used for this included both the non-carcinogen and carcinogen-treated groups.

It is clear from Tables 25a & b that distinct differences exist between the faecal compositions of the different dietary groups. The animals receiving the high fat, low fibre diet have the highest levels of sterols, fatty acids and bile acids of any of the groups. The animals receiving the low fat, high fibre diet have the lowest levels of these substances of the three groups. This difference is seen whether or not the animal had received the carcinogen. The most emphatic difference is seen in the total faecal fatty acid content which is between 14 and 20 times greater in the high fat, low fibre group than in the low fat, high fibre group. The sterol and bile acid concentrations of the high fat group are three times those of the low fat diet.

In the animals which did not receive the carcinogen (Table 25a) the total sterol concentration of animals on the high fat, low fibre diet was $9.85 \pm 2.65 mg.g^{-1}$. This was significantly higher than either of the other two dietary groups ($p<0.005$). The total faecal bile acid concentration of the high fat, low fibre dietary group was $4.59 \pm 2.62 mg.g^{-1}$ which was significantly greater than the low fat, high fibre group ($1.58 \pm 1.12 mg.g^{-1}$, $p<0.005$) but not different from animals on their standard diet.

In the animals which received carcinogen the total sterol concentration was significantly higher in the high fat, low fibre group than in either of the other two diet groups. ($9.00 \pm 1.65 mg.g^{-1}$ vs $2.28 \pm 0.73 mg.g^{-1}$ & $4.33 \pm 2.64 mg.g^{-1}$; high fat vs low fat & standard diet respectively, $p<0.005$) Similarly, the total faecal bile acid concentration of the high fat, low fibre dietary group ($4.77 \pm 2.45 mg.g^{-1}$) was significantly higher than either of the other two dietary groups. (low fat, high fibre diet = $1.39 \pm 0.36 mg.g^{-1}$; standard diet = $1.72 \pm 2.05 mg.g^{-1}$; $p<0.005$)

In the animals which did not receive carcinogen (Table 25a) the levels of total sterols and total bile acids were greater in the animals on their standard diet than those on the the low fat, high fibre diet. (Sterols: 6.02 ± 1.39 vs $3.61 \pm 3.02 mg.g^{-1}$; bile acids: 4.09 ± 2.35 vs $1.58 \pm 1.12 mg.g^{-1}$; $p<0.005$ for both comparisons) However, the faecal fatty acid composition of animals on their standard diet was no different to that of animals on a low fat, high fibre diet. (Table 25a) When

the animals received carcinogen (Table 25b) the faecal sterols and fatty acids of the animals on their standard diet were significantly greater than those of animals on the low fat, high fibre diet. (Sterols: $4.33 \pm 2.64 mg.g^{-1}$ vs $2.28 \pm 0.73 mg.g^{-1}$; fatty acids: $4.74 \pm 2.29 mg.g^{-1}$ vs $1.93 \pm 1.48 mg.g^{-1}$; standard diet vs low fat, high fibre diet, $p<0.05$) The total bile acid concentration of the animals on their standard diet and receiving carcinogen was lower than those not receiving carcinogen (1.72 ± 2.05 vs 4.09 ± 2.35 $mg.g^{-1}$; $p<0.025$) and was no different from animals on the low fat, high fibre diet.

Study of the individual sterols, fatty acids and bile acids reveals that the differences between the diets were due to alterations in all components of the totals. However the elevated total bile acids of the high fat, low fibre group were largely due to an increase in the concentrations of deoxycholic acid and muricholic acids. (fig 4) The animals on their standard diet seemed to have a relative excess of unsaturated fatty acids in comparison to the other dietary groups. (fig 4)

Reviewing the raw data for these results it was clear that the effect of the diet on the faecal constituents was so dominant that it was impossible to distinguish if any of the other treatments produced an effect on the faecal composition.

TUMOUR INDUCTION

Histological assessment of the colonic mucosa was carried out routinely in all animals under study. Additional samples of any macroscopic mucosal abnormality seen at the time of sacrifice were taken. All the histological examination was carried out by one individual.

In the animals not receiving carcinogen treatment there were no histological effects of any of the drug treatments at any of the time periods examined. In particular, no evidence of tumour formation was seen.

In the animals receiving the carcinogen treatment no tumours were seen at the 16 weeks stage (the end of the course of carcinogen injections.) Tumours were seen in all dietary groups at 24 weeks and these are detailed in table 26. This table lists all the histologically confirmed lesions and their nature. Colonic lesions were recorded at a distance from the ileocaecal valve. Table 27 shows the number of animals with a tumour in each group . All neoplastic lesions are counted as representing tumour formation since colonic lesions are essentially never seen in the untreated rat colon. The subsequent table (Table 28) lists the numbers of tumours developed by each group. The typical appearance of the tumours is shown in figure 5.

Documented colonic lesions included tubular adenomas (17), tubulovillous adenomas (3), villous adenomas (2) and adenocarcinomas (32). Fifteen of the adenocarcinomas were classified as being signet-ring cell type. All degrees of

invasion of carcinomas were seen from dysplastic foci with carcinoma-in-situ to carcinomatosis peritonei. (Figure 6) Adenocarcinomas were seen arising in adenomas. Primary tumours of the small bowel and duodenum were also seen - 6 adenocarcinomas and 1 tubulovillous adenoma.

In total 62 primary neoplastic lesions were documented. These lesions occurred in 46 rats. Tumours developed more commonly in the the animals receiving the high fat, low fibre diet than in the animals on the low fat, high fibre diet. (X^2 = 4.140, $p < 0.05$). The animals receiving their standard diet developed most tumours with a marginally raised frequency over those on the high fat diet. (44% vs 41%, N.S.)

As regards the different treatment subgroups three differences emerged. Firstly, no tumours developed in the animals receiving the ethanol vehicle which is significantly different from the untreated controls or saline vehicle controls in any of the dietary groups. (X^2 = 10.91, $p < 0.05$) (Table 27)

Secondly, in the group receiving the low fat, high fibre diet and aprotinin only 2 out of 12 animals developed tumours which is less than the frequency of tumour development in the saline controls taken as a group (X^2 = 3.84, $p < 0.05$). If the untreated controls are included in this analysis then the difference is still apparent (X^2 = 5.74 , $p < 0.05$).

Thirdly, also in the low fat, high fibre group, none of the animals receiving indomethacin developed tumours. This is not different from the ethanol control group but does differ from

the other controls ($X^2 = 5.31$, $p < 0.05$).

Apart from the absence of tumours in the previously noted groups there were no significant differences in the number of tumours developing per animal. (Table 28)

The situation of the tumours was examined by measuring their distance from the ileocaecal valve. (Table 26; fig 7) Tumours in the animals on the high fat, low fibre diet occurred more frequently in the proximal colon (at 9.3 ± 4.8cm from ileocaecal valve) than did tumours in the animals on either of the other diets. (Low fat, high fibre diet 12.6 ± 6.7cm; standard diet 14.8 ± 5.2cm ; high fat vs low fat and standard diet, $p < 0.05$, U-test.)

In summary, tumour development was only seen in the animals receiving carcinogen treatment and after 24 weeks of the experiment. All varieties of neoplasm were encountered. There was a distinct dietary effect on tumour incidence which was reduced in the low fat, high fibre diet groups. There was also a suggestion that some of the drug treatments reduced the tumour incidence. Aprotinin reduced tumour incidence in the low fat, high fibre diet group and no animal receiving ethanol developed a tumour. The effect of indomethacin was therefore not apparent. The distribution of the tumours in the colon was affected by the diet being more proximal in the high fat, low fibre group.

Finally, in an effort to relate the studies on the autacoid measurements and the occurrence of tumours the tissue kallikrein-like amidase activity and the PGE_2 content of the

colon was compared between the animals which actually developed tumours and those which did not. This was only done for the 24 week period since tumours were only seen at this stage and Table 29 shows the results. Since the numbers in each subgroup become small all the dietary groups and treatment groups are considered together. There is no significant difference between the measured levels of colonic TKLA or PGE_2 when the tumour-bearing animals are compared with the non-tumour bearing animals in either the caecum or distal colonic segments.

INDOMETHACIN MEASUREMENT

Indomethacin was measured by the method described in a random selection of plasma samples taken from the 14 different rats at the time of sacrifice. By this method indomethacin was shown to be present in the plasma of the animals at the time of sacrifice in a concentration of between 0.5 and 4 $mcg.ml^{-1}$. (mean indomethacin concentration $1.00 \pm 0.9 mcg.ml^{-1}$, Table 30).

TABLES 12 -30

TABLE 12a

GROWTH OF THE ANIMALS ON THE VARIOUS DIET AND DRUG COMBINATIONS

NO CARCINOGEN ADMINISTERED

DIET	TIME	DRUG TREATMENT NONE	APROTININ	INDOMETHACIN	SALINE	ETHANOL	MEAN
HIGH FAT LOW FIBRE DIET	0	279±37	249±23	273±37	262±21	241±17	263±32
	n =	20	30	30	13	13	106
	16	364±16	351±24	362±41	368±26	371±11	360±28
	n =	16	20	19	9	4	68
	24	382±31	390±22	391±27	400±23	401±13	392±23
	n =	4	10	10	4	4	32
LOW FAT HIGH FIBRE DIET	0	240±23	240±29	231±30	242±18	249±19	239±26
	n =	16	30	30	13	13	102
	16	323±19	315±13	305±24	321±13	314±13	314±18
	n =	12	20	19	9	9	69
	24	352± 7	304±11	306±20	334±15	312±11	316±22
	n =	4	10	9	4	4	31
STANDARD DIET	0	244±20	247±12	266±31	242±11	239± 8	247±30
	n =	30	30	30	18	13	121
	16	338±34	351±15	357±30	345±14	345±18	348±26
	n =	20	20	18	9	9	96
	24	341±19	365±13	391±54	370±23	377±29	365±34
	n =	10	9	7	4	4	34

Values are mean weights (g)±SD; Time in weeks;

Column headed "Mean" shows mean weight regardless of treatment

TABLE 12b

GROWTH OF THE ANIMALS ON THE VARIOUS DIET AND DRUG COMBINATIONS

CARCINOGEN ADMINISTERED

DIET	TIME	DRUG TREATMENT NONE	APROTININ	INDOMETHACIN	SALINE	ETHANOL	MEAN
HIGH FAT LOW FIBRE DIET	0	252±29	265±12	280±15	294±22	234±18	261±24
	n =	16	22	22	10	10	80
	16	320±26	323±14	328±35	323±10	315±20	323±25
	n =	15	11	21	10	10	67
	24	329±32	351±18	353±24	347± 6	333±18	344±24
	n =	9	10	12	5	5	41
LOW FAT HIGH FIBRE DIET	0	243± 9	269±30	252±37	251±37	248±30	255±31
	n =	16	22	22	10	10	80
	16	284±26	292±19	293±26	288±39	287±21	289±26
	n =	15	20	20	10	10	75
	24	288±18	308±20	328±18	300±20	326±20	311±24
	n =	9	12	12	5	5	43
STANDARD DIET	0	243±26	234±24	232±32	247±25	225±26	236±27
	n =	22	22	22	10	10	86
	16	318±32	304±24	306±20	319± 8	296±24	309±26
	n =	22	21	22	9	10	84
	24	348±21	335± 9	328±17	358±12	349±24	341±20
	n =	12	11	12	5	5	45

Values are mean weights (g)±SD; Time in weeks;

Column headed "Mean" shows mean weight regardless of treatment

TABLE 13

CONSUMPTION OF DIET AND WATER BY THE ANIMALS

DIET	DRUG TREATMENT				
	NONE	APROTININ	INDOMETHACIN	SALINE	ETHANOL
HIGH FAT, LOW FIBRE DIET					
DIET	13.6±1.5	13.2±2.4	11.8±1.4	22.9±1.0	21.4±0.9
	22.8±1.0	*23.6±1.1*	*21.6±0.9*	*19.5±0.9*	*18.8±0.7*
WATER	26.9±5.1	22.7±7.1	24.1±7.1	24.2±4.0	22.5±2.8
	26.7±8.6	*24.2±4.1*	*21.4±4.7*	*19.0±4.9*	*21.1±3.1*
LOW FAT, HIGH FIBRE DIET					
DIET	29.2±2.0	27.6±1.6	29.8±1.2	27.5±1.9	27.4±1.6
	20.2±1.2	*19.9±1.1*	*22.8±1.0*	*17.8±1.7*	*18.7±1.3*
WATER	31.4±4.2	26.5±3.5	26.8±1.9	30.8±4.9	30.9±3.6
	21.9±4.2	*27.0±3.4*	*27.4±3.0*	*23.2±3.8*	*23.5±3.0*
STANDARD DIET					
DIET	19.6±1.9	19.9±0.9	21.4±2.1	23.8±0.6	23.1±0.7
	23.3±1.1	*23.9±0.6*	*22.7±1.4*	*19.8±2.1*	*22.0±2.1*
WATER	32.0±3.8	25.1±4.9	28.9±5.4	30.0±5.6	30.0±5.6
	25.5±3.2	*24.4±2.9*	*23.3±2.8*	*18.1±1.0*	*21.2±2.7*

Values in italics are for animals receiving carcinogen treatment.

Diet consumption is in g.animal day^{-1}

Water consumption is in ml.animal day^{-1}

Values are mean ± SD

TABLE 14a

KALLIKREIN ACTIVITY IN THE RAT COLON

DIET: High Fat, Low Fibre diet

COLON SEGMENT:		CAECUM		PROX. COLON		MID COLON		DIST. COLON		RECTUM		n	
CARCINOGEN	:	NO	YES	NO	YES	NO	YES	NO	YES	NO	YES	NO	YES
DRUG	TIME												
NIL	4	118±34	------	46±22	-----	33±13	-----	56±27	-----	32± 7	-----	4	-
	16	164±41	210±48	81±32	98±38	50±15	46±16	63±20	67±25	32± 7	34± 5	8	6
	24	38±23	167±46	34± 7	65±27	23±6	48±30	57±25	52±17	19± 7	29±10	4	9
APROTININ	4	78±48	------	35±15	-----	32±11	-----	31±14	-----	12± 5	-----	7	-
	16	119±32	141±102	26± 7	72±67	21± 4	57±50	25± 8	69±60	16± 7	51±38	6	6
	24	104±72	170±39	48±31	58±35	25± 8	23± 9	27± 6	40±12	20±12	24± 9	6	5
INDOMETHACIN	4	132±36	------	36±18	-----	30±10	-----	42±13	-----	19± 7	-----	8	-
	16	190±66	176±56	74±41	111±82	43±15	61±67	45±14	72±52	23± 7	39±35	6	5
	24	145±46	181±45	46±17	57±41	41± 5	26±12	37±12	28±12	31±16	33± 9	6	6
SALINE	4	172±55	------	64±28	-----	50±14	-----	63±24	-----	26± 4	-----	4	-
VEHICLE	16	139±20	182±38	24± 5	35±14	22± 3	34± 6	24± 6	49± 8	10± 4	21± 7	5	5
	24	125±69	217±66	66±44	54±19	60±15	31±11	66±25	37±21	50±26	26±12	4	5
ETHANOL	4	161±29	------	50±20	-----	22± 8	-----	57± 7	-----	23± 6	-----	4	-
VEHICLE	16	158±18	180±30	22± 5	38±16	24± 4	36±11	26± 9	48±20	12± 3	21± 4	5	5
	24	222±15	202±87	98±40	46±28	75±15	32± 9	90±26	56±13	67±34	29±15	4	6

Values shown are mean ± standard deviation

Kallikrein activity expressed as $nmolpNA.min^{-1}.g^{-1}$

Time expressed as weeks

TABLE 14b

KALLIKREIN ACTIVITY IN THE RAT COLON

DIET: Low Fat, High Fibre diet.

COLON SEGMENT:		CAECUM		PROX. COLON		MID COLON		DIST. COLON		RECTUM		n	
CARCINOGEN	:	NO	YES	NO	YES	NO	YES	NO	YES	NO	YES	NO	YES
DRUG	TIME												
NIL	4	135±17	------	57±21	-----	40±14	-----	45±22	-----	24± 9	-----	3	-
	16	132±50	185±47	51±33	48±18	45±32	37± 6	63±55	36± 8	28±26	33± 5	8	6
	24	152±53	160±24	101±26	35±14	56±12	33± 9	47±17	42± 8	32±15	23±10	4	6
APROTININ	4	114±75	------	30±17	-----	18± 4	-----	23±10	-----	24±10	-----	8	-
	16	162±32	136±53	24± 9	44±17	25± 2	26±10	36±11	31±13	19±10	30±18	6	6
	24	173±47	164±80	34±11	54±41	31±13	55±51	30±10	48±37	22± 7	36±23	6	6
INDOMETHACIN	4	146±65	------	48±30	-----	30±12	-----	48±16	-----	24± 6	-----	4	-
	16	63±28	34±20	31± 4	28± 9	26± 7	24± 5	27± 8	26± 9	20± 7	12± 4	6	4
	24	149±36	148±31	44±12	42±12	48±15	28±10	42±19	50±27	21± 7	29±22	6	6
SALINE	4	130±20	------	32±13	-----	21±10	-----	57±35	-----	18± 6	-----	4	-
VEHICLE	16	198±18	125±27	70±20	24± 6	33±10	33±12	42±16	26± 6	24± 9	11± 3	5	5
	24	160±20	211±15	72±26	87±40	54±14	60±45	49±18	94±67	67± 3	62±44	4	5
ETHANOL	4	152±29	------	54±26	-----	27±10	-----	65±38	-----	22± 8	-----	4	-
VEHICLE	16	154±33	148±60	27± 9	29± 7	22± 2	25± 7	25± 3	37±10	14± 4	19± 6	5	5
	24	146±65	193±78	82±45	42±13	61±22	37±10	62±25	56±32	32±17	32±21	4	5

Values shown are mean ± standard deviation

Kallikrein activity expressed as $nmolpNA.min^{-1}.g^{-1}$

Time expressed as weeks

TABLE 14c

KALLIKREIN ACTIVITY IN THE RAT COLON

DIET: Standard Laboratory diet.

COLON SEGMENT:		CAECUM		PROX. COLON		MID COLON		DIST. COLON		RECTUM		n	
CARCINOGEN	:	NO	YES	NO	YES	NO	YES	NO	YES	NO	YES	NO	YES
DRUG	TIME												
NIL	4	182±53	------	54±26	-----	54±10	-----	60±25	-----	42±24	-----	9	-
	16	217±94	212±33	52±12	39±13	42±11	38±15	62±27	39± 4	34±12	24± 6	6	6
	24	115±61	146±51	36±11	95±39	34±10	47±10	34±16	56±11	22±15	41±13	6	6
APROTININ	4	134±38	------	43±36	-----	34±21	-----	24±10	-----	10± 5	-----	5	-
	16	137±22	145±41	30±15	36±15	26± 7	20± 7	22± 6	32±22	17± 5	17±10	6	6
	24	119±21	105±41	32±22	41±35	19± 3	19± 5	28± 8	32±12	19± 5	21±10	4	5
INDOMETHACIN	4	152±68	------	29±10	-----	40±36	-----	32± 6	-----	20± 7	-----	6	-
	16	93±34	146±35	30± 9	36±11	26± 9	31± 9	22± 7	38±20	16± 6	14± 3	6	6
	24	141±50	163±65	43±27	58±21	32± 9	34± 6	42±26	40±12	23± 7	43±14	5	6
SALINE	4	125±60	------	48±36	-----	23± 9	-----	24± 3	-----	17± 4	-----	8	-
VEHICLE	16	174±38	199±56	33±11	41±25	33± 5	29± 6	30± 4	39±14	25±14	21±12	5	4
	24	207±56	156±52	40±23	44±13	24±12	44±16	40±17	48±15	23±14	30±12	4	5
ETHANOL	4	94±43	------	42±19	-----	14± 2	-----	20± 4	-----	14± 4	-----	4	-
VEHICLE	16	164±32	177±44	43±43	39± 6	27± 6	31± 9	29± 8	47±20	21± 7	22± 5	5	5
	24	160±39	142±39	76±25	47±11	20± 5	38±12	29±14	56±37	18± 6	30± 7	2	5

Values shown are mean ± standard deviation

Kallikrein activity expressed as $nmolpNA.min^{-1}.g^{-1}$

Time expressed as weeks

TABLE 15

KALLIKREIN ACTIVITY IN RAT COLON

The effects of diet and drug treatment by segment of colon.

DIET	CARCINOGEN TIME	SEGMENT		NO DRUG NO	NO DRUG YES	SALINE VEHICLE NO	SALINE VEHICLE YES	ETHANOL VEHICLE NO	ETHANOL VEHICLE YES	APROTININ NO	APROTININ YES	INDOMETHACIN NO	INDOMETHACIN YES
	4 & 16	A		149±43	210±48	154±40	182±39	160±22	180±30	105±53	141±102	157±57	189±59
HIGH FAT	4 & 16	B-E		52±15	61±15	33±19	35± 7	30±12	36± 6	24±12	62±52	38±23	75±65
LOW FIBRE			n	12	6	9	5	9	5	13	6	14	5
DIET	24	A		38±23	167±46	125±69	217±66	222±15	202±87	123±62	170±39	145±46	181±45
	24	B-E		33± 6	49±16	60±26	37±12	83±12	41± 9	30±20	36±21	39±14	36±25
			n	4	9	4	5	4	5	6	5	6	6
	4 & 16	A		133±43	185±47	168±40	125±27	153±29	148±60	135±64	136±53	106±66	34±20
LOW FAT	4 & 16	B-E		45±31	38± 6	38±11	23± 4	31±14	28± 4	25±11	33±16	31±15	22± 9
HIGH FIBRE			n	11	6	9	5	9	5	14	6	10	4
DIET	24	A		152±53	160±24	160±20	211±15	146±65	193±78	173±47	164±80	149±36	148±31
	24	B-E		59±11	33± 8	51± 8	76±47	59±23	42±18	29±11	48±37	39±17	37±20
			n	4	6	4	5	4	5	6	6	6	6
	4 & 16	A		191±71	212±33	144±57	199±56	133±51	177±44	136±27	145±41	122±60	146±35
	4 & 16	B-E		50±21	35±12	29±18	32±16	27±19	35±14	26±17	26±16	27±15	30±15
STANDARD			n	16	6	13	4	9	5	12	6	12	6
DIET	24	A		115±61	146±51	207±51	156±52	146±33	142±39	119±21	105±41	138±45	163±65
		B-E		32±14	60±30	32±17	42±14	37±27	43±21	24±12	28±19	37±20	44±16
			n	6	6	4	5	4	5	6	5	6	6

Kallikrein values expressed as nmolpNA.min^{-1}.g tissue^{-1} (Mean ± SD)

Segment A = Caecum; Segment B - E = Distal Colon segments; Time in weeks

TABLE 16

COMPARISON OF THE PLACEBO GROUPS: probabilities.

Tissue kallikrein-like amidase activity.

Comparison	p - value
Diet : High fat vs Low fat	0.4190
Placebo groups vs no drug	0.7620
Time: 4 & 16 vs 24 weeks	0.1160
No carcinogen vs carcinogen	0.0500
Segment: A vs B - E	0.0000

These comparisons are only for the placebo groups - i.e. the saline-treated and ethanol-treated animals and the animals receiving no drugs.

The comparisons indicate that there is no systematic effect of diet, treatment (saline, ethanol or no drug) or time on the levels of TKLA measured in the control animals.

The effect of carcinogen is very marginal and is not considered significant by this analysis. The effect of segment is clear and is discussed in the text.

For further analysis the placebo groups were merged for analysis with respect to diet and drug treatment.

TABLE 17

KALLIKREIN ACTIVITY IN RAT COLON

The effects of diet and drug treatment by segment of colon.

DIET	CARCINOGEN TIME	SEGMENT		POOLED CONTROL NO	POOLED CONTROL YES	APROTININ NO	APROTININ YES	INDOMETHACIN NO	INDOMETHACIN YES
	4 & 16	A		153±35	192±38	105±53	141±102	157±57	189±59
HIGH FAT	4 & 16	B-E		40±24	45±26	24±12	62±52	38±23	75±65
LOW FIBRE			n	30	16	13	6	14	5
DIET	24	A		128±80	189±61	123±62	170±39	145±46	181±45
	24	B-E		58±32	44±22	30±20	36±21	39±14	36±25
			n	12	19	6	5	6	6
	4 & 16	A		150±38	155±48	135±64	136±53	106±66	34±20
LOW FAT	4 & 16	B-E		38±27	30±12	25±11	33±16	31±15	22± 9
HIGH FIBRE			n	29	16	14	6	10	4
DIET	24	A		153±42	186±46	173±47	164±80	149±36	148±31
	24	B-E		56±28	49±35	29±11	48±37	39±17	37±20
			n	12	16	6	6	6	6
	4 & 16	A		161±64	196±40	136±27	145±41	122±60	146±35
	4 & 16	B-E		37±22	34±13	26±17	26±16	27±15	30±15
STANDARD			n	38	15	12	6	12	6
DIET	24	A		150±57	148±42	119±21	105±41	138±45	163±65
		B-E		33±18	49±24	24±12	28±19	37±20	44±16
			n	14	16	6	5	6	6

Kallikrein values expressed as nmolpNA.min^{-1}.g tissue^{-1} (Mean ± SD)

Segment A = Caecum; Segment B - E = Distal Colon segments; Time in weeks

TABLE 18

KALLIKREIN ACTIVITY IN THE RAT COLON

The effect of drug treatment

Pooled diets - high fat, low fibre and low fat, high fibre.

Pooled placebo groups

CARCINOGEN		POOLED CONTROL		APROTININ		INDOMETHACIN	
		NO	YES	NO	YES	NO	YES
TIME	SEGMENT						
4 & 16	A	152±38	173±49	116±58	139±78	132±65	113±85
4 & 16	B-E	39±20	38±15	25± 6	48±37	35±12	49±44
	n	*59*	*32*	*27*	*12*	*24*	*9*
24	A	140±70	188±57	139±68	167±62	147±39	164±40
24	B-E	58±21	46±24	30± 5	43±29	39±10	37±13
	n	*24*	*35*	*12*	*11*	*12*	*12*

Kallikrein values are in nmolpNA.min^{-1}.g^{-1}

Time expressed in weeks.

Segment A = Caecum

Segment B - E = Distal colonic segments

TABLE 19a

PROSTAGLANDIN CONTENT OF THE RAT COLON.

DIET: high fat, low fibre diet

COLON SEGMENT:		CAECUM		PROX. COLON		MID COLON		DIST. COLON		RECTUM		n	
CARCINOGEN		NO	YES	NO	YES	NO	YES	NO	YES	NO	YES	NO	YES
DRUG	TIME												
NIL	4	55±19	-----	51±29	-----	96±46	-----	101±36	-----	108±41	-----	3	-
	16	120±44	122±53	252±114	138±91	313±196	184±91	316±192	294±238	225±95	124±44	6	4
	24	34±11	107±54	38±16	143±94	128±92	203±102	116±24	173±48	184±52	186±55	4	9
APROTININ	4	103±15	-----	163±77	-----	172±77	-----	198±48	-----	170±67	-----	7	-
	16	42±37	73±16	158±37	68± 6	279±76	142±56	520±459	106±28	366±392	74±66	4	2
	24	71±20	107±36	166±53	153±67	137±52	230±172	230±114	232±69	209±70	163±65	6	6
INDOMETHACIN	4	56±13	-----	77±29	-----	111±70	-----	99±34	-----	115±51	-----	8	-
	16	69±13	80±26	98±64	71±18	106±76	156±52	154±26	142±23	143±58	146±34	2	4
	24	48± 8	106±70	54±30	128±77	64±40	168±67	111±90	146±74	129±61	194±54	6	-
SALINE	4	104±22	-----	112±59	-----	159±56	-----	172±63	-----	173±87	-----	4	-
VEHICLE	16	-----	202±102	-----	200±19	-----	298±34	-----	328±156	-----	248±24	-	2
	24	65	86±14	56	84±26	192	151±33	108	128±35	175	140±59	1	5
ETHANOL	4	76±17	-----	93±19	-----	148±33	-----	173±64	-----	145±20	-----	4	-
VEHICLE	16	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-	-
	24	58± 4	112±34	94± 2	131±37	96±32	277±87	98±18	315±67	78± 7	224±39	2	5

Values shown are mean ± standard deviation

Prostglandin content expressed as $ng.g^{-1}$ PGE_2

Time expressed as weeks

TABLE 19b

PROSTAGLANDIN CONTENT OF THE RAT COLON.

DIET: low fat, high fibre diet.

COLON SEGMENT:		CAECUM		PROX. COLON		MID COLON		DIST. COLON		RECTUM		n	
CARCINOGEN :		NO	YES	NO	YES	NO	YES	NO	YES	NO	YES	NO	YES
DRUG	TIME												
NIL	4	66± 8	-----	37±15	-----	130±44	-----	178±63	-----	182±16	-----	3	-
	16	119±18	132±51	116±38	88±57	233±97	199±117	256±146	223±67	196±68	171±73	6	3
	24	55	74±20	71	127±67	222	150±58	79	199±40	125	222±53	1	6
APROTININ	4	73±16	-----	86±61	-----	147±28	-----	137±34	-----	145±54	-----	8	-
	16	-----	110± 8	-----	98±32	-----	122±13	-----	167±65	-----	128± 5	-	2
	24	-----	209±112	-----	157±50	-----	202±20	-----	245±57	-----	282±26	-	4
INDOMETHACIN	4	102±56	-----	98±22	-----	161±14	-----	176±64	-----	179±92	-----	4	-
	16	110±14	110±30	133±58	124±43	200±54	188±36	228±78	168±76	206±79	166±55	6	4
	24	68±37	59±17	52±29	96±46	62±16	115±35	81±51	134±29	137±47	140±78	3	4
SALINE	4	90±17	-----	84±28	-----	206±121	-----	201±83	-----	221±103	-----	4	-
VEHICLE	16	-----	297±173	----	232±74	-----	411±108	-----	471±107	-----	466±45	-	4
	24	70±24	157±52	121±48	137±67	182±83	278±123	128±30	376±178	128±37	238±54	4	4
ETHANOL	4	77±28	-----	92±59	-----	171±90	-----	170±38	-----	154±43	-----	4	-
VEHICLE	16	-----	-----	-----	-----	-----	-----	-----	-----	-----	-----	-	-
	24	46±14	110± 8	78±27	262±163	117±24	220±42	135±62	193±30	140±41	200±34	4	2

Values shown are mean ± standard deviation

Prostglandin content expressed as $ng.g^{-1}$ PGE_2

Time expressed as weeks

TABLE 19c

PROSTAGLANDIN CONTENT OF THE RAT COLON.

DIET: standard laboratory diet.

COLON SEGMENT:		CAECUM		PROX. COLON		MID COLON		DIST. COLON		RECTUM		n	
CARCINOGEN		NO	YES	NO	YES	NO	YES	NO	YES	NO	YES	NO	YES
DRUG	TIME												
NIL	4	66±32	-----	70±45	-----	132±94	-----	130±90	-----	134±96	-----	9	-
	16	62±37	-----	104±87	-----	266±168	-----	218±190	-----	107±69	-----	6	-
	24	76±12	57±15	74±57	45±14	162±59	78±19	187±84	102±41	167±40	107±41	6	6
APROTININ	4	66±26	-----	85±43	-----	96±37	-----	133±75	-----	133±32	-----	6	-
	16	-----	70±17	------	100±23	------	147±19	------	171±35	-----	142±25	-	2
	24	79±30	107±55	101±34	105±38	156±44	185±90	162±69	193±93	151±62	199±67	6	5
INDOMETHACIN	4	48±19	-----	53±30	-----	70±52	-----	68±38	-----	88±48	-----	6	-
	16	95±54	78±19	110±58	88±44	151±53	166±14	154±53	183±51	153±43	150±47	6	4
	24	56±21	86±47	79±29	90±45	153±48	124±76	175±83	123±80	159±55	152±99	6	3
SALINE	4	171±68	-----	151±70	-----	216±70	-----	424±237	-----	296±105	-----	6	-
VEHICLE	16	-----	-----	------	-----	-----	-----	-----	-----	-----	-----	-	-
	24	142±38	85±43	210±101	98±19	326±199	214±38	297±49	188±44	223±35	158±30	4	4
ETHANOL	4	110±27	-----	162±96	-----	192±55	-----	200±75	-----	274±67	-----	4	-
VEHICLE	16	-----	-----	------	-----	-----	-----	-----	-----	-----	-----	-	-
	24	141±28	78±17	143±27	93±37	257±111	153±22	242±42	141±48	260±72	189±49	4	5

Values shown are mean ± standard deviation

Prostglandin content expressed as $ng.g^{-1}$ PGE_2

Time expressed as weeks

TABLE 20

PROSTAGLANDIN CONTENT OF THE RAT COLON

The effects of diet and drug treatment by segment of colon.

DIET	CARCINOGEN TIME	SEGMENT		NO DRUG NO	NO DRUG YES	SALINE VEHICLE NO	SALINE VEHICLE YES	ETHANOL VEHICLE NO	ETHANOL VEHICLE YES	APROTININ NO	APROTININ YES	INDOMETHACIN NO	INDOMETHACIN YES
	4 & 16	A		98±48	122±53	104±22	202±102	76±17	------	113±34	73±16	63±17	80±26
HIGH FAT	4 & 16	B-E		214±138	185±109	154±62	268±37	140±16	------	234±196	97±46	108±50	129±46
LOW FIBRE			n	9	4	4	2	4		11	2	10	4
DIET	24	A		34±11	107±54	65	86±14	58± 3	112±34	71±20	107±36	48± 8	106±70
	24	B-E		116±39	176±67	133	133±35	92±14	237±40	171±88	195±104	89±63	159±68
			n	4	9	1	5	2	5	6	6	5	6
	4 & 16	A		101±30	132±51	90±17	297±173	77±27	------	73±16	110± 8	106±33	110±30
LOW FAT	4 & 16	B-E		177±74	170±61	178±67	395±15	146±51	------	128±51	129±39	180±76	161±55
HIGH FIBRE			n	9	3	4	4	4		8	2	10	4
DIET	24	A		55	74±20	70±24	157±52	45±14	110± 8	------	209±112	68±37	39±18
	24	B-E		124	174±22	140±37	257±95	117±22	219±14	------	222±61	83±47	121±49
			n	1	6	4	4	4	2		4	3	4
	4 & 16	A		66±32	------	171±68	------	110±27	------	66±26	70±17	72±46	78±19
	4 & 16	B-E		141±115	------	272±166	------	207±79	------	112±51	140±34	106±59	147±52
STANDARD			n	15		6		4		6	2	12	4
DIET	24	A		76±12	57±15	142±38	75±44	141±28	78±17	79±30	107±55	56±21	74±45
	24	B-E		148±73	83±38	267±115	162±57	225±80	144±51	142±56	170±79	141±65	97±59
			n	6	6	4	5	4	5	6	5	6	6

Prostaglandin content expressed as $ng.g^{-1}$ PGE_2 (Mean±SD)

Segment A = Caecum; Segment B - E = Distal Colon segments; Time in weeks

TABLE 21

COMPARISON OF THE PLACEBO GROUPS: probabilities.

Tissue PGE_2.

Comparison	p - value
Diet : High fat vs Low fat	0.2439
Placebo groups vs no drug	0.0668
Time: 4 & 16 vs 24 weeks	0.0000
No carcinogen vs carcinogen	0.0000
Segment: A vs B - E	0.0000

These comparisons are only for the placebo groups - i.e. the saline-treated and ethanol-treated animals and the animals receiving no drugs.

The comparisons indicate that there is no systematic effect of diet or treatment (saline, ethanol or no drug) on the levels of PGE_2 measured in the control animals.

There are clear effects of time, carcinogen administration and segment on the PGE_2 levels measured and for further analysis the placebo groups will be merged with respect to diet and drug treatment but considered separately for other comparisons.

TABLE 22

PROSTAGLANDIN CONTENT OF THE RAT COLON

The effects of diet and drug treatment by segment of colon.

DIET	CARCINOGEN TIME	SEGMENT		POOLED CONTROL NO	POOLED CONTROL YES	APROTININ NO	APROTININ YES	INDOMETHACIN NO	INDOMETHACIN YES
	4 & 16	A		93±35	149±62	113±34	73±16	63±17	80±26
HIGH FAT	4 & 16	B-E		179±119	186±83	234±196	97±46	108±50	129±46
LOW FIBRE			n	17	6	11	2	10	4
DIET	24	A		48±15	103±39	71±20	107±36	48± 8	106±70
	24	B-E		113±59	179±83	171±88	195±104	89±63	159±68
			n	7	19	6	6	5	6
	4 & 16	A		93±26	239±128	73±16	110± 8	106±33	110±30
LOW FAT	4 & 16	B-E		170±90	186±83	128±51	129±39	180±76	161±55
HIGH FIBRE			n	17	7	8	2	10	4
DIET	24	A		59±18	108±45	------	209±112	68±37	39±18
	24	B-E		129±50	209±99	------	222±61	83±47	121±49
			n	9	10		4	3	4
	4 & 16	A		97±58	540±257	66±26	70±17	72±46	78±19
	4 & 16	B-E		182±134	584±265	112±51	140±34	106±59	147±52
STANDARD			n	25	2	6	2	12	4
DIET	24	A		114±38	69±26	79±30	107±55	56±21	74±45
	24	B-E		203±99	126±58	142±56	170±79	141±65	97±59
			n	14	16	6	5	6	6

Prostaglandin content expressed as ng.g^{-1} PGE_2 (Mean±S.D.)

Segment A = Caecum; Segment B - E = Distal Colon segments; Time in weeks

TABLE 23

PROSTAGLANDIN CONTENT OF THE RAT COLON.

The effect of drug treatment

Pooled diets - high fat, low fibre and low fat, high fibre.

Pooled placebo groups

CARCINOGEN		POOLED CONTROL		APROTININ		INDOMETHACIN	
		NO	YES	NO	YES	NO	YES
TIME	SEGMENT						
4 & 16	A	94±32	191±125	98±34	92±24	83±36	95±31
4 & 16	B-E	176±88	259±117	188±124	113±19	141±57	145±31
	n	*34*	*13*	*19*	*4*	*20*	*8*
24	A	52±20	105±44	71±20	148±87	56±23	87±59
24	B-E	121±30	191±66	171±51	206±54	87±36	144±46
	n	*16*	*31*	*6*	*10*	*8*	*10*

Prostaglandin content expressed as $ng.g^{-1}$ PGE_2

Time expressed in weeks.

Segment A = Caecum

Segment B - E = Distal colonic segments

TABLE 24a

CRYPT CELL PRODUCTION RATES

High fat, low fibre diet

DRUG	CARCINOGEN	TIME	CAECUM	MID COLON	DIST. COLON	RECTUM
			COLON SEGMENT			
	NO	4	0.248±0.023	0.250±0.074	0.100±0.071	0.195±0.041
		16	0.191±0.024	0.238±0.053	0.128±0.010	0.159±0.037
NONE		24	0.221±0.038	0.326±0.034	0.203±0.033	0.134±0.015
	YES	16	0.233±0.017	0.475±0.087	0.445±0.080	0.102±0.037
		24	0.323±0.037	0.176±0.044	0.128±0.041	0.123±0.009
	NO	4	0.174±0.035	0.152±0.039	0.251±0.064	0.179±0.124
		16	0.129±0.019	0.191±0.029	0.335±0.031	0.290±0.041
APROTININ		24	0.081±0.071	0.033±0.024	0.145±0.073	0.300±0.062
	YES	16	0.131±0.036	0.087±0.024	0.247±0.102	0.232±0.061
		24	0.143±0.033	0.267±0.076	0.359±0.124	0.261±0.064
	NO	4	0.277±0.115	-----------	0.097±0.057	0.089±0.072
		16	0.203±0.145	0.145±0.121	0.136±0.021	0.209±0.094
INDOMETHACIN		24	0.149±0.032	-0.049±0.036	0.160±0.067	0.031±0.046
	YES	16	-----------	-----------	-----------	-----------
		24	0.184±0.039	0.108±0.048	0.246±0.036	0.068±0.051

Values show the slope of the line of regression ± standard error.

The slope represents the crypt cell production rate in cells.min^{-1}.crypt^{-1}.

Time in weeks

TABLE 24b

CRYPT CELL PRODUCTION RATES

Low fat , high fibre diet

DRUG	CARCINOGEN	TIME	COLON SEGMENT: CAECUM	MID COLON	DIST. COLON	RECTUM
	NO	4	-----------	-----------	-----------	-----------
		16	0.107±0.031	0.057±0.026	0.145±0.073	0.264±0.033
NONE		24	0.255±0.065	0.105±0.046	0.193±0.013	0.278±0.080
	YES	16	0.185±0.049	0.193±0.060	0.529±0.059	0.204±0.052
		24	0.155±0.028	0.050±0.018	0.129±0.028	0.092±0.024
	NO	4	0.218±0.026	0.039±0.087	0.191±0.038	0.128±0.068
		16	0.177±0.055	0.137±0.023	0.158±0.032	0.165±0.028
APROTININ		24	0.177±0.022	0.090±0.030	0.016±0.033	0.091±0.017
	YES	16	0.163±0.054	0.065±0.063	0.176±0.054	0.238±0.048
		24	0.121±0.042	0.098±0.050	0.137±0.038	0.161±0.028
	NO	4	0.136±0.067	0.089±0.031	0.082±0.015	0.137±0.035
		16	0.185±0.030	0.117±0.020	0.101±0.025	0.074±0.046
INDOMETHACIN		24	0.113±0.022	0.151±0.012	0.130±0.031	0.148±0.062
	YES	16	0.159±0.078	0.006±0.055	0.299±0.080	0.133±0.108
		24	0.217±0.045	0.212±0.024	0.143±0.049	0.098±0.038

Values show the slope of the line of regression ± standard error.

The slope represents the crypt cell production rate in cells.min^{-1}.crypt^{-1}.

Time in weeks

TABLE 24c

CRYPT CELL PRODUCTION RATES

Standard laboratory diet

DRUG	CARCINOGEN	TIME	COLON SEGMENT: CAECUM	MID COLON	DIST. COLON	RECTUM
	NO	4	0.150±0.071	0.137±0.022	0.078±0.050	0.049±0.014
		16	0.194±0.039	0.136±0.031	0.117±0.040	0.115±0.039
NONE		24	0.212±0.066	0.256±0.043	0.260±0.061	0.141±0.031
	YES	16	0.187±0.055	0.300±0.061	0.279±0.034	0.092±0.022
		24	0.142±0.055	0.158±0.039	0.148±0.034	0.155±0.035
	NO	4	0.213±0.062	0.125±0.034	0.255±0.041	0.366±0.092
		16	0.063±0.021	-0.017±0.062	0.026±0.012	0.131±0.051
APROTININ		24	0.136±0.034	0.062±0.015	0.072±0.017	0.153±0.032
	YES	16	0.284±0.030	0.173±0.019	0.182±0.056	0.221±0.016
		24	0.097±0.043	0.170±0.048	0.164±0.039	0.158±0.056
	NO	4	0.186±0.066	0.230±0.029	0.176±0.037	0.080±0.034
		16	0.056±0.048	0.080±0.062	0.143±0.093	0.299±0.168
INDOMETHACIN		24	0.090±0.040	0.063±0.035	0.116±0.017	0.041±0.017
	YES	16	0.010±0.049	0.006±0.012	0.183±0.099	0.097±0.054
		24	0.098±0.062	0.077±0.024	0.165±0.056	0.158±0.020

Values show the slope of the line of regression ± standard error.

The slope represents the crypt cell production rate in cells.min^{-1}.crypt^{-1}.

Time in weeks

TABLE 25a

RAT FAECAL CONSTITUENTS

Animals not receiving carcinogen

	High fat, Low fibre diet	Low fat, High fibre diet	Standard diet
NEUTRAL STEROIDS			
Coprostanol	1.38±0.74	0.80±1.60	0.50±0.17
Cholesterol	2.57±0.84	0.73±0.54	1.04±0.29
Total plant sterols	5.90±1.82	2.08±1.08	4.47±1.03
TOTAL STEROLS	9.85±2.65*	3.61±3.02	6.02±1.39+
LONG CHAIN FATTY ACIDS			
Palmitic acid	12.73±3.15	0.79±0.43	0.85±0.23
Unsaturated F.A.	10.71±3.74	1.02±1.02	1.85±1.26
Stearic acid	12.42±2.89	0.69±0.27	0.43±0.15
TOTAL FATTY ACIDS	35.86±8.41*	2.49±1.51	3.13±1.43
FREE BILE ACIDS			
Lithocholic acid	0.30±0.07	0.22±0.13	0.22±0.09
Deoxycholic acid	2.04±0.74	0.67±0.47	1.37±0.90
Chenodeoxycholic acid	0.20±0.10	0.14±0.13	0.17±0.11
Hyodeoxycholic acid	0.49±0.27	0.38±0.24	1.16±1.11
Muricholic acids	1.07±1.00	0.20±0.29	1.07±0.87
TOTAL BILE ACIDS	4.59±2.62+	1.58±1.12	4.09±2.35+
n =	13	11	13

Notes

1. Values shown are expressed in $mg.g^{-1}$ of dry faeces (mean ± SD).

2. Totals may not equal the sum of the preceding components since the total value was calculated from the individual points.

3. Unsaturated F.A. = linoleic, linolenic and oleic acids.

4. * = greater than other diets, $p<0.005$.

   + = greater than low fat, high fibre diet, $p<0.005$

TABLE 25b

RAT FAECAL CONSTITUENTS

Animals receiving carcinogen

	High fat, Low fibre diet	Low fat, High fibre diet	Standard diet
NEUTRAL STEROIDS			
Coprostanol	1.40±0.53	0.23±0.06	0.87±0.25
Cholesterol	2.92±0.62	0.50±0.06	0.87±0.56
Total plant sterols	4.78±1.10	1.55±0.66	2.59±1.85
TOTAL STEROLS	9.00±1.65*	2.28±0.73	4.33±2.64+
LONG CHAIN FATTY ACIDS			
Palmitic acid	13.71±4.81	0.54±0.45	1.24±0.63
Unsaturated F.A.	14.09±5.43	0.62±0.37	1.77±1.49
Stearic acid	12.60±4.33	0.77±1.01	1.73±0.32
TOTAL FATTY ACIDS	40.41±14.22*	1.93±1.48	4.74±2.29+
FREE BILE ACIDS			
Lithocholic acid	0.25±0.09	0.16±0.04	0.23±0.10
Deoxycholic acid	1.68±0.79	0.49±0.23	0.79±0.52
Chenodeoxycholic acid	0.19±0.07	0.06±0.02	0.15±0.07
Hyodeoxycholic acid	0.80±0.48	0.37±0.21	0.37±0.40
Muricholic acids	1.26±1.14	0.29±0.20	0.22±1.18
TOTAL BILE ACIDS	4.77±2.45*	1.39±0.36	1.72±2.05
n =	10	10	9

Notes

1. Values shown are expressed in $mg.g^{-1}$ of dry faeces (mean ± SD).

2. Totals may not equal the sum of the preceding components since the total value was calculated from the individual points.

3. Unsaturated F.A. = linoleic, linolenic and oleic acids.

4. * = greater than other diets, $p<0.005$.

   + = greater than low fat, high fibre diet, $p<0.05$

TABLE 26

TUMOURS IN RATS

DIET/DRUG GROUP	ANIMAL NO.	SITE OF LESION	HISTOLOGY
GROUP 50	84/274	colon - 16cm	tubular adenoma
		duodenum	adenocarcinoma
STANDARD	84/275	colon - 14cm	adenocarcinoma
DIET	84/279	colon - 11cm	adenocarcinoma
		colon - 16cm	tubulovillous adenoma
NO DRUGS	84/280	colon - 0cm	adenocarcinoma
	84/281	colon - 16cm	tubular adenoma
24 WEEKS	84/282	colon - 18cm	tubular adenoma
		ileum	adenocarcinoma
	84/284	colon - 15cm	tubular adenoma
		colon - 16cm	tubular adenoma
	84/285	colon - 18cm	adenocarcinoma
GROUP 53	84/306	colon - 12cm	tubular adenoma
	84/309	colon - 20cm	tubulovillous adenoma
STANDARD		ileum	adenocarcinoma
DIET	84/312	colon - 10cm	tubular adenoma
		colon - 17cm	tubular adenoma
APROTININ	84/313	colon - 12cm	tubular adenoma
		colon - 24cm	tubular adenoma
24 WEEKS		colon - 25cm	dysplastic focus
	84/316	jejunum	adenocarcinoma
	84/317	colon - 10cm	adenocarcinoma
		colon - 15cm	adenocarcinoma
		colon - 20cm	adenocarcinoma
GROUP 56	84/338	colon - 10cm	adenocarcinoma
	84/343	colon - 10cm	adenocarcinoma
STANDARD		ileum	adenocarcinoma
DIET	84/348	colon - 20cm	adenocarcinoma
	84/349	colon - 10cm	adenocarcinoma
INDOMETHACIN 24 WEEKS			

TABLE 26 (continued)

TUMOURS IN RATS

DIET/DRUG GROUP	ANIMAL NO.	SITE OF LESION	HISTOLOGY
GROUP 58	84/354	colon - 13cm	tubular adenoma
		ileum	adenocarcinoma
STANDARD DIET	84/356	colon - 18cm	adenocarcinoma
SALINE VEHICLE 24 WEEKS			
GROUP 60	NO TUMOURS		
STANDARD DIET			
ETHANOL VEHICLE 24 WEEKS			

TABLE 26 (continued)

TUMOURS IN RATS

DIET/DRUG GROUP	ANIMAL NO.	SITE OF LESION	HISTOLOGY
GROUP 63	84/379	colon - 8cm	tubular adenoma
	84/902	colon - 0cm	adenocarcinoma
HIGH FAT	84/903	colon - 0cm	adenocarcinoma
LOW FIBRE		colon - 10cm	adenocarcinoma
DIET	84/904	colon - 10cm	adenocarcinoma
NO DRUGS			
24 WEEKS			
GROUP 66	84/405	colon - 15cm	villous adenoma
	84/407	colon - 11cm	adenocarcinoma
HIGH FAT		colon - 16cm	tubular adenoma
LOW FIBRE	84/411	colon - 6cm	tubular adenoma
DIET	84/412	colon - 13cm	tubular adenoma
	84/414	colon - 10cm	adenocarcinoma
APROTININ			
24 WEEKS			
GROUP 69	84/437	colon - 11cm	adenocarcinoma
	84/439	colon - 10cm	adenocarcinoma
HIGH FAT	84/441	colon - 10cm	adenocarcinoma
LOW FIBRE	84/443	colon - 11cm	adenocarcinoma
DIET			
INDOMETHACIN			
24 WEEKS			
GROUP 71	84/451	colon - 0cm	adenocarcinoma
		colon - 8cm	adenocarcinoma
HIGH FAT	84/453	colon - 13cm	adenocarcinoma
LOW FIBRE	84/454	colon - 16cm	tubular adenoma
DIET	84/455	colon - 8cm	adenocarcinoma
SALINE VEHICLE			
24 WEEKS			
GROUP 73	NO TUMOURS		
HIGH FAT			
LOW FIBRE			
DIET			
ETHANOL VEHICLE			
24 WEEKS			

TABLE 26 (continued)

TUMOURS IN RATS

DIET/DRUG GROUP	ANIMAL NO.	SITE OF LESION	HISTOLOGY
GROUP 76	84/474	colon - 11cm	adenocarcinoma
	84/476	colon - 20cm	adenocarcinoma
LOW FAT		duodenum	tubulovillous adenoma
HIGH FIBRE	84/478	colon - 16cm	villous adenoma
DIET	84/911	colon - 17cm	tubular adenoma
	84/912	colon - 0cm	adenocarcinoma
NO DRUGS 24 WEEKS			
GROUP 79	84/506	colon - 17cm	adenocarcinoma
	84/511	colon - 18cm	tubulovillous adenoma
LOW FAT HIGH FIBRE DIET			
APROTININ 24 WEEKS			
GROUP 82	NO TUMOURS		
LOW FAT HIGH FIBRE DIET			
INDOMETHACIN 24 WEEKS			
GROUP 84	84/550	colon - 15cm	adenocarcinoma
	84/551	colon - 9cm	adenocarcinoma
LOW FAT HIGH FIBRE DIET			
SALINE VEHICLE 24 WEEKS			
GROUP 86	NO TUMOURS		
LOW FAT HIGH FIBRE DIET			
ETHANOL VEHICLE 24 WEEKS			

TABLE 27

TUMOURS IN RATS

THE DISTRIBUTION OF ANIMALS WITH TUMOURS.

DRUG TREATMENT	HIGH FAT, LOW FIBRE DIET	LOW FAT, HIGH FIBRE DIET	STANDARD DIET
NONE	4 (9)	5 (9)	8 (12)
APROTININ	5 (10)	2 (12)	6 (11)
INDOMETHACIN	4 (12)	0 (12)	4 (12)
SALINE VEHICLE	4 (5)	2 (5)	2 (5)
ETHANOL VEHICLE	0 (5)	0 (5)	0 (5)
TOTAL FOR DIET	17 (41)	9 (43)	20 (45)

Figures show the number of animals with tumours

Figures in brackets indicate the number of animals in the group

For high fat, low fibre diet versus low fat, high fibre diet:
$X^2 = 4.140$, $p < 0.05$

TABLE 28

TUMOURS IN RATS

THE DISTRIBUTION OF TUMOURS IN ANIMALS.

DRUG TREATMENT	HIGH FAT, LOW FIBRE DIET	LOW FAT, HIGH FIBRE DIET	STANDARD DIET
NONE	5 (4)	6 (5)	12 (8)
APROTININ	6 (5)	2 (2)	12 (6)
INDOMETHACIN	4 (4)	0	5 (4)
SALINE VEHICLE	5 (4)	2 (2)	3 (2)
ETHANOL VEHICLE	0	0	0
TOTAL TUMOURS	20 (17)	10 (9)	32 (20)

Figures represent the total number of tumours in each group

Figures in brackets represent the number of animals with tumours.

TABLE 29

TISSUE KALLIKREIN-LIKE AMIDASE ACTIVITY
AND PGE_2 CONTENT
IN ANIMALS WITH AND WITHOUT COLONIC TUMOURS.

Colonic tissue kallikrein-like amidase activity

COLON SEGMENT	ANIMALS WITH TUMOURS	ANIMALS WITHOUT TUMOURS
Caecum (A)	165 ± 51 (33)	169 ± 58 (52)
Distal colon (B-E)	42 ± 24 (33)	45 ± 28 (52)

Colonic PGE_2 content

COLON SEGMENT	ANIMALS WITH TUMOURS	ANIMALS WITHOUT TUMOURS
Caecum (A)	99 ± 70 (33)	94 ± 45 (44)
Distal colon (B-E)	164 ± 101 (33)	166 ± 73 (44)

TKLA expressed as nmol $pNA.mg^{-1}.min^{-1}$

PGE_2 expressed as $mg.g^{-1}$

Values are mean ± S.D. (n)

TABLE 30

PLASMA INDOMETHACIN IN RATS

SAMPLE NUMBER	INDOMETHACIN CONCENTRATION
1	1.0
2	0.8
3	1.0
4	1.0
5	4.2
6	0.5
7	0.4
8	0.6
9	0.6
10	0.8
11	0.9
12	1.1
13	0.6
14	0.6

Indomethacin concentrations shown in $mcg.ml^{-1}$ of plasma.

Mean concentration is 1.00 $\pm$ $0.9 mcg.ml^{-1}$.

Each sample came from a different animal.

FIGURES

FIGURE 1

THE SYNTHESIS OF PROSTAGLANDINS.

The chart shows the synthesis of the major prostaglandins from arachidonic acid by cyclo-oxygenase. The source of the fatty acids is shown and the pathway of formation of the leukotrienes is indicated.

Indomethacin blocks the activity of cyclo-oxygenase.

Synthesis of Prostaglandins

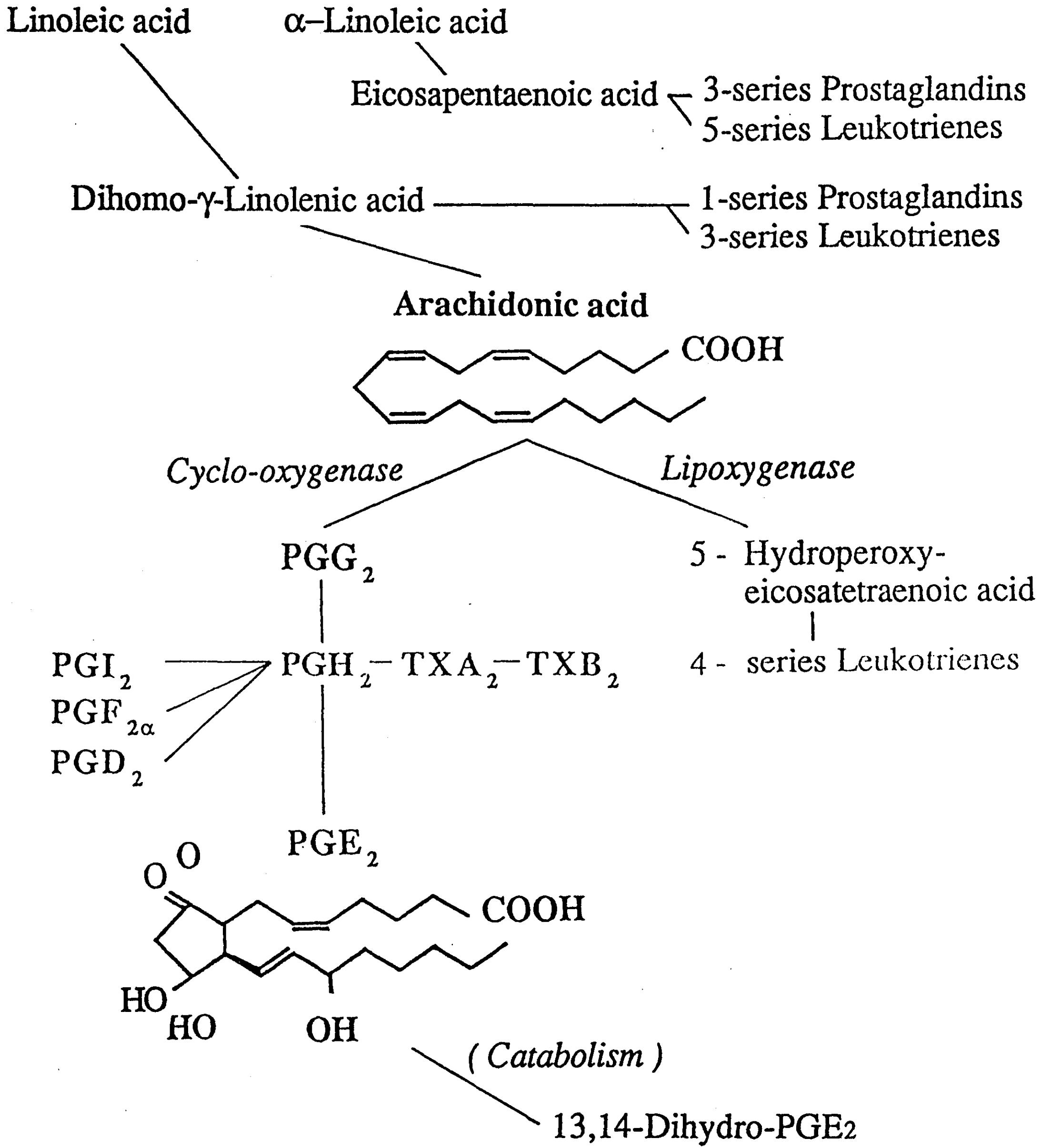


FIGURE 2

EXPERIMENTAL PLAN

Animals were entered into the study at about 10 weeks of age. At this time, having been weaned on their standard laboratory diet, they commenced either of the specialised diets or continued with their standard diet. Any drug treatment was also commenced at this point.

Weekly injections of azoxymethane began at the fourth week and were given for twelve consecutive weeks.

Groups of animals were sacrificed for study at 4, 16 and 24 weeks after entry into the experiment.

(see pages 51-56 for more details)

Experimental Plan

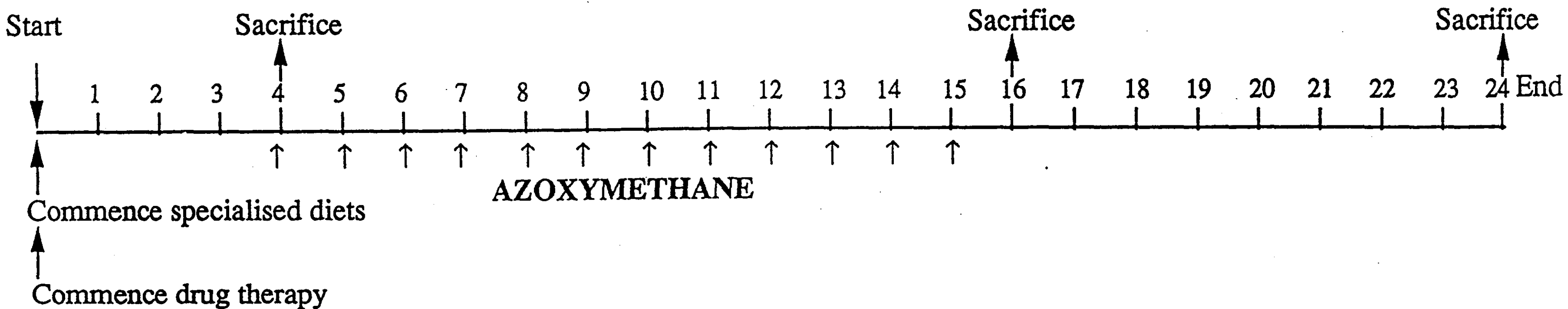
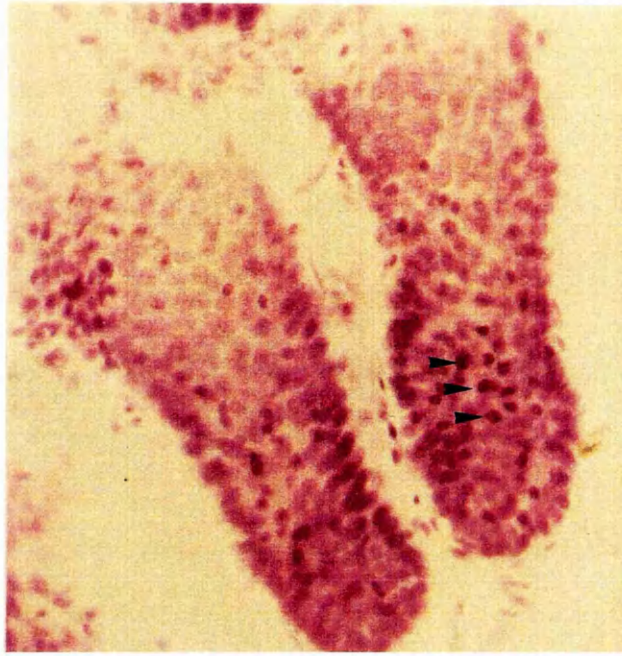


FIGURE 3

EXAMPLES OF CRYPT CELL MICRODISSECTIONS.

The upper figure shows crypts from the caecum; the lower figure shows crypts from the distal colon. The greater length of the distal crypts is evident. The metaphase figures appear as dense points of staining in the lower crypt. (indicated by arrowheads)

(original magnification x100)



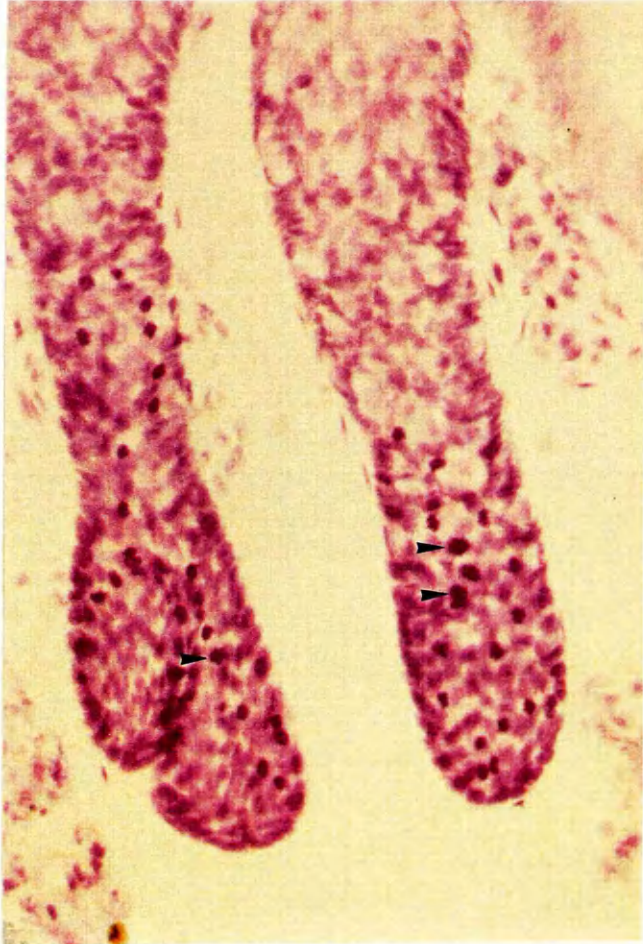


FIGURE 4

PROPORTIONS of NEUTRAL STEROIDS, FATTY ACIDS and BILE ACIDS in FAECES

Each of the faecal components is shown as a percentage of the total neutral steroids, fatty acids or bile acids.

Each diet is presented separately.

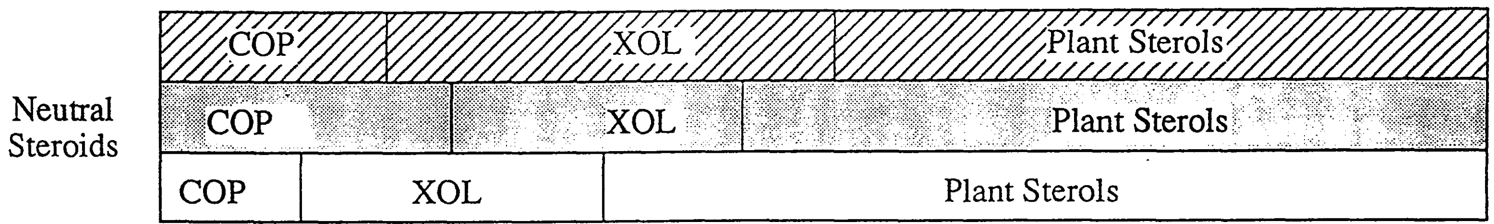
Abbreviations:

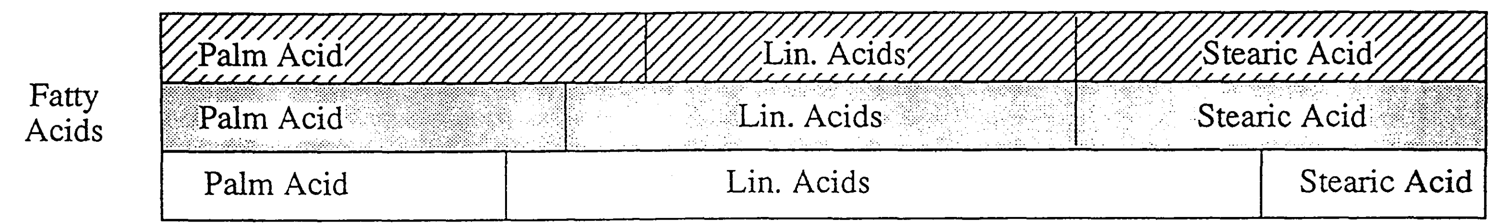
COP = coprostanol
XOL = cholesterol

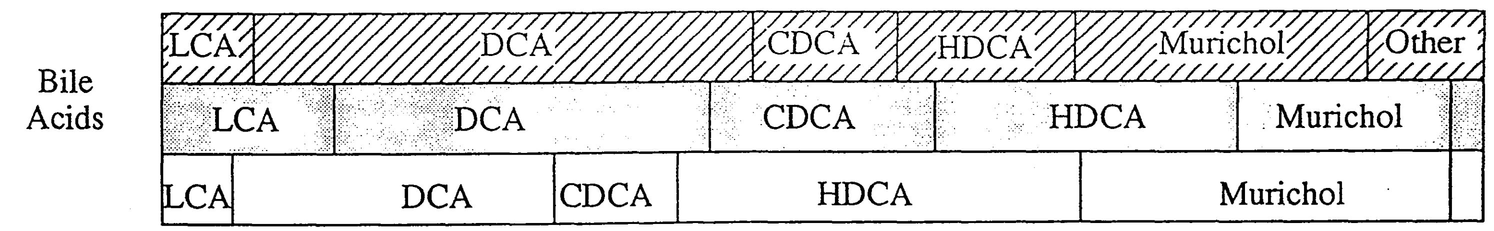
Palm. acid = palmitic acid
Lin. acids = linoleic, linolenic and oleic acids

LCA = lithocholic acid
DCA = deoxycholic acid
CDCA = chenodeoxycholic acid
HDCA = hyodeoxycholic acid
Murichol = muricholates

Proportions of Neutral Steroids, Fatty Acids and Bile Acids







- High fat, low fibre diet
- Low fat, high fibre diet
- Standard diet

For abbreviations see facing notes

Each component expressed as % of total

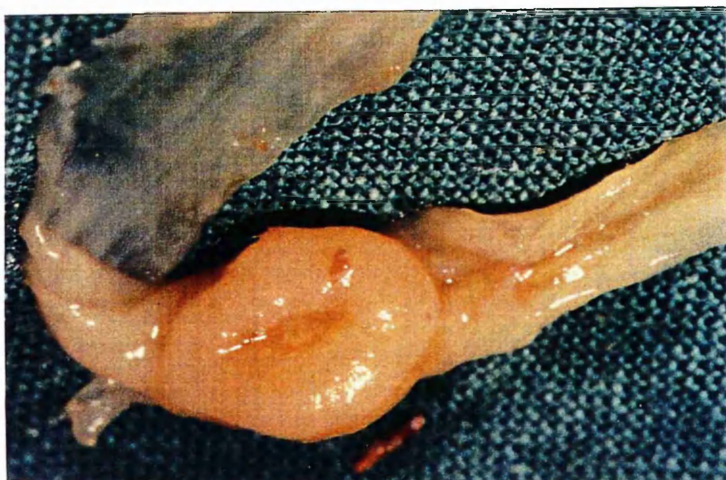
FIGURE 5

EXAMPLES OF COLONIC TUMOURS

The two figures opposite show the macroscopic appearance of the colonic tumours induced in the rat colon after treatment with azoxymethane.

The upper figure shows a sessile tumour of the distal colon and the lower figure shows a polypoid tumour at the caecal pole.

(scale in lower figure is 1cm)



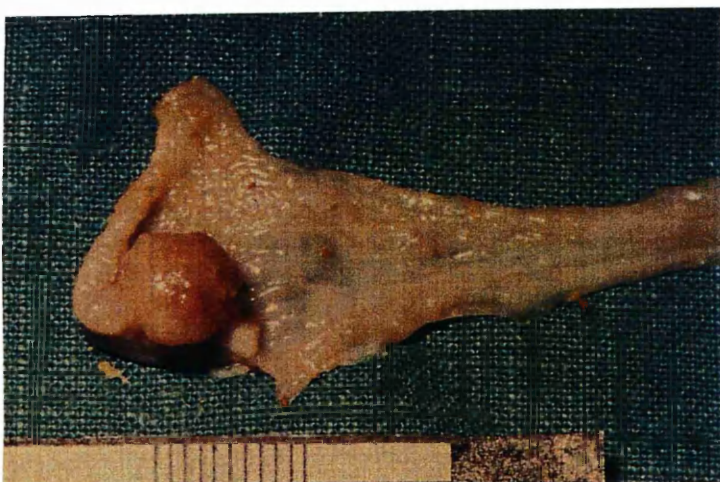


FIGURE 6

This figure illustrates the abdominal viscera from an animal with carcinomatosis. The primary tumour is shown at * and has been opened. Multiple peritoneal and mesenteric tumour deposits are evident. Liver metastases were also present. (not shown)

This appearance was exceptional at the stage examined (24 weeks) and was only seen in animals on the high fat, low fibre diet.

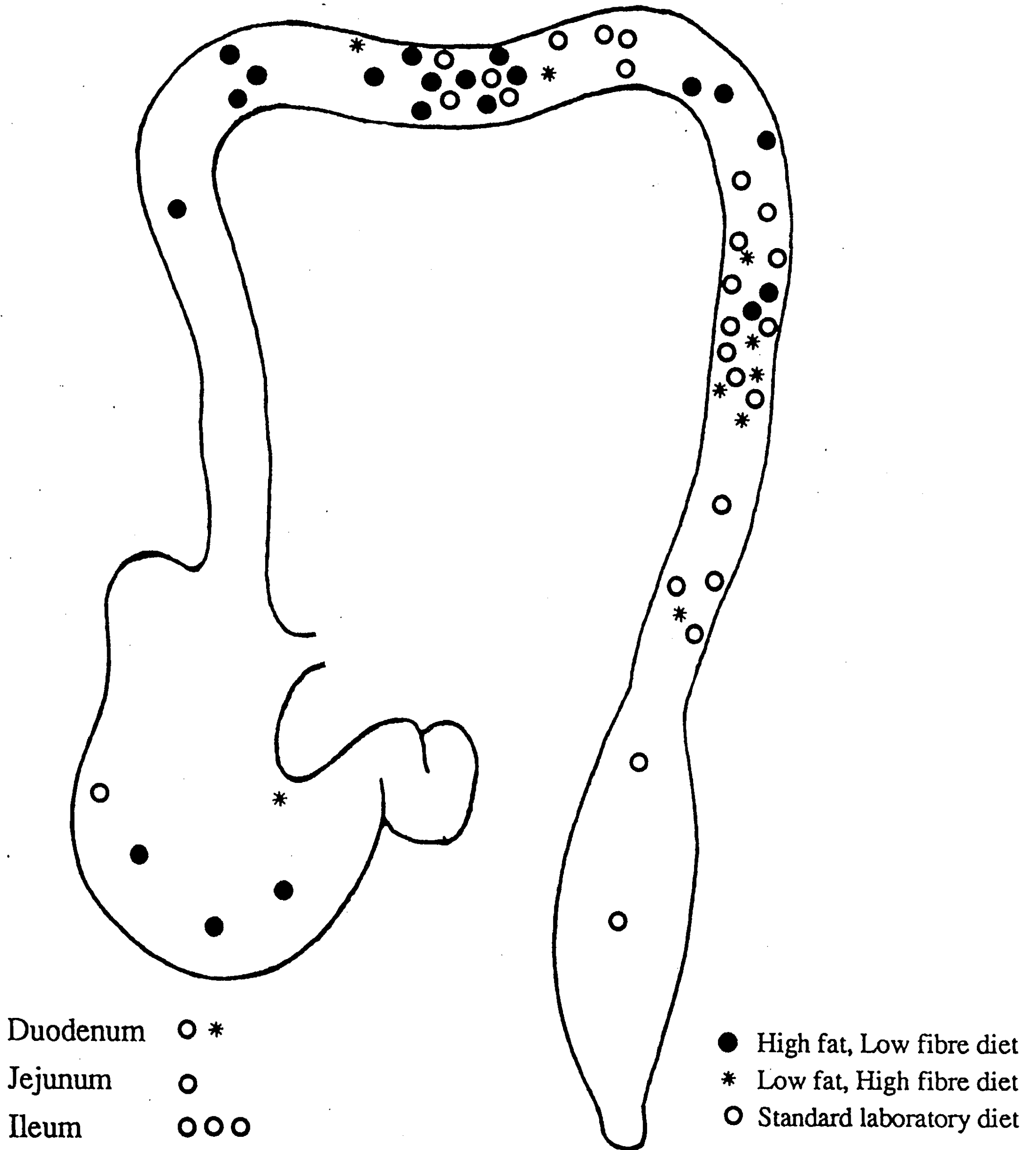
FIGURE 7

DISTRIBUTION OF TUMOURS IN THE COLON

Each tumour which developed in the animals was recorded and is illustrated in this diagram. At the time of sacrifice the distance of the tumour from the ileo-caecal junction junction was recorded and expressed as a percentage of the total length of the colon. The site of the tumour was then plotted onto this diagram by proportion.

The more proximal location of tumours from aniamls on the high fat, low fibre diet is apparent when compared with those on the other two diets.

Distribution of Colon Tumours in the Rat



DISCUSSION

There is little doubt that the development of colorectal cancer whether experimental or naturally occurring is conveniently and correctly regarded as a multistep process. (Maskens, 1976, 1978 & 1981) In the animal model it appears that colonic carcinogenesis is a two-step process. The first step involves initiation of the tumour by administration of the carcinogen. The second step involves the promotion of the tumour by multiplication of the transformed cells. This study is an examination of tumour promotion - the study of agents which are not of themselves carcinogenic but which aid the development of a tumour. In the rat model used in this study the initiator is clearly defined and the moment of its exposure known. This contrasts with the situation in the human where neither the initiating agent nor the time of its exposure are known.

The principles governing current thinking in colorectal carcinogenesis may be conveniently summarised : " A particular substrate is acted on by bacteria in the bowel to produce a carcinogenic product. Diet is important in determining the concentration of the substrate and will also affect the composition of the bacterial flora. Thus cholesterol and bile acids may be acted on by colonic bacteria to produce carcinogens that act locally - or may be absorbed to act elsewhere. Bowel transit time, affected by fibre, will affect exposure to any active compounds produced." (Berry, 1988) These few sentences distil a great deal of the knowledge and work which has accumulated on colonic carcinogenesis and are remarkable by their

generality rather than their brevity. The author leaves open the question of whether the "products" are initiators or promoters but it is recognised that the search for tumour initiators in the diet for human colon cancer has been a barren field.

The current study has endeavoured to go beyond simple observation of dietary influence on tumour induction and bile acids to make some attempt to establish the mechanisms by which such alterations in colonic environment may alter tumour promotion. The results obtained will be discussed with regard to the levels of local mediators - autacoids - distributed in the colon and to the bearing this may have on tumorigenesis in this model and more generally.

TISSUE KALLIKREIN-LIKE AMIDASE ACTIVITY (TKLA)

The methodology used for the assay of TKLA is well-established and known to produce reliable results. This was found in this study also with the coefficient of variation being around 2%. The only aberrant feature was the inability to establish an optimum working pH. This has not been previously noted and a broad pH optimum of pH 8 - 9 is usually described (Claeson, Friberger, Knos et al., 1978; Amundsen, Putter, Friberg et al., 1979). Despite this the assay performed predictably in our hands in all other ways and in particular showed inhibition by protease inhibitors in a standard pattern with activity being completely abolished by aprotinin but only partly inhibited by soybean trypsin inhibitor. There is no record of instability of the substrate S-2266 at alkaline pH (Kabi - personal

communication) and TRIS buffer has been satisfactorily used in this pH range previously. The problem was not confined to colon as gastric and small bowel tissues would not demonstrate an optimal pH either. Nonetheless using the suggested pH of 8.2 provided results which were compatible with other workers in terms of activity measured. (Al-Dahir & Zeitlin, 1983). Therefore there was no reason to mistrust the assay and its use here was entirely identical with other documented workers. The most likely explanation is that pH curve has a rather flat peak and this obscures the optimal pH as assessed here. The sharp increase in measured activity at high, non-physiological alkalinity may not reflect enzymic activity on the substrate and may be alkaline hydrolysis. The substrate, S-2266 is thought to be the most sensitive for the detection of human urinary kallikrein (its original use). (Fiedler, Gieger, Hirschauer et al., 1978)

Tissue kallikrein-like amidase activity (TKLA) in the colon

The pattern of kallikrein-like activity along the colon has not been previously described in detail. It has been appreciated that kinin-forming activity varies along the gastrointestinal tract and was higher in the caecum than in the proximal or distal colon which had similar levels of activity. (Frankish & Zeitlin, 1980) The underlying belief is that kallikrein distribution as measured by this assay is a reflection of intrinsic kinin-forming activity. It seems that the tissue kallikrein-like amidase activity of the caecum is considerably

higher than that of the rest of the colon. This presumably reflects the natural function of the caecum in water and electrolyte resorption but it must also be appreciated that the rat caecum is not analogous to the human and seems to have special, but as yet poorly defined functions. This study has assessed the activity of the whole thickness of the colon and takes no account of the source of the activity which may be in any of the layers of the bowel. It should not be from extrinsic sources since the colon was perfused blood-free prior to assay. Zeitlin and Smith (1973) showed in human colon that the kininogen (kallikrein substrate) was in the muscle of the colon and the kallikrein activity was in the mucosa (a pattern which changed with inflammatory bowel disease). No comparable analysis exists in the rat but for reasons to be discussed below it is tempting to consider this possibility in the rat also since other autacoids (PGE_2, cAMP and cGMP) have a variable distribution between mucosa and submucosa. It is possible to localise kallikrein-like activity histochemically (Kimura, Takagi, Igari et al., 1982) using a peptide ester with simultaneous coupling to an azo dye and this may be a means to identifying the site of kallikrein activity.

That there is a role for kinins in the colonic mucosa was clearly demonstrated by Al-Dhahir and Zeitlin (1982 & 1983) since the reversal of bile salt-induced mucosal damage was effected by the antiproteinase aprotinin and accompanied by a reduction in the activity of the previously raised tissue kallikrein-like

amidase activity.

Frankish and Zeitlin (1980) described an effect of diet on the colonic kinin-forming activity with a fall in the kinin-forming activity in the caecum and a concomitant rise in the distal colon with starvation or feeding a 5% glucose diet for 60 hours. However they point out that these results related to the "fullness" of the bowel rather than the dietary content. In the current study no effect of diet was observed on the colonic tissue kallikrein-like amidase activity. This is probably not surprising since the animals were allowed to consume their diet until the time of sacrifice and the colons were invariably "full"! It might be argued that increasing the dietary fat content would increase the bile acid production which, on the basis of the work of Al-Dhahir & Zeitlin (1983) may have been expected to increase the tissue kallikrein-like amidase activity. While considerable changes in the concentrations of faecal bile acids were demonstrated the situation is not analogous to directly perfusing the colon with high concentration bile acid solution.

It was not unexpected that there would be no change in the tissue kallikrein-like amidase activity with time but changes in the rat colon with senescence are defined particularly with relation to cell turnover (Holt & Yeh, 1988). Considerable effort was made to eliminate diurnal changes by sacrificing the animals at the same time of day on every occasion.

The Effect of Aprotinin

Aprotinin produced a fall in the colonic tissue kallikrein-like amidase activity (TKLA) which is explicable by its antiprotease activity. However, when its ability to abolish TKLA in vitro is taken into account then the observed reduction in activity was actually quite small. This was underlined by the prompt significant fall seen in the pilot experiment when aprotinin was given for a period of two weeks. It could be suggested that the dose given was inadequate but the dose used in this experiment was clearly effective in the short term and appeared to be the maximum safe dose which could be used for these animals. The LD_{50} has been documented at 2.5×10^6 $KIU.kg^{-1}$ for rats (Fritz & Wunderer, 1983) which is 100 times greater than the dose used here but the aim in this experiment was long-term survival. Protracted injections at 5 times the final dose level used were associated with great morbidity and mortality. Alternatively, the dose could have been given more frequently to produce a prolonged suppression period but this raises many practical difficulties.

Aprotinin forms a relatively stable complex with tissue kallikrein (Fritz & Wunderer, 1983) but it also inhibits other proteases. None of the animals showed any evidence of this - in particular there was no evidence of a haemorrhagic diathesis. Aprotinin is metabolized in the rat kidney microsomes and this appears to be a fairly rapid process. Therefore the paucity of the aprotinin effect could be attributed to either:

1. inadequate dosage
2. doses which were too infrequent
3. the ability of the animals to synthesise new enzyme to replace that which was inhibited by the aprotinin
4. induced metabolism of aprotinin.

The third of these possibilities is perhaps the most intriguing and worthy of further assessment.

Neither carcinogen nor indomethacin had an effect on TKLA. No interaction between TKLA and azoxymethane or indomethacin has been previously shown.

TISSUE PROSTAGLANDIN E_2 (PGE_2).

The distribution of PGE_2 in the colon has never been examined in the detail described in this study (nor for any eicosanoid). The method used to estimate the colonic PGE_2 follows accepted methodology and uses a well-established, validated assay using commercial reagents of predictable purity and high quality. In particular the antibody showed little cross-reactivity with other similar eicosanoids. The levels of PGE_2 in the tissue obtained by the methods used here produce results which are of very similar magnitude to other workers who have attempted to do the same. (Bennett & DelTacca, 1975; Karmali, Thaler & Cohen, 1983, Bennett, Charlier & McDonald, 1976; Sharon, Ligumsky, Rachmilewitz et al., 1978; Minoura, Takata, Sakaguchi et al., 1988) The results of Attallah, Lee, Ambrus et al., (1984) in a variety of normal and neoplastic tissues produce PGE_2 contents on average one fiftieth of those quoted elsewhere. These results

were obtained by either bioassay or radioimmunoassay following simple extraction or chromatographic separation.

The large variability of some of the results in this study is difficult to explain and is not simply a technical problem. The interassay variation was shown to be around the 20% level which is acceptable for this method. All samples were assayed in duplicate to minimise random error and the most extreme values were on occasion analysed a second time with no change in the result. Additionally the extreme values were obtained during assays which produced "normal" results in the same batch. This variability has been noted by other workers but is rarely commented on. It would seem that the assay is giving a true reflection of the PGE_2 in the vial. The method of preparation was designed to prevent undue formation of prostanoid and this was shown to be effective. The preparation used in this study was probably more cautious than others as both low temperatures and indomethacin were used to prevent prostanoid production. The other studies cited use one or other method as a rule. By separating the homogenised sample immediately then loss of prostanoid content by metabolism or increase in the content by continued formation from its precursors were both avoided. The experiments on overnight storage showed that the latter situation could have been a problem for homogenates left for a significant amount of time before separation. Since the tissue was perfused blood-free then large variations in exogenous prostanoid from activated platelets or other blood components would be unlikely.

It did appear that the aberrant values did affect the colon of the animal in a general way i.e. all segments examined had higher levels. These observations would all tend to point to the observed "extreme" values being a true reflection of the colonic PGE_2 at the time of sacrifice. It is possible that the trauma of sacrifice may influence the final values assayed. Granstrom (1978) captured the essence of this problem when he said "... the accuracy of many radioimmunoassays unfortunately seems to be very low when they are applied to biologic material ... there are often enormous discrepancies between data obtained by this kind of methodology and those from the more specific gas chromatographic - mass spectrophotometric methods....No source of error seems to exist to explain sometimes unphysiologically high 'PG levels'... the most likely explanation is misinterpretation [of the assay]."

Tissue PGE_2 in the colon.

The distribution of PGE_2 along the colon with relatively low levels proximally and higher levels more distally has never been described. The significance of this is purely conjectural but in relation to the the present study it is perhaps worthwhile reflecting that colonic tumours in this model tend to occur in the distal colon and that this distribution is the inverse of the colonic tissue kallikrein-like amidase activity.

There did not appear to be any significant effect of diet on the colonic content of PGE_2 or its distribution in the colon. A considerable body of work describes colonic prostanoids, the

effects of tumorigenesis and the actions of a selection of reagents. There has been no work relating the diet to colonic prostanoid content until recently. Minoura and coworkers (1988) fed rats a diet supplemented with either linoleic acid or eicosapentaenoic acid (EPA) and demonstrated that the colonic PGE_2 content as measured by radioimmunoassay was significantly lower in the animals on the EPA-augmented diet. Hence the composition of the diet affected the colonic PGE_2 content and the inhibitory effect of EPA was thought to represent inhibition of conversion of linoleic acid to arachidonic acid. The high fat diet used in the current study had 2.5% linoleic acid and 7.5% oleic acid (compared with 2.1% total mixed fat for the low fat diet) and may therefore have been expected to show a significant alteration in the colonic PGE_2 if a simple increase in substrate flow were all that influenced the tissue levels of PGE_2. While PGE_2 is the most abundant prostanoid in the colon there is good evidence that the gastrointestinal tissues synthesise the whole spectrum of cyclo-oxygenase and lipoxygenase products. (Dreyling, Hoppe, Peskar et al., 1986; Craven, Saito & DeRubertis, 1983) It is therefore quite possible that assay of one component is a very blunt instrument and only a panoramic analysis would reveal the true extent of the changes occurring with dietary alteration.

Colonic PGE_2 and Indomethacin.

The effect of indomethacin was reasonably unambiguous and produced a significant reduction in colonic content of PGE_2 as would be expected from its inhibition of cyclo-oxygenase. It was

interesting that administration of the carcinogen resulted in a rise in the PGE_2 content for two reasons. Firstly, this rise was occurring in colonic tissue which did not necessarily have tumour formation evident and secondly it was not really apparent until the 24 week stage. It may be said therefore, that the raised PGE_2 content did not occur during the phase of tumour initiation and was only evident after the phase of tumour promotion. This change was not simply localised to areas of frank neoplastic change but was in fact a field change affecting apparently normal mucosa. Additionally, the raised levels of PGE_2 induced by the carcinogen were reduced by the administration of indomethacin.

Inhibition of colonic PGE_2 production by indomethacin has been demonstrated by several workers. (Boughton-Smith, et al., 1983; Craven, Saito et al., 1983; Rampton et al., 1981;) While elevated levels of prostaglandin production have been clearly associated with tumours of the colon (Bennett et al., 1977) and elsewhere (Bennett et al., 1975; Rolland et al., 1980; Karmali, Welt et al., 1983; Smith et al., 1983; Attallah et al., 1984) an effect of carcinogen administration such as described here has not been previously noted. In view of the observable changes which occur in the mucosa by histological, histochemical and ultrastructural assessment on the administration of a carcinogen or in the areas adjacent to a tumour (page 22) it is not surprising to find a widespread alteration in PGE_2 levels. This must lead to the reappraisal of those results which use "normal" tissue adjacent to a tumour as the control as this may be of

itself abnormal.

While it is tempting to attribute the beneficial effects of indomethacin on tumorigenesis to its ability to inhibit prostaglandin synthesis (Powles et al., 1973; Humes et al., 1974; Lynch et al., 1979; Bockman, 1983; Pollard et al., 1983; Kollmorgan et al., 1983; Narisawa et al., 1984) it must always be remembered that inhibition of one pathway necessarily alters another and the role of other prostanoids and the leukotrienes remains to be established. The significance of the lipoxygenase pathway in inflammatory bowel disease only became apparent when the deleterious effect of indomethacin was properly assessed. (Rampton & Sladen, 1981) Indeed, completely different systems may be involved - indomethacin can inhibit the effects of bradykinin which result in a rise in cGMP in lung tissue. (Stoner, Manganiello & Vaughan, 1973)

Colonic PGE_2 -further considerations.

Another problem which confronts this type of research is to identify the source of the metabolite under study. In this study the whole thickness of the colonic wall has been used to measure the prostanoid levels - a manoeuvre which necessarily measures the prostanoid content of a myriad of different cell types including the one of interest - the colonic epithelial cell. It has been shown that the virtually all colonic PGE_2 is synthesised by the submucosa and that the epithelial cells possess 71% of the prostanoid degradative capacity of the colon. While the intact colon produces mainly PGE_2 the epithelial cells

produce mainly PGF_{2a} and only small quantities of PGE_2. (Craven and DeRubertis, 1983; Dreyling et al., 1986) Furthermore, it appears that superficial epithelial cells produce more prostanoid and have a greater capacity to metabolise prostanoid than do the proliferative cells. These observations must be compared with the observations that colonic kallikrein activity is lodged in the mucosa while its substrate is in the muscle (vide supra). The overall view would suggest that there is a flux of autacoids which bathes the proliferative compartment of the colonic epithelium and are degraded elsewhere.

All of which poses yet another problem - what relevance has the measurement of an aggregate prostanoid tissue content of a large area of tissue from a single moment in time? The true answer probably is that it is the best which can be done. Some workers have tried to circumvent this problem by measuring the synthetic capacity of the colon given specified substrates (e.g. Boughton-Smith et al., 1983) or simply to measure the release of prostanoid in tissue culture (e.g. Dreyling et al., 1986). The curious finding is that while the algebra may alter, the net results are much the same.

This study has demonstrated that the levels of PGE_2 in the rat colon depend on the site of the colon where the sample is made, are reduced by indomethacin and raised by the administration of the carcinogen azoxymethane. These changes are largely consistent with previous observations on prostanoids in carcinogenesis but it has not been possible to demonstrate an

association with dietary change. The relevance of these changes to the development of colorectal tumours in this model will be discussed below.

CRYPT CELL PRODUCTION RATE

The metaphase arrest technique for the estimation of the crypt cell production rate is a satisfactory one especially when used with crypt cell squash preparations as were used here. This eliminates the problem of cell size variation and altered position with mitosis with estimates from histological sections and also avoids the necessity of having to calculate a crypt size in terms of cell population. Wright (Wright & Appleton, 1980; Wright, 1982) has compared stathmokinetic analysis with thymidine labelling techniques and concludes that the metaphase arrest technique gives an estimate of a useful parameter, the cell birth rate. This study has used this parameter alone to measure the crypt cell production rate which requires no derivation from the measured values and is therefore as accurate as any method for this purpose.

Despite fulfilling the necessary criteria laid out by Wright this study failed to demonstrate any significant effect of site of colon, diet or carcinogen treatment on the crypt cell production rate. The values obtained were of the same magnitude as Galloway (1986) and more recently by Savage et al. (1988). In spite of the lack of effect of site, diet and carcinogen both of the drugs given to the rats - aprotinin and indomethacin - appeared to result in a generalised reduction in the crypt cell

production rate.

Previous work by a variety of authors has presented evidence that administration of a carcinogen results in a series of changes in the crypt involving the proliferative compartment - principally a shift in the site of proliferation to the surface of the crypt with subsequent failure of cellular differentiation and a general increase in cell proliferation. (Lipkin, 1971, Maskens and Deschner, 1977; Deschner, 1982 & Wright, 1983) It has also been appreciated that the pattern of cellular proliferation differs from one area of colon to the next being generally slower in the distal colon than elsewhere (Lipkin, 1971; Sunter, Wright & Appleton, 1978 and Sunter, Watson, Wright et al., 1979) and it is therefore important to compare like areas of the colon. Furthermore the proportion and site of the proliferating component of the crypt also varies by region. (Sunter, Appleton, Wright et al., 1978) With this degree of variability, studying changes associated with tumour development becomes very involved. Wright (1983) suggests in discussing the rat-DMH model that the carcinogen produces progressive hyperplasia in the colon with increased proliferative rates and that out of that generalized hyperplasia evolves a slowly cycling transformed cell which gives rise to the tumour. This has no bearing on the proliferation rate of the induced tumour which may be increased over normal. Sunter (1984) proposes a similar scenario. Tutton and Barkla (1976) measured cell proliferation in the descending colon of the rat following treatment with dimethylhydrazine and found the cell

cycle time to be reduced with an accompanying increase in proliferation in the carcinogen treated animals.

Deschner and coworkers (Deschner, Long, Hakissian et al., 1983) showed in mice that the susceptibility to colonic tumour induction by dimethylhydrazine was directly related to the rate of cellular proliferation in the colonic crypt as assessed by thymidine labelling - a distinction which was still present after administration of the carcinogen. Deschner and Maskens (1982) also showed characteristic changes in the labelling of the colonic crypts in patients and animals with tumours.

This sort of evidence makes it more difficult to explain why this study was unable to discern any differences in the crypt cell production rates between carcinogen and non-carcinogen treated animals since it would appear that this sort of transformation is easiest to detect. While the technique used is reliable and was correctly executed consideration must be given as to whether sufficient animals were studied, sufficient crypts counted in each preparation and whether mitotic figures were correctly assessed. The experimental protocol ensured that the numbers of animals and crypts counted fell within accepted guidelines. While the counting of mitotic figures can vary significantly between individuals, when counted by one individual is very reproducible. (Own observations, unpublished.) All samples in this study were counted by one person.

By examining tissue which was not necessarily adjacent to tumours or in animals which had had carcinogen but had not

developed tumours, then it is quite possible that significant alterations in the crypt cell production rate were overlooked. Indeed the spread of the results may suggest that the wide variation is due to sampling animals with widely varying crypt cell production rates due to whatever cause and not just random variation.

With the failure to demonstrate any effect of carcinogen on the crypt cell production rate it is perhaps not surprising that no effect of diet could be discerned. The significance of the reduction by the drug therapy is therefore perhaps more statistical than practical as the levels of significance achieved are not high. It has to be borne in mind that aprotinin did reduce tumour incidence in one group and that no tumours were seen in the groups receiving indomethacin and ethanol. The altered crypt cell production rate may be a reflection of these observations. A reduction in crypt cell production rate would be consistent with reduced kinin formation as might be produced by aprotinin. (Pisano, 1975) A reduction in crypt cell production would not be consistent with previous observations on the effects of prostaglandins on cell turnover. (Tutton & Barkla, 1980b) However, it would be remarkable if such weak effects showed through when dietary effects did not apparently effect the crypt cell production rate.

A recent study (Savage et al., 1988) showed a significant alteration in tumorigenesis following small bowel resection but could only demonstrate an alteration in the crypt cell production

rate after transection at the duodenum. This manoeuvre is already known to produce hyperplasia in the colon (Williamson & Rainey, 1984) and the failure to demonstrate changes in the crypt cell production rate in this situation highlights the problems of this kind of study.

The study most similar to the current one is that of Galloway (1986) in that it uses in the same animals, diets and methodology. Even this study which examined the effect of diet on the crypt cell production rate found the results difficult to assimilate but arrived at the conclusion that in animals fed a tumour promoting diet the crypt cell production rate was reduced - an effect which was properly considered to allow "...initiated cells to more readily establish their carcinogenic advantage" which is reminiscent of the comments of Wright. However the dietary effects on the crypt cell production rate were by no means clear-cut and the problem of variability was noted.

Hence, while the current study does not immediately support conventional wisdom and is difficult to understand and unify as no clear pattern emerges it is not invalidated and merely points to the depth of confusion which exists and to the necessity for careful evaluation of future work.

Control of epithelial turnover.

A remarkable variety of agents have been shown to influence the proliferative activity of the rat colon. Jacobs (1984) found that feeding bran to rats produced an increase in the proliferative activity of the crypt. Deschner & Raicht (1979)

and Deschner et al., (1981) showed that bile acids had a strong trophic effect on colonic cell proliferation whether administered directly or in the diet. Tutton and Barkla (1980a,b & c ; 1981 & 1982) demonstrated the dependence of colonic epithelial cell proliferation on cAMP, prostanoids, neurotransmitters, glucocorticoids and androgens. Oscarson (1982) adds gastrin and prolactin to this list.

Eastwood (1977) has said that gastrointestinal epithelial renewal is "...a complex process which involves the interaction of numerous extracellular factors with the intracellular control mechanisms for proliferation, migration and differentiation " . Current research has barely described the events of epithelial renewal let alone deal with the controlling factors. Yet something is known of these controlling processes as has been outlined above.

Moreover it can be shown that some of these controlling mechanisms are influenced by carcinogens. DeRubertis and Craven (1977 & 1980) have shown that carcinogens can activate cyclic nucleotide metabolism to effectively increase the cGMP:cAMP ratio which is associated with enhanced cell growth and loss of contact inhibition - a characteristic of transformed cells. Further studies (Craven, Saito et al., 1983) showed that PGE_2 would decrease thymidine uptake in vitro and increase the cAMP levels; indomethacin produced the converse result when given in vivo but in vitro did not alter thymidine uptake. This indicated that inhibition of colonic prostaglandin synthesis and the concurrent

reduction in mucosal cAMP were insufficient alone to stimulate mucosal DNA synthesis. The net effect of prostaglandin was to inhibit proliferative activity.

Tutton and Barkla (1980b & c) also found that colonic cell proliferation was reduced by cAMP but could demonstrate no effect of a prostaglandin analogue or indomethacin on colonic crypt turnover. Curiously, the prostaglandin analogue increased jejunal cell proliferation but inhibited the proliferation of adenocarcinomas. On the other hand indomethacin has also been shown to inhibit the growth of some cell lines in vitro. (Bayer & Beaven, 1979) The inhibition seems to occur at the G1 phase of the cell cycle which may imply an effect on the synthesis of RNA or proteins. (Johnson & Wolberg, 1971)

To this list of controlling factors must be added the expanding field of growth factors. Growth of normal cells is largely controlled by the interplay between several polypeptide hormones and hormone-like growth factors that are present in tissue fluid. Malignant cells do not seem to be subject to the same controls. (Sporn & Todaro, 1986) The factors may be produced by the cells themselves and the control exerted in an autocrine fashion. The presence of such growth factors has been demonstrated in human colonic cancer cell lines. (Coffey, Shipley & Moses, 1986) In the context of the current study it should be pointed out that bradykinin is a small peptide which may well have the properties of a growth factor and certainly functions in an autocrine fashion as do the prostanoids. The recent

demonstration of the control of human colonic cancer cell line growth by peptide hormones lends more weight to the study of agents such as the kinins. (Smith & Solomon, 1988)

This digression into the mechanisms of control of cellular growth and differentiation has been an attempt to relate the findings of the current work to established knowledge. It is abundantly clear from all which has preceded that the understanding of the cellular growth and differentiation is embryonic and the current work underlines the volatile nature of the results which can be obtained. The essential problem with much of this kind of work is separating cause and effect. As regards cellular proliferation it must be asked when is a cell which is proliferating abnormally neoplastic? Since neoplastic change is a disease of the genes then it is easily understood that the genetic change must occur long before any alteration in the detectable changes in cellular proliferation - at least a whole colonic crypt needs to be repopulated for a change in the crypt cell production rate to be noticed. Similarly, if the changes in the surrounding autacoids are being assessed then are these changes the result of tumour development or are they simply epiphenomena which have no direct role in the tumour development? Or are they causative and what causes them? The answers to these questions are not simple and will be returned to later.

FAECAL BILE ACIDS

The results of the study on the faecal bile acids distinctly showed that the faecal bile acid concentration depended significantly on the diet the animals consumed. The high fat, low fibre diet resulted in higher levels of total faecal bile acids, total neutral sterols and total long chain fatty acids than in the animals on the low fat, high fibre diet. Moreover, the pattern of the bile acids was altered. The rise in the faecal bile acids was principally due to a rise in the levels of deoxycholate and muricholate. The rise in the sterols and fatty acids was due to a rise in all components. It is interesting to note that the fatty acids present did not simply reflect the dietary input and must therefore imply that these are derived by metabolism.

Diet and Faecal Bile Acids.

The effect of dietary fat on faecal bile acids in the experimental animal has been studied at some length with fairly consistent results similar to those in this study. (Reddy et al. 1974; Reddy et al., 1984; Reddy et al., 1986; Sakaguchi et al., 1986; Galloway et al. 1986). These results confirm the original hypothesis of Hill and colleagues (Hill, 1983) who made their deductions from epidemiological and human studies. Some workers have shown that the actual changes produced depend on the type of dietary fat used with polyunsaturated fats having the most pronounced effects on the faecal bile acids and sterols. (Reddy et al., 1984; Sakaguchi et al., 1986) Some authors have suggested

that administration of a carcinogen is sufficient to alter the faecal bile acid profile but there is no good evidence of that in this study. (Reddy et al., 1974; Nigro et al, 1974)

The effect of fibre on the faecal components has been much less-well assessed. Brydon et al. (1980) suggested that a diet low in fibre resulted in increased levels of cholate, chenodeoxycholate and muricholate in the rat colon which would accord with the present study and corroborate the findings in humans. (Aries et al., 1971; Cummings et al., 1979). The work of Galloway et al. (1986) examined combinations of high and low fat and fibre and found that the faecal bile acid levels were best correlated with the fibre level of the diet - a low fibre diet resulted in higher levels of bile acids. However, as Galloway correctly points out the faecal bile acid profiles of the rat are considerably different to those of man - the principal products are muricholate and hyodeoxycholate (Brydon et al., 1980) - and this makes it "..impossible..to draw analogies between the rat model and human colonic carcinogenesis." The final pattern of tumour induction in that series of experiments reflected the faecal bile acid concentrations and that is true here also. However, it must be said that the tumour incidence in the animals receiving their standard laboratory diet was as high as that in the animals on the specifically high fat, low fibre diet. The faecal bile acid composition of standard diet animals did not resemble the high fat group at all except in the levels of faecal deoxycholate and muricholate which appeared similar. The animals

on their standard diet also had a higher level of polyunsaturated fatty acids than those on the low fat diet but the level in no way matched that of the the high fat group. While the groups are not strictly comparable this may imply that the combination of fat and fibre produces a maximum effect at a specific concentration. More simply, the relationships between fat, fibre and tumour incidence are non-linear and may be inter-dependent. It may also be deduced that some other component of the faeces is in fact the important agent. The faeces are mutagenic by themselves and may contain any number of active or potentially active substances. (Venitt, 1982; Thompson, 1983 & 1984)

Bile Acids and Tumour Development.

The promoting effect of bile acids on experimental rat tumorigenesis has been shown several times with convincing results. (Narisawa et al., 1974; Reddy et al., 1977 & 1979; Cohen, Raicht, Deschner et al., 1980). The promotional properties have been principally attributed to the secondary bile acids although the primary ones are also effective. A recent study using intestinal resection as a means of stimulating tumorigenesis did not demonstrate a relationship with the bile acid levels, (Savage et al. 1988) but an earlier study in the same vein did show the expected alterations in the bile acids (Koga et al., 1982).

The role of bile acids in the scheme of carcinogenesis in man is to act as substrates for the faecal microbes to form either carcinogenic or promotional agents. In the animal model

they can only act as promoters since the carcinogen is defined. As to how the bile acids perform this promotion is not established. This work has found no direct interaction between bile acids and the endogenous mediators bradykinin and prostaglandin E_2 as assessed by the drug-based experiments. Bile acids may act directly on the epithelial cell to cause promotion and the occurrence of bile acid receptors in tumours may support this idea (Summerton et al., 1983) but it has been pointed out that receptor induction may also be an indecisive epiphenomenon. (Berry, 1988) It seems more likely that there is at least one further link in the chain. Recent evidence has added a new dimension to this. The induction of ornithine decarboxylase in the mouse skin by the tumour promoter 12-O-tetradecanoylphorbol acetate seems to be mediated by the prostaglandins (Verma, Ashendel & Boutwell, 1980). In the rat colon, azoxymethane and bile acids will stimulate the activity of ornithine decarboxylase - the hallmark of promotion - and this also seems to mediated by prostaglandins. (Takano, Matsushima, Erturk et al., 1981; Narisawa, Hosaka & Niwa, 1985; Luk, Hamilton, Yang et al., 1986). It has been appreciated for years that bile acids can stimulate the activity of phospholipase A_2 which is necessary to liberate arachidonic acid for prostanoid formation. (Creutzfeld & Schmidt, 1970) Raised levels of phospholipase A_2 have been demonstrated in tumours (Rillema, Osmialowski & Linebaugh, 1980). Bradykinin can be shown to stimulate cellular production of prostanoid by governing one or more phospholipases. (Bell,

Baenziger & Majerus,1980; Hofman & Majerus, 1982; Nielson, Bukhave, Ahnfelt-Ronne et al., 1988) Hence both of the mediators studied here may yet have a role in bile acid-associated tumour promotion which this study has not demonstrated.

DIET AND TUMOUR INDUCTION.

The overall tumour induction rate in this model was somewhat lower than is usually seen in comparable studies. Galloway (1986) noted this also. The dose of carcinogen was certainly adequate by any standard and the time interval satisfactory at 6 months. Susceptibility to the carcinogen is recognised to vary between and within species (e.g. Deschner et al., 1983) and it may be that the breed of rat used here (Albino Swiss) is more resistant to the carcinogen than the species most commonly used (Sprague-Dawley, Wistar & Fisher F344). Since tumours did develop there can be no doubting the potency of the carcinogen and the tumour yield would probably have increased if the animals had been left for another month.

The defined diets produced the expected dichotomy in tumour induction with the high fat, low fibre diet producing more animals with tumours. This accords with the work of Galloway et al. (1986). Quite unexpectedly, an equivalent number of the animals on their usual laboratory diet developed tumours when given the carcinogen. While it is appreciated that these diets are not strictly comparable this observation does serve to illustrate that consideration of aetiological factors should perhaps be more wide-ranging. The standard laboratory diet

differs in many ways from the defined diets and opens up the possibilities that one or more of these differences may be responsible for its tumour promoting potential. As has been described above in the examination of the bile acid results it does seem possible that the "final common path" of the promotional event may be through the bile acids since the profile in these animals resembles the high fat, low fibre diet.

It has also become apparent with further research that not only the quantity but the quality of the dietary fat is of importance. It would seem that polyunsaturated fats have a greater carcinogenic potential than saturated fats (Sakaguchi et al., 1984 & 1986; Reddy et al. 1984; Minoura et al, 1988) an effect which is not confined to colonic tumours. (Hopkins & West, 1976; Hillyard & Abraham, 1979) Not every study has been able to make this distinction (Reddy et al., 1976a & 1977) and the difference may be more subtle with altered distribution of tumours or more aggressive histology. (Nauss et al., 1983 & Lockniskar et al., 1985) The fatty acid composition of the standard laboratory diet is not available but animals consuming this diet had a relative excess of faecal polyunsaturated fats. In this study it is interesting to note that the tumours were differently distributed in the colon for each of the dietary groups and that while the high fat, low fibre diet produced more, proximal tumours the low fat, high fibre diet produced tumours which were usually more distally located. Histological variation was not a prominent feature. This is similar to Nauss et al.,

(1983) and bears comparison with the work of Koga et al., (1982) who found that resection of small bowel led to more tumours in the "left" colon associated with higher bile acid concentrations where the bile acids would accumulate. Presumably this is true in the high fibre diet in this study and the shift to the "right" in the high fat diet reflects higher concentrations of bile acids entering the colon. The standard diet produced tumours in a distribution more resembling the low fat, high fibre diet and this would suggest that the effect of the diet is more subtle than it first appears. The dose of carcinogen also determines the final tumour distribution with high doses producing more distal lesions. (Ward et al., 1973; LaMont & O'Gorman, 1978)

The effect of dietary fibre is much less predictable as has already been outlined (page 15). Most frequently dietary fibre has been associated with a protective effect and that was seen here since the lowest tumour incidence occurred in the group with the high fibre content. By analogy with Galloway's study it would appear from this study that dietary fibre accords with the tumour risk better than the dietary fat assuming that the relationship between these variables (risk, fat and fibre content) is linear. One explanation for the disparate results seen by some workers may be the fact that there is an "ideal" fat and fibre combination which promotes tumorigenesis better than one or other factor alone. Most studies have examined only the effect of one variable on tumour development and only studies such as those of Wilson et al. (1977), Trudel et al., (1983) and

Galloway et al., (1986) go any distance towards examining both variables simultaneously.

Another source of confusion in studies on dietary fibre is the definition of the fibre itself. The term dietary fibre covers a multitude of chemicals and is probably best defined as "the sum of lignin and non-alpha glucanpolysaccharides" (Southgate, 1982). From a practical standpoint this is equivalent to lignin, cellulose and a variety of other polysaccharides. Studies into the effect of dietary fibre have to settle for either adding one or two defined components of this mixture to the diet in known quantity or giving a mixture (e.g. bran) of essentially unknown and variable composition. The fibre in the diet of this study comprises almost entirely of lignin and cellulose.

Dietary fibre can have one or more of a variety of effects on the colon. It will increase faecal weight and defaecation frequency while reducing transit time, diluting the intestinal contents and reducing the faecal pH (Kritchevsky, 1986) but these effects depend on the type of fibre (Hillman, Peters, Fisher et al., 1983) and cellulose may be the most potent. Fibre will also adsorb organic and inorganic substrates and may increase microbial growth (Kritchevsky, 1986; Cummings & Branch, 1982) but the activity of some microbial enzymes such as *B*-glucuronidases, *B*-glycosidases and mucinases may be decreased. (Shiau et al., 1983; Prizont 1984) Dietary fibre is also associated with decreased dehydroxylation of bile acids and

the production of short chain fatty acids by cellulose fermentation. (Cummings, 1982 & 1984) Short chain fatty acids can have effects on cell structure and proliferation which are thought to be protective both directly and by their ability to reduce colonic pH. (Cummings et al., 1982; Kim, Tsao, Morita et al., 1982; Samuelson, Nelson & Nyhus, 1985; Kritchevsky, 1986) The binding of bile acids per se does not seem to be beneficial (Kritchevsky, 1986) and the dilutional effect of fibre is less convincing as a a protective mechanism. (Calvert, Klurfeld, Subramaniam et al., 1987) It would appear that effects on the colonic mucins, transit time, colonic bulk or the effect of fibre on energy availability may be more important than the obvious ones. Kritchevsky (1986) concludes that "There is no consistent thread of evidence through either human or experimental data which would confer an unequivocally protective role on fibre in general or on some specific fibre component in particular."

Dietary fat, faecal bile acids, prostanoids and tumours.

By comparison, the mechanism of action of dietary fat is thought to be reasonably well understood since there appears to be a more direct line from fats to bile acids to tumours. However, there is growing evidence that the effect may not be as straightforward as originally visualised.

A direct caloric effect of fat on tumour promotion has never been adequately demonstrated but it has to be noted from this study that the animals receiving a high fat, low fibre diet did have a significantly greater caloric intake than the animals

on the low fat, high fibre diet when given carcinogen. When no carcinogen was given the caloric intakes of these two groups were indistinguishable. The differences in the growth patterns of the two groups reflected the differences in caloric intake and it was obvious that the animals on the high fat diet tolerated the carcinogenic insult better than those on the low fat diet. It is difficult to determine if the observed changes in caloric intake which occurred were the indicators of impending tumour development or the result of the animals developing tumours.

Several studies have noted that dietary fats can modulate the immune response in an inhibitory fashion and that polyunsaturated fats are more effective than saturated ones. (Hopkins & West, 1976; Bansal, Rhoads & Bansal, 1978; Kollmorgan, Sansing, Lehman et al., 1979; Erickson, McNeill, Gershwin et al., 1980; Vitale and Broitman, 1981) It is widely appreciated that immunosuppression facilitates the development and metastasis of tumours and some observations in humans give support to the experimental situation by showing alterations in lipid profiles in malignancy and increased invasiveness of tumours with immunosuppressive manoeuvres. (Wood, Habib, Thomson et al., 1985; Blumberg, Agarwal & Chuang, 1985) As to how the dietary lipids may influence the corporate immunity is not understood but it seems likely that they will alter the composition and fluidity of cell membrane, alter the levels of the ubiquitous cAMP, alter macrophage function and cholesterol synthesis. (Vitale & Broitman, 1981) With respect to the colonic cancer model one

group of authors has demonstrated that the risk of the animal developing a colonic tumour is directly related to the relative proportions of polyunsaturated fats present (the Hartz ratio) the principal determinant of this ratio being arachidonic acid (Sakaguchi et al., 1984). When dealing with changes in polyunsaturated fats in the context of the current study it is difficult not to be led to immediately consider whether the prostanoids may be involved. There is good evidence that they may also mediate the immune response and seem on the whole to inhibit both T and B cell activity although this is by no means uniform. (Pelus & Strausser, 1977; Harvey, Allegra, Demers et al., 1977; Goodwin & Webb, 1980; Goldyne & Stobo, 1981; Mertin & Stackpole, 1981) It is clear that prostanoids do not initiate immune responses but modulate a response initiated elsewhere and this modulation may vary depending on the cell type and its stage of differentiation or activation. (Goldyne et al., 1981) The evidence of prostanoid involvement in immunoinhibition and the other evidence of their involvement in tumours and tumour promotion necessarily implies that an inhibitor of prostanoid production such as indomethacin would most likely have a beneficial effect effect on tumour development. This was not seen in this study although the protective effect of indomethacin has been shown elsewhere. (Humes et al., 1974; Lynch et al., 1979; Pollard et al., 1981a & b; Narisawa et al., 1981; Bennett et al., 1982; Carter et al., 1983; Kollmorgan et al., 1983; Pollard et al., 1983; Metzger, Meier, Uhlschmid et al., 1984) Indomethacin

was given in the drinking water in a dose comparable to the other studies and no previous study has demonstrated the presence of indomethacin in the serum of the animals at the time of sacrifice in levels which are thought to be therapeutic. (Hucker et al., 1966)

If the results obtained for tumour induction in the animals receiving standard diet were unexpected then the results for animals given ethanol were startling since not one animal showed any sort of neoplasm. This observation is completely without precedent since all the previous evidence suggests that ethanol in high doses will enhance tumour development - predominantly in the rectum. (Seitz, Czygan, Waldherr et al., 1984; Hamilton, Hyland, McAvinchey et al., 1987) and may do this by enhancing the crypt cell turnover in the rectum. (Simanowski, Seitz, Baier et al., 1986) Others who gave the ethanol as a control medium (as in this study) demonstrated no effect on tumour incidence (Lynch et al., 1978, Carter et al., 1983). This immediately draws critical attention to the other works suggesting indomethacin was protective.

Humes et al., (1974) administered indomethacin by subcutaneous injection on alternate days in a short-term model. Lynch et al., (1978) administered indomethacin in drinking water with ethanol as a solvent which appears to have been properly controlled. However the dose of indomethacin used (120mcg/day at maximum) was rather small. Pollard and Luckert (1981a & b) calculated that they gave their rats 3 mg/kg in a day via

drinking water but did not use ethanol and only changed the water every three days which suggests that the rats may have been receiving little or any drug of consequence since the stability of their solution was not demonstrated and indomethacin is virtually insoluble in water. Their study in 1983 was conducted on similar lines. Bennett et al., (1982) gave the drug as a syrup but only in a short term system. The studies of Narisawa et al.,(1981), Carter et al., (1983), and Kollmorgan et al., (1983) are more convincing demonstrations of the inhibitory capacity of indomethacin on tumour development but the latter two of these pertain to rat mammary tumorigenesis. It is pertinent to note that the Kollmorgan group calculated the final dosage of indomethacin to be 2.5 - 3.0mg/kg.day which is exactly the dose used in this study. The study by Metzger et al. (1984) is interesting in that a protective effect of indomethacin is demonstrated (at a low dose of 200 - 500 $mcg.kg^{-1}.d^{-1}$) on colonic carcinogenesis but another cyclo-oxygenase inhibitor - meclofenamate - was without effect. As in some other studies, the indomethacin was dissolved in water directly and this must raise doubts as to the delivery to the animal. The discrepancy between the two drugs may underline this and pose the probability that a non-specific effect has occurred. In direct contrast, Danzi, Ferulano, Abate et al., (1984) could demonstrate no protective effect of indomethacin on tumour development and suggested that it might even enhance the progression of the disease. However this study is not analogous to the current study since the drug

was not administered until after tumours were proven to be present at an exploratory laparotomy.

It could therefore be argued that the evidence for indomethacin protection is much less than it first appears since some of the studies are poorly controlled and give no indication of the dose of indomethacin used. Only the study of Narisawa et al., (1984) resembles the current research and was inconclusive also.

It has been shown that ethanol can influence the production of prostanoids by a mechanism which is not clear but which may involve prostanoids other than those examined here. (Manku, Oka & Horrobin, 1979) It is therefore possible that the apparent inhibitory effect of ethanol on tumour production is mediated by prostanoids.

The evidence linking prostanoids and neoplasia has already been presented. (page 42) It is worthwhile recalling that a considerable body of evidence has positively identified the prostanoids as being intimately involved with classical tumour promotion in other systems and that they may also be a part of tumour initiation by acting as co-oxygenators. Moreover they have roles in cellular communication and differentiation. (Droller, 1981; Honn, Bockman & Marnett, 1981; Levine, 1981; Karmali, 1983; Fischer, 1985; Trosko, Aylsworth, Jone et al., 1985) To this must be added the concept of cytoprotection and their ability to alter mucus thickness. (Bickel, 1981; Miller & Jacobson, 1979; Robert, 1981) Despite this a true "...causal relationship between

arachidonic acid transformation and cancer has yet to be demonstrated." (Karmali, 1981)

Aprotinin and tumours.

Aprotinin produced a mild protective effect on tumour development in the animals receiving the low fat, high fibre diet and this would be consistent with work already reviewed showing an inhibitory capacity of aprotinin on tumour development. The hypothesis under study has suggested that this might be due to its ability to inhibit bradykinin formation as assessed by measurement of the tissue kallikrein-like amidase activity in the colon. This was undoubtedly reduced by aprotinin in a small way but not in a pattern which could be related directly to carcinogenesis. However it is interesting that the effect was apparent when the tumour incidence was generally low. This may imply that local kinin activity is only one influential factor and that its effect can be completely swamped by other factors such as dietary fat or fibre. The spectrum of activity of aprotinin is so broad that it is not thought to act by just one channel. Other mechanisms of inhibition have been suggested - inhibition of macrophage lysosomal enzymes (Thomson, Tweedie, Pugh-Humphreys et al., 1978) alterations in the cell surface (Latner & Sherbet, 1979) metabolism of "Metastatic factors" (Stein-Werblowsky, 1980) and improvement of host immunity. (Fritz & Wunderer, 1983) It is clear that whatever mechanism is involved then a great deal of further work is required to fully assess the role of aprotinin in tumour development.

GENERAL DISCUSSION AND CONCLUSIONS.

It is a great but not insurmountable step from the experimental animal to man. The inconstant nature of the results seen in this study - both within the study and in comparison with others - only serves to remind that generalised deductions from this kind of work are intrinsically flawed no matter the care taken. None the less, if the results are taken as pointers rather than destinations then it may be suggested that the study within these pages gives support to the hypothesis linking diet and cancer of the colorectum. In very general terms it would appear that dietary fat increases the risk of tumour development while dietary fibre protects against it. The relationship between these two components and their interaction is uncertain and it has been suggested, on the basis of the results obtained in animals on their standard diet, that these two components may not produce a directly linear relationship with risk and that there may be an "ideal" combination which promotes the development of experimental colonic tumours best. The place of other dietary components has also been considered although no information is available on this.

Taken further the study supports, by association, the role of faecal bile acids in the promotion of this tumour. The original aim of the study was to seek expectantly into the place of the autacoids in this scheme by selecting two which seemed on a priori grounds to be of some importance. These results are much more problematical and the role of the kinins and the prostanoids

is by no means established. Nor is it refuted. While the present study has not demonstrated a role for kinins and prostanoids in experimental colonic tumours this may not necessarily reflect the events occurring at or in the colonic epithelium. As has been already suggested, any detectable changes which available methodology can recognise may at best be the sum of a myriad of undetectable alterations occurring at the cellular level. Failure to demonstrate a substantial role for these autacoids may not necessarily mean they are inactive in carcinogenesis. The pointers are that kinins and prostanoids both have a place but the problem lies in defining and measuring that place. Both of these substances are by their nature rather intangible in the body and much has been said in the preceding dialogue about the problems of measurement and the relevance of such measurement to the questions under study. In the final analysis, until such times as we can place ourselves at the heart of the cell during its life we may never properly understand what controls it. The realisation that cancer ".. is at heart a genetic disease." (Bishop, 1986) and the relentless assault on the gene may be the first step in that understanding.

The relevance of this study to human disease must be the final consideration. The colorectal cancer animal model has been universally acclaimed as a good representation of human tumours and their controlling factors. This is true only within the confines of the experiment being conducted and it is clear that too many workers take a rather literal interpretation of the

animal model. A brief consideration of the technical aspects reveals one very fundamental difference - in the rat, the primary carcinogen is known; in the human it is not. Furthermore, observations which suggest that rat colonic tumours may be arising principally from areas of specialised epithelium over lymphoid aggregates and that there may be two separate neoplastic processes at work only serve to highlight the differences. (Nauss, Locniskar, Pavlina et al., 1984) If human colorectal cancer is a two-stage process then it might reasonably be asked when the initiation process occurs. It is difficult to see a human situation whereby a single, unique exposure to a carcinogen will occur which has never previously occurred and which will never recur. On the other hand it may not be necessary to have an initiator: considering the millions of cell divisions which occur daily then a spontaneous error could easily be promoted. Most errors may be inconsequential; many will not survive or will be corrected. One may be promoted and be unique by resisting all of man's inbuilt safeguards by a legion of mechanisms of which changes in autacoids may only be one facet. The logical extension of that would be that a high rate of epithelial turnover would generate more errors and thereby more tumours; more important may be the ability of the cell to resist mobilisation and express its carcinogenic advantage to the full in a more slowly cycling cell population. While it is relatively easy to develop concepts of large bowel tumorigenesis none of the hypotheses can adequately explain the most impressive observation of all - that adjacent

small bowel is highly resistant to tumour development in any situation. Surely, an answer to this conundrum would shed more light on tumour development elsewhere.

When Doll and Peto published their article on the causes of cancer in 1981 they cited five broad headings by which the diet may influence the development of a tumour. Thus the diet may provide the carcinogen directly, may provide for the production of carcinogen within the body, may alter the activation or inactivation of the carcinogen, affect the promotion of cells or simply increase risk by obesity. This study has examined only one small facet of that range of possibilities in a purely experimental situation and has demonstrated that the diet does influence the susceptibility to tumour development in the colon and that the effect of the diet may be mediated by the actions of local hormones such as the kinins or prostanoids but these effects are difficult to quantify. This is borne out by the many other studies with similar results. In spite of this wealth of knowledge it is still true that "..the science of diet and cancer is immature." (Doll & Peto, 1981)

APPENDICES

APPENDIX 1

COMPOSITION OF THE DIETS.

CONSTITUENT	OXOID BREEDING DIET	HIGH FAT LOW FIBRE DIET	LOW FAT HIGH FIBRE DIET
CRUDE FAT	4.0%	25.2%	2.1%
CRUDE PROTEIN	22.9%	14.9%	14.8%
CRUDE FIBRE	3.56%	2.1%	26.9%
ASH	N/K	4.1%	7.5%
NITROGEN FREE EXTRACTIVES	N/K	43.9%	38.7%
DIGESTIBLE CRUDE FAT	N/K	22.6%	1.6%
DIGESTIBLE CRUDE PROTEIN	N/K	13.6%	13.4%
TOTAL DIETARY FIBRE	N/K	6.7%	35.5%
STARCH	N/K	35.5%	25.8%
SUGARS	N/K	3.2%	3.7%
GROSS ENERGY	N/K	20.3 MJ/kg	14.7 MJ/kg
DIGESTIBLE ENERGY	N/K	17.9 MJ/kg	8.2 MJ/kg
METABOLIZABLE ENERGY	12.0 MJ/kg	16.1 MJ/kg	7.4 MJ/kg
VITAMIN A	50000 i.u.	6000 i.u.	6000 i.u.
VITAMIN D_3	3000 i.u.	1000 i.u.	1000 i.u.
VITAMIN E	60.0 mg	50.0 mg	50.0 mg
VITAMIN B_1	6.0 mg	5.0 mg	5.0 mg
VITAMIN B_2	10.0 mg	5.0 mg	5.0 mg
VITAMIN B_6	5.0 mg	5.0 mg	5.0 mg
VITAMIN B_{12}	35.0 mcg	5.0 mcg	5.0 mcg
MENADIONE	N/K	5.0 mg	5.0 mg
FOLIC ACID	1.0 mg	0.5 mg	0.5 mg
NICOTINIC ACID	10.0 mcg	10.0 mg	10.0 mg
CALCIUM PANTOTHENATE	N/K	10.0 mg	10.0 mg
CHOLINE CHLORIDE	1500.0 mg	400.0 mg	400.0 mg
LYSINE	1250.0 mg	2000.0 mg	2000.0 mg
METHIONINE	470.0 mg	1500.0 mg	1500.0 mg
CALCIUM	810.0 mg	4700.0 mg	2000.0 mg
PHOSPHORUS	650.0 mg	3500.0 mg	4000.0 mg
SODIUM CHLORIDE	230.0 mg	5000.0 mg	5000.0 mg
MAGNESIUM	180.0 mg	1000.0 mg	1000.0 mg
IRON	10.0 mg	20.0 mg	20.0 mg
COPPER	25.1 mg	7.0 mg	7.0 mg
MANGANESE	102.6 mg	20.0 mg	20.0 mg
ZINC	95.5 mg	10.0 mg	10.0 mg
COBALT	3.0 mg	0.3 mg	0.3 mg
IODINE	1.5 mg	0.5 mg	0.5 mg

[Unless otherwise specified, all values are for 1 kg of diet.]

[For definitions see notes on page 190]

COMPOSITION OF THE DIETS.

Fatty acid composition of High fat, low fibre diet.

FATTY ACID	CHEMICAL COMPOSITION	% COMPOSITION OF DIET
Lauric acid	$CH_3(CH_2)_{10}COOH$	0.1
Myristic acid	$CH_3(CH_2)_{12}COOH$	0.9
Palmitic acid	$CH_3(CH_2)_{14}COOH$	5.2
Stearic acid	$CH_3(CH_2)_{16}COOH$	3.6
Linoleic acid	$CH_3(CH_2)_4CH{=}CHCH_2CH{=}CH(CH_2)_7COOH$	2.6
Linolenic acid	$CH_3(CH_2CH{=}CH)_3(CH_2)_7COOH$	0.4
Oleic acid	$CH_3(CH_2)_7CH{=}CH(CH_2)_7COOH$	7.5
Palmitoleic acid	$CH_3(CH_2)_5CH{=}CH(CH_2)_7COOH$	0.3
Myristoleic acid	$CH_3(CH_2)_3CH{=}CH(CH_2)_7COOH$	0.01
Clupanodonic acid	$CH_3(CH_2)_3(CH{=}CHCH_2)_5(CH_2)_2COOH$	0.03
Arachidonic acid	$CH_3(CH_2)_4(CH{=}CHCH_2)_4(CH_2)_2COOH$	0.2

APPENDIX 1 (continued)

NOTES

1.Crude fat is the material extracted with petroleum-ether under continuous reflux-Soxhlet Extraction

2.Crude protein is the total nitrogen content determined by Kjeldahl multiplied by the factor 6.25 to convert to protein concentration

3.Crude fibre (essentially cellulose and lignin) is the residue left after treatment with boiling dilute acid and alkali.

4.Ash is the residue left after incineration at 500^{0}C.

5.Nitrogen Free Extractives (essentially crude carbohydrate) is the difference of the sum of the percentages of moisture, fat, protein, fibre and ash from 100%

6.Digestible values are found by experiment and free digestibility factors are used to indicate the amount of digestible nutrient. Usually obtained from pigs or poultry.

7. Total dietary fibre includes pectins and hemicelluloses which are soluble in the crude fibre assay and are therefore included in the N.F.E. value.

8. Starches and sugars are obtained by analysis.

9. Gross energy is the energy derived from bomb calorimetry and reasonably is the sum of the gross energies of the fat, protein, fibre and carbohydrate contents of the diet. Metabolizable energy is taken as 90% of digestible energy.

APPENDIX 2

THE USE OF AZOXYMETHANE.

Azoxymethane was purchased from Ashe Stevens Inc. Detroit, USA in aliquots of 10 grams. This was made into a stock solution of 0.2g/ml with saline and stored at $4^{o}C$.

An aliquot of the stock solution was diluted prior to use to give a solution of azoxymethane in saline of 10mg/ml. The animals were injected subcutaneously in the scapular area with a dose of 10mg/kg. This dose was repeated at weekly intervals for 12 weeks. The first dose of carcinogen was given at the fourth week of the experiment.

Animals receiving the carcinogen were kept separate from those which were not receiving it. While their diet, water and drugs came from a common source, at no time did the carcinogen treated animals and non-carcinogen treated animals mix. All animal waste was disposed of separately.

The use and storage of the carcinogen conformed to the guidlines laid out in the memorandum "Working with Carcinogens - Code of Practice." (University of Glasgow, 1981)

APPENDIX 3

ASSAY OF TISSUE KALLIKREIN-LIKE AMIDASE ACTIVITY.

PREPARATION

1. Prepare TRIS (Sigma, Poole) buffer 0.2M, pH 8.2 with SBTI 250mcg/ml (Sigma)
2. Prepare sodium lauryl sulphate 20% (BDH, Analar grade)
3. Prepare acetic acid 50% (BDH)
4. Water bath at 37°C

METHOD

1. Thaw tissue extract
2. Place 500mcl TRIS buffer in tubes and incubate for 10 minutes in water bath
3. Add 400mcl of extract
4. Mix and incubate for 5 minutes
5. Add 100mcl of substrate (S-2266, Kabi Diagnostica) and incubate at 37°C for 45 minutes.
6. Add 100mcl of acetic acid to stop the reaction
7. Centrifuge for 15 minutes to remove sediment (2000g)
8. Take 900mcl of supernatent and place in fresh incubation vial
9. Add 100mcl of sodium lauryl sulphate
10. Mix and allow to stand for 5 minutes
11. Measure optical density on spectrophotometer at 405nm, slit width 0.3mm with distilled water as the blank

All samples assayed in duplicate.

Assay blanks are prepared in the same way but the steps are: 1,6,2,3,4,5,7,8,9,10,11.

APPENDIX 4

CALCULATION OF THE TISSUE KALLIKREIN-LIKE AMIDASE ACTIVITY OF RAT COLON.

Let A = Absorbance at 405nm

e = Extinction coefficient

V_T = Volume of incubate before centrifuging (ml)

V_S = Volume of supernatent taken (ml)

V_F = Volume of final solution for assay (ml)

W = Wet weight of tissue (g)

V_H = Volume of water used to homogenise (ml)

D = Dilution of extract before assay expressed as:

$$\frac{\text{volume of concentrate}}{\text{volume of final solution}}$$

t = Time of incubation

V_E = Volume of extract used

V_E contains $V_E \times W/V_H$ g of tissue

(If this is diluted before assay then V_E contains $V_E \times W/V_H \times D$ grams of tissue)

Activity is produced by this tissue and is assessed by formation of pNA. The concentration of pNA is measured in the final solution V_F. This pNA is entirely derived from the supernatent taken - V_S. Therefore the concentration of the pNA in the original incubate was :

$$V_F/V_S \times A/e \text{ (m mol of pNA)}$$

APPENDIX 4 (continued)

The original solution had a volume V_T, and therefore the number of moles of pNA produced by incubation was:

$$V_F/V_S \times A/e \times V_T \times 10^{-3} \quad \text{(since vol is in ml)}$$

This activity is produced in time t by the tissue extract and therefore the rate of production of pNA per gram of tissue per minute is

$$V_F/V_S \times A/e \times V_T \times 10^{-3} \times 1/t \times \frac{1}{(V_E \times W/V_H \times D)}$$

This reduces to

$$\frac{V_F \times A \times V_T \times V_H \times 10^{-3}}{V_S \times e \times t \times V_E \times W \times D} \quad \text{mol pNA.g-1.min-1.}$$

For the usual values of $V_T = 1.1$

$V_S = 0.9$

$V_F = 1.0$

$V_E = 0.4$

$e = 10343.367$

this becomes :

$$295.41208 \times (A \times V_H)/(W \times D \times t) \text{ nmolpNA.g-1.min-1.}$$

APPENDIX 5

S-2266

DATA SHEET AS PREPARED BY KABI DIAGNOSTICA, STOCKHOLM, SWEDEN.

S-2266 is a chromogenic substrate for glandular kallikreins.

Chemical Name : H-D-Valyl-L-leucyl-L-arginine-p-nitroanilide dihydrochloride.

Formula : H-D-Val-Leu-Arg-NH-⟨O⟩-NO_2·2HCl

Molecular Wt. : 579.6

Principle : H-D-Val-Leu-Arg-pNA $\xrightarrow{enzyme}$ H-D-Val-Leu-Arg-OH + p-NA

The method for the determination of activity is based on the difference in the optical density (absorbance) between the pNA formed and the original substrate. The rate of pNA formation is proportional to the enzymatic activity.

S-2266 is supplied in vials containing 25mg of substrate and was made up in 28.8ml of fresh distilled water to give a solution of $1.5mmol.l^{-1}$. This was stored in aliquots of 1ml at $-40^{o}C$ until use.

The data supplied with the reagent suggests that it is stable in solution for at least 2 months at temperatures of $2 - 8^{o}C$. All of the experiments here used the diluted substrate well within that time.

APPENDIX 6

RADIOIMMUNOASSAY FOR PGE_2

The individual steps for the assay are shown below.

1. Place 4ml of ethyl acetate into 18 tubes which will be used for the standard curve and evaporate to dryness in the vacuum oven at 37^oC
2. Thaw all samples and mix.
3. Take at least two aliquots (250mcl) from each sample and place in extraction tubes.

EXTRACTION

4. Add 50mcl of ^3H-PGE_2 (0.02mcl/ml) to each sample tube and to two of four blank tubes (internal standard for the estimation of recovery). Add 50mcl of phosphosaline buffer to the two remaining blanks.
5. Place 50 mcl of ^3H-PGE_2 (0.02mcCi/ml) in each of four scintillation vial inserts and add 5ml of scintillant to each. (Total counts for the estimation of recovery).
6. Add sodium citrate buffer to all tubes (half the volume already present in each tube = 150mcl) and mix. Prostanoids will be non-ionised at mildly acidic pH.
7. Add 2ml ethyl acetate to each tube and mix (Multivortex shaker) for 5 minutes.

APPENDIX 6 (continued)

8. Allow organic and inorganic phases to settle. Freeze the inorganic layer by placing in dry ice/acetone up to interface. Decant the ethyl acetate into assay tubes.
9. Place assay tubes in the vacuum oven and evaporate to dryness at 37°C.
10. Allow the samples to thaw and repeat steps 7, 8 and 9.

STANDARD CURVE

11. Set up standard curve using the tubes which had 4ml of ethyl acetate evaporated from them. Prepare in duplicate tubes for T, B, O, (empty) and then 3, 10, 30, 100, 300 and 1000 pg of prostanoid by pipetting the appropriate stock solution into the tubes. Evaporate to dryness in the vacuum oven.

RECONSTITUTION

12. Add phosphosaline buffer to all tubes - 200mcl to the standards and 250mcl to the samples. Mix.
13. Remove 50mcl from each sample tube and place in a scintillation vial insert. Add 5ml of scintillant (Biofluor) to the insert. (For estimation of recovery).

APPENDIX 6 (continued)

INCUBATION

14. Add 50mcl of ^{3}H-PGE_2 (0.2mcCi/ml) to each tube and mix.
15. Add 50mcl of anti-PGE_2 antibody solution to all tubes except T and B. Add 50mcl of phosphosaline buffer to T and B.
16. Incubate overnight at $4^{o}C$.

SEPARATION

17. Add 50mcl of gelatin to every tube. Mix.
18. Add 200mcl of Dextran-coated charcoal suspension to all tubes except T. Add 200mcl of phosphosaline buffer to T. Mix.
19. After 10 minutes centrifuge at 1000g for 2 minutes.
20. Take 250mcl of supernatant from each tube and place in a scintillation vial insert. Add 5ml of scintillant.
21. Count all recovery and assay vials on scintillation counter.

APPENDIX 6 (continued)

THEORY.

The samples are assayed in duplicate. After acidification they are extracted twice with ethyl acetate and the recovery measured by use of the tracer dose of $^{3}H\text{-}PGE_2$ (0.02mcCi/ml). After reconstitution in phosphosaline buffer the antibody and the tritiated prostanoid are added for the incubation to proceed. The bound and unbound $^{3}H\text{-}PGE_2$ are separated by using Dextran-charcoal and a reasonable sample of the bound $^{3}H\text{-}PGE_2$ measured by scintillation counting. From the standards the scintillation counts are converted into pg of PGE_2 in the original sample.

The blanks: T measures the total activity of the tritiated solution used for the assay and was in the order of 4000 cpm. (mean 4362 ± 1060)

B was used to give an estimate of the non-specific binding by the charcoal in the assay. This non-specific binding was expressed as B/T x 100% and was less than 3%.

The '0' tube gave an estimate of the initial binding of the assay and was expressed as ''0'/T x 100%. This was in the order of 25 - 38%.

The IC_{50} for the reaction was the amount of PGE_2 to reduce the '0' value by 50% and ranged from 40 - 140pg (mean 74 $\pm$ 41pg) depending on the total activity of the ^3H-PGE_2.

The detection limit was defined as the amount of PGE_2 which would reduce the '0' value by by 10% and ranged from 5 - 15 pg. (mean 9 $\pm$ 5pg)

Standard Curve Fitting

The standard curve has the form $Y = [K3/(X + K2)] - K1$ and was fitted by an iterative programme on an Apple computer. In this case Y = the cpm and X the amount of PGE_2 in pg.

APPENDIX 6 (continued)

Calculation

Let P_M = pg of PGE_2 measured by RIA

P = pg of PGE_2 in the original sample

R = the recovery (%)

V_E = volume of extract assayed (mcl)

W_E = weight of tissue in sample assayed (mg)

W = weight of original colon sample (mg)

V_H = volume of PSB used to homogenise the tissue (ml)

The amount of PGE_2 measured in the assay derives from 200mcl of reconstituted sample which represents 4/5 of the total sample therefore :

the amount of PGE_2 in the original sample $= \frac{5}{4} \times P_M \times \frac{100}{R} = P$

This PGE_2 derived from V_E mcl of extract which contains W_E mg of tissue.

$$W_E = V_E \times \frac{W}{V_H \times 10}$$ since the sample was diluted tenfold before assay.

Therefore the $[PGE_2]$ of the original sample extract

$$= P/W_E = (5/4 \times P_M \times 100/R)/(V_E \times W/(V_H \times 10))$$

$$= \frac{1250 \times P_M \times V_H}{V_E \times R \times W} \quad ng.g^{-1}$$

In this case $P_M = \frac{K_3}{(C + K_1)} - K_2$ where C = cpm

APPENDIX 6 (continued)

SOLUTIONS FOR PROSTAGLANDIN ASSAY

Sodium Citrate Buffer 0.15M pH 4.0

Solution 1	27.5ml
Solution 2	22.5ml
Distilled water to 100 ml	

Solution 1 = Citric acid $C_6H_8O_7.1H_2O$ 21g (BDH Chemicals, Poole)

Sodium hydroxide 1M 200ml (BDH)

Distilled water to 1000ml

Solution 2 = Hydrochloric acid 0.1M (BDH)

(All reagents were Analar grade)

Phosphosaline Buffer 0.15M pH 7.4

Solution A	10.12ml
Solution B	2.37ml
Sodium chloride	2.25g (BDH)
Gelatin	0.25g (BDH)
Thiomersal	0.012g (BDH)
Distilled water to 250 ml	

Solution A = 0.2M Na_2HPO_4 = 28.3g/l

Solution B = 0.2M NaH_2PO_4 = 31.3g/l

(All reagents were Analar grade)

APPENDIX 6 (continued)

SOLUTIONS FOR PROSTAGLANDIN ASSAY (continued)

Gelatin Solution

Gelatin	250mg (BDH Chemicals)
Phosphosaline buffer	25ml

Dextran-coated Charcoal Suspension

Charcoal (activated)	500mg (Sigma Chemical Co. Ltd., Poole)
Dextran T70	50mg (Sigma)
Phosphosaline buffer to	50ml

(solution used on ice with continuous stirring)

Scintillation Counting

Scintillation counting was carried out on a Packard Tri-carb 460C using a tritium programme. The counting time was 5 minutes for each sample. This included the automatic caluation of a quench index parameter by analysis of the spectral index of the sample.

APPENDIX 7

REAGENTS FOR PROSTAGLANDIN RADIOIMMUNOASSAY

Manufacturers' data

ANTISERA TO PGE_2 (Institut Pasteur, 3,Bd Raymond Poincare, BP3)

Origin: rabbit   Immunogen: PGE_2-BSA

Presentation: lyophilised (Reconstituted in phospholsaline buffer, 5.5ml per vial)

Binding parameters: Reconstituted antiserum binds 45% of 10pg of ^3H-PGE_2

Sensitivity = 8 - 20pg (IC_{50}) Affinity: $K_a = 8.2 \times 10^{10}\ M^{-1}$

Cross - reactivity:

PGA_1	,0.04%	PGA_2	0.30%
PGB_1	<0.01%	PGB_2	<0.01%
PGD_1	<0.01%	PGD_2	<0.01%
PGE_1	10.70%	PGE_2	100.00%
DH-PGE_1	0.01%	DH-PGE_2	2.1%
15K-PGE_1	0.16%	15K-PGE_2	13.2%
15K,DH-PGE_1	0.03%	15K,DH-PGE_2	0.6%
PGF_1	0.01%	PGF_2	0.11%
DH-PGF_1	----	DH-PGF_2	<0.01%
15K-PGF_1	<0.01%	15K-PGF_2	<0.01%
15K,DH-PGF_1	<0.01%	15K,DH-PGF_2	<0.01%
19-OH-PGE_1	<0.01%	19-OH-PGE_2	----
6K-PGF_1	<0.01%	19-OH-PGF_2	0.019%
		TXB_2	<0.01%
		6,15K-PGE_2	0.01%

APPENDIX 7 (continued)

TRITIATED PGE_2 (Amersham International plc, Amersham HP7 9LL)

Structure: [5,6,8,11,12,14,15(n) - ^{3}H]PGE_2

Specific Activity: 160Ci/mmol, 438mCi/mg

MW = 352 (= 363 at this specific activity)

Radioactive concentration: 0.1mCi/ml

Radiochemical purity (HPLC) : 94.8%

Storage: At -20°C; decomposition not greater than 3%/month for 2 months but may accelerate thereafter typical of tritiated compounds.

Supplied in ethanol:water mixture containing 25mcCi in 250mcl of solution.

Stock solution: 50mcl of supplied solution has 4.95ml of ethanol added to give a solution of 1mcCi/ml. (stored at -20°C)

Working solution: 1.4ml of stock solution (=1.4mcCi) has solvent evaporated and the 3H-PGE_2 reconstituted in 7ml of phosphosaline buffer (0.2mcCi/ml). A further 1 in 10 dilution is made to give a 0.02mcCi/ml solution for the estimation of recovery.

PGE_2 Standards

PGE_2 was obtained from Upjohn Co., Kalamazoo. A stock solution of 1ng/ml was prepared in ethanol and stored at 4°C. In the assay varying volumes of the standard solution were assayed to create the standard curve for the assay (3 - 1000 mcl of solution = 3 - 1000 ng of PGE_2)

APPENDIX 8

PREPARATION OF COLONIC TISSUE FOR STATHMOKINETIC ANALYSIS.

1. Fix in Carnoy's solution 4 hours. (Drury & Wallington, 1980)
2. Transfer to 70% ethanol for storage.
3. Rehydrate through 70%, 50% & 30% ethanols - 10 mins each.
4. Immerse in N HCl at 60°C for controlled hydrolysis. (10 mins)
5. Stain with Schiff's reagent (Sigma, Poole) for 1 hour.
6. Dehydrate in sequential alcohols; store in absolute alcohol.
7. Suspend tissue in a drop of 5% acetic acid on a microscope slide.
8. Strip off mucosa using dissecting microscope.
9. Microdissect mucosal crypts using needles/blade to tease apart.
10. Resuspend in a drop of 5% acetic acid.
11. Squash preparation with a coverslip.
12. Soak in methylated spirit to free coverslip with preparation attached.
13. Dip coverslip in xylol.
14. Mount on a clean slide with clear polyester resin (Araldite) with clamp to maintain squash.
15. Count metaphase figures in ten crypts.
16. Plot metaphase count with time to calculate regression line.

Method after Ferguson et al., 1977 & Cooke et al., 1984

APPENDIX 9

MEASUREMENT OF FAECAL BILE ACIDS.

Faecal bile acids were identified and quantified by the method of Owen, Thompson and Hill. (1985)

Samples of the freeze-dried rat faeces were subjected to continuous extraction in a Soxhlet apparatus using organic solvents. Initial extraction with petroleum ether yielded a 'neutral fraction' comprising mainly cholesterol, coprostanol, coprostanone, plant sterols and up to 10% free bile acids especially lithocholic acid. Subsequent extraction with methanol and chloroform (1:1 v/v) yielded an extract containing free bile acids and their conjugates and sulphates. This was fractionated using a lipophilic anion exchanger (diethylamino-hydroxypropyl Sephadex LH-20) and the steroids then identified by computerised gas-liquid chromatography - mass spectrometry.

APPENDIX 10

THE DETERMINATION OF INDOMETHACIN IN PLASMA BY SPECTROFLUORIMETRY

(Hucker et al., 1966)

Method

1. To a stoppered test tube add
   - 1.0ml plasma
   - 1.0ml citrate buffer
   - 0.5ml water
   - 5.0ml n-heptane/iso-amyl alcohol
2. Extract by mechanical shaking - 15mins.
3. Centrifuge at 2000 rpm
4. Transfer heptane phase (supernatant, 4ml) to a separate tube.
5. Add 5ml 0.1M sodium hydroxide.
6. Extract by mechanical shaking - 15mins.
7. Centrifuge at 2000 rpm
8. Aspirate organic phase
9. Place aqueous phase in a quartz cuvette
10. Measure fluorescence - excitation 295nm
    - emission 385nm
    - slit 8nm

Solutions

Citrate buffer pH 5.0

n-heptane 3% v/v iso-amyl alcohol (Sigma, Poole)

0.1M Sodium Hydroxide.

APPENDIX 10 (continued)

Principle

Indomethacin is extracted into heptane. It is then deacylated by sodium hydroxide to a fluorescent product which is measured. The amount of indomethacin in the plasma is calculated from known standards assayed at the same time. The relationship between the fluorescence and the indomethacin concentration is known to be linear.

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