Investigations into the Multistep Process of Colon Carcinogenesis as Affected by Dietary Calcium

by

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INVESTIGATIONS INTO THE MULTISTEP PROCESS OF COLON CARCINOGENESIS AS AFFECTED BY DIETARY CALCIUM

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree

DOCTOR of PHILOSOPHY

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ABSTRACT

The main objective of this dissertation was to explore the growth modulating effect of dietary calcium (Ca) on the azoxymethane induced multistep process of colon carcinogenesis in Sprague Dawley rats. Number and growth features of microscopic aberrant crypt foci (ACF) and colonic tumors were used as the biological end points. Risk markers such as proliferative indices, protein kinase C (PKC) and lipid composition were also investigated. The diets containing low (0.1%), normal (0.5%) or high (1.0 and 2.0%) Ca and low (5%) or high (20%) fat were fed one week after azoxymethane or saline administration. There were significant fat * Ca interactions for the number of ACF with advanced growth characteristics ($p=0.006$), PKC activity ($p=0.0001$), and the content of polyunsaturated fatty acids in phosphatidylcholine ($p=0.0001$), phosphatidyl-ethanolamine ($p=0.0001$), phosphatidylserine ($p=0.001$), and phosphatidylinositol ($p=0.0006$). Dietary intervention was carried out in a group of rats, 12 weeks after azoxymethane treatments, harboring preneoplastic lesions in their colons. Diets varying in Ca were fed for 12 weeks and both ACF and tumors were evaluated. The growth restrictive effect of increasing the level of Ca was more evident on primal ACF ($p=0.05$) than their advanced counterparts. Analyses of tumor outcome by region revealed a growth restrictive effect of 2.0% Ca in the distal region and a permissive effect in the proximal region . Dietary Ca affected the lipid composition of neutrophils ($p=0.05$) and their ability to produce leukotrienes ($p=0.05$). These findings attest to the multiple and complex effect of dietary Ca on cellular responses. The novel and important findings of this dissertation are: 1) dietary Ca, at varying levels, elicits different responses in the presence of a low or high fat diet; 2) a positive correlation was found between alteration in PKC activity and ACF with advanced growth features under the same dietary regime; 3) preneoplastic lesions at different developmental stages respond differently to dietary Ca in different colonic regions; and 4) the ability of Ca to modulate the lipid composition and leukotriene production of neutrophils suggests that Ca mitigates, in part, its biological effect by a systemic route.

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LIST OF ABBREVIATIONS

AA	arachidonic acid
AC	aberrant crypt
AC/focus	aberrant crypts per focus
AC1...AC≥4	focus with 1....≥4 aberrant crypts
ACF	aberrant crypt focus(foci)
AIN-76A	American Institute of Nutrition -76A
AOM+	azoxymethane treated
AOM-	non-azoxymethane treated
ATP	adenosine triphosphate
b.w.	body weight
BrdU	bromodeoxyuridine
CaCarb	calcium carbonate
CaGluca	calcium glucarate
CaGluco	calcium gluconate
CaLact	calcium lactate
CaPhos	calcium phosphate
DAG	diacylglycerol
HETE	hydroxyeicosatetraenoate
HPETE	hydroperoxyeicosatetratenoate
HF	high fat
LA	linoleic acid
LF	low fat
LI	labeling index
LTB4	leukotriene B4
MI	mitotic index
OA	oleic acid
PA	phosphatidic acid
PBS	phosphate buffered saline
PC	phosphatidylcholine
PCNA	proliferating cell nuclear antigen
PE	phosphatidylethanolamine
PI	phosphatidylinositol
PKC	protein kinase C
PMSF	phenyl methyl sulfonyl fluoride
PS	phosphatidylserine
PUFA	polyunsaturated fatty acid
PZ	proliferative zone
SA	stearic acid
SAS	statistical analysis software
SATFA	saturated fatty acid
s.c.	subcutaneous
S.E.	standard error
Sphing	sphingomyelin
TI	tumor incidence

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Section I

INTRODUCTION

Chapter 1. INTRODUCTION

> Hee is a better physician that keepes diseases off us, than hee that cures them being on us. Prevention is so much better than healing because it saves us the labour of being sick.
>
> Thomas Adams, 17th Century

Cancer is defined as a cellular malignancy whereby transformed cells have unregulated growth, lack of differentiation, and possess the ability to invade neighboring tissues and metastasize (Berkow & Fletcher, 1987). Colorectal cancer, prevalent in industrialized countries, is of worldwide importance. In Canada, the top three cancers account for more than 50% of all cancer related deaths and colorectal cancer is second only to lung cancer (National Cancer Institute of Canada, 1996). This disease appears gender unbiased with incidence rates of approximately 13% for both men and women (National Cancer Institute of Canada, 1996). In 1993, $3.5 billion was funneled to cancer related costs, 80% directed to hospitals and only 2% to research (National Cancer Institute of Canada, 1996). As a result, the disease has significant economic impact such that resources expended could be allocated elsewhere in the absence of the disease (National Cancer Institute of Canada, 1996).

Increasing attention has been given to research efforts in the area of cancer causation and prevention. Indeed the concept of prevention is very attractive as it would free up financial obligations and "save us the labour of being sick". It is thought that a large proportion of human cancer (50-80%) is preventable as causative factors have been found to be largely environmental (Doll & Peto, 1981; Ries et al., 1991; Weinstein, 1991). Primary prevention involves the identification of exogenous factors important in the development of cancer. This is an important step as these exogenous factors provide the most malleable variable in a majority of human cancers (Weinstein, 1991). The

elucidation of mechanism(s) by which nutrients impact the carcinogenic process is paramount to disease prevention.

The study of dietary constituents on colon carcinogenesis has been hampered by limitations in markers of disease development (Bird, 1989). Tumorigenesis studies reveal little information as to the effect of the nutrient at various stages of prior to cancer development. Risk markers which can be measured at early time points are associated with increased risk but do not represent the disease itself. Again this provides little information regarding the effect of the nutrient during the carcinogenic process. Precursor lesions of colon cancer, first identified by Bird (1987), provide early indicators of the role of dietary factors on the disease process itself. This system has been intensely scrutinized and supports the use of aberrant crypt foci (ACF) as end points in colon cancer risk assessment or in the identification of modulators of the disease process (Bird, 1995).

The stimulus of this dissertation involved interest in research literature regarding calcium as a potential modulator of carcinogenesis and subsequent use in human clinical trials. The calcium "soap hypothesis" suggested that benefit was derived from the binding of free and bile acids which were toxic to the colonic epithelium (Newmark et al., 1984). However, findings that calcium modulated hyperproliferation of the mammary gland (Zhang et al., 1987) and the mixed findings of calcium tumorigenesis studies (Pence & Buddingh, 1988; McSherry et al., 1989; Wargovich et al., 1990; Karkare et al., 1991) suggested that the effect of calcium might be more complex than first thought. Together these studies indicated that further research in understanding the role of calcium in the multistage nature of colon cancer was needed.

The hypothesis to be tested in the present dissertation is that the cancer modulating effect of dietary calcium would depend on the level of fat in the diet and that preneoplastic lesions would respond differently to dietary calcium depending on their

developmental stages. Based on this hypothesis, the two main objectives were: (a) to systematically evaluate diet composition relevant to calcium investigations; and (b) to assess the efficacy of calcium intervention on different developmental stages in the colon carcinogenic process as well as related biochemical and cellular events. In order to address these objectives, the specific objectives of each study were as follows:

(i) assess the effect of dietary constituents pertinent to calcium investigations on the growth of ACF and colonic epithelial proliferation (Chapter 4 & 5);

(ii) assess whether the growth pattern of ACF and colonic epithelial proliferation are modulated in an expected dose response at an early time point in disease development to increasing levels of calcium in the presence and absence of high fat (Chapter 6);

(iii) assess whether increasing levels of calcium in the presence and absence of high fat modulate cellular signaling pathways through enzyme activity and membrane lipid composition at an early time point in disease development (Chapter 7 & 8);

(iv) assess the ability of calcium to modulate the growth of established ACF and eventual tumor outcome (Chapter 9);

(v) assess a mechanistic role for calcium as systemic modulator by evaluating the response of neutrophil leukotriene B_4 production to altered calcium intake (Chapter 10).

Section II

LITERATURE REVIEW

Chapter 2. LITERATURE REVIEW

A. Colon Cancer

Malignant tumors of the colon and rectum can be either primary or metastatic. Malignancies of the colorectal region are most commonly primary sites with the largest proportion of these being adenocarcinomas (Winawer et al., 1992). Anatomic clustering of human colorectal cancers occurs predominantly in the rectosigmoid region which accounts for approximately 55% of the malignant tumors (Schottenfeld & Winawer, 1982). Risk factors include increasing age, diet, inflammatory bowel disease, colorectal polyps, and hereditary factors (Fry et al., 1989). First degree relatives of persons with colorectal cancer have a two to four fold increased risk for disease development (Woolf, 1958; Lovett, 1974). In addition, individuals with: polyposis coli/familial polyposis or Gardner's syndrome (characterized by >100 colorectal adenomatous polyps); Turcot's syndrome (characterized by adenomatous colorectal polyps in conjunction with malignant central nervous system tumors); or the cancer family syndrome (characterized by adenocarcinomas of the endometrium or colon) develop colorectal cancer with increased frequency (Fry et al., 1989). Dietary risk factors are of great interest as changes in diet may be made with relative ease and these factors identify areas of preventive intervention. Dietary constituents implicated include: type and amount of fat; type and amount of fiber; caloric intake; and individual nutrients such as carotenes, retinoids, and calcium (Wargovich et al., 1988; Kritchevsky, 1990). The concept of prevention is very alluring, however, in order to effectively determine preventive actions one must first understand the normal tissue biology as well as critical steps in carcinogenesis. The focus of this literature review will be to explore current knowledge in the colon carcinogenic process including intermediate biological endpoints and the role of calcium as a potential preventive agent.

1. Biology of the Normal Colon

The four main tissue layers of the colon are the mucosa, submucosa, muscularis externa, and the serosa. In terms of carcinogenesis the mucosal layer is the most important tissue type (Ward, 1974). A single layer of epithelial cells line the folds of the mucosa forming finger-like projections known as the crypts of Lieberkuhn (Lev, 1990). The lamina propria fills the space between and beneath the crypts and is separated from the epithelium by the basement membrane. Supporting the lamina propria is the muscularis mucosa.

In the normal colon, crypts exist in a steady state in terms of production, migration, and loss where cell production in the basal one-third of the crypt is balanced by surface epithelial cell exfoliation (Chang, 1984). The entire mucosa is replaced every 4 to 8 days in humans (Cohen et al., 1989) and 3 to 5 days in rats (Maskens & Dujardin-Loitus, 1981b). Five critical events to maintain this steady state include: (1) sufficient stem cell replacement; (2) differentiation of these stem cells to columnar, goblet mucous, or enteroendocrine (argentaffin) cells; (3) transition of these differentiated cells from proliferative to non-proliferative status; (4) progression of differentiated epithelial cells from proliferative epithelial cells toward the luminal surface; and (5) sloughing off of terminally differentiated cells from the luminal surface (Chang, 1985).

2. Multistage Nature of Carcinogenesis

Animal models have contributed a great deal to our understanding of carcinogenesis. Two basic observations made from animals studies have led to the belief in the stepwise development of carcinogenesis. First, a time delay of about one-half to two-thirds the animal's lifespan was observed between carcinogenic insult and ultimate manifestation of the disease (Farber & Cameron, 1980; Rous & Kidd, 1941). Second, focal non-cancerous lesions appeared prior to cancer development and while some lesions

disappeared others became the site of eventual cancer development (Farber and Cameron, 1980; Rous and Kidd, 1941). These observations led to the tenet that carcinogenesis was a stepwise process thought to be discontinuous in that only a small proportion of the altered cell population continued to the next step (Farber and Cameron, 1980; Foulds, 1975; Rous & Beard, 1935). This process is driven by carcinogen induced damage in susceptible cells which acquire a selective growth advantage and undergo clonal expansion until one population expresses the three phenotypic characteristics of cancer (Harris, 1991). Thus each emerging cell population represent precursor lesions until the ultimate manifestation of the disease. The long time interval between carcinogenic insult and the emergence of a clone of fully malignant cells provides the opportunity for intervention strategies to prevent or retard the progression from precursor lesion to frank cancer (Tubiana, 1991)

Presently, carcinogenesis has been broadly organized into three stages: initiation; promotion; and progression. In the first stage cells are exposed to a carcinogenic agent of chemical, microbial, or physical origin (Harris, 1991). This causes genetic changes in the initiated cell which alter its responsiveness to internal and external regulators providing these and subsequent progeny with a selective growth advantage (Pitot, 1993). The exact changes which define an initiated cell are not clear, however, alterations in DNA are thought to be the primary cause. A plausible explanation is the inappropriate activation or deactivation of protooncogenes and tumor suppressor genes, respectively, leading to increased neoplastic transformation (Harris, 1991). It is believed that for a cell to be fixed as an initiated cell it must have undergone at least one or two rounds of cell division in the presence of the initiating agent or with unrepaired DNA damage (Pitot, 1993). Promotion involves the survival and propagation of initiated cells as compared to or at the expense of the normal cell population (Harris, 1991). The distinguishing characteristic of promotion compared to initiation and progression is its operational reversibility (Pitot, 1993).

Tumor promoting agents themselves have little if any carcinogenic effect, but instead they enhance tumor number and growth when introduced in temporal proximity to the carcinogen (Weinstein, 1988). The tumor promoters are thought to produce primarily epigenetic effects by inducing DNA synthesis, selectively enhancing cell proliferation in a population of initiated cells, and possibly inhibiting apoptosis (Pitot, 1993; Weinstein, 1988). Progression involves continued phenotypic changes in the malignant cell population (Foulds, 1975) eventually resulting in metastasis. The reversible feature of the intermediate stage of promotion, which presumably involves alterations in gene expression, suggests that cancer prevention efforts focused in this stage of carcinogenesis would be most effective (Pitot, 1993).

3. Cancer of the Colon

Cancer of the colon is predominantly (99%) epithelial in origin (Hermanek & Karrer, 1983) and is distinguished from benign tumors by the infiltration of the muscularis mucosa (Morson & Sorbine, 1976). Prior to infiltration the tumor is considered benign (adenoma) with malignant potential (Winawer et al., 1992). One of the key characteristics of malignancy is the ability to metastasize and thus due to the fact that there are no lymphatics above the muscularis mucosa, infiltration into this layer is critical for metastasis (Lane et al., 1977). The primary human malignant tumors are adenocarcinoma, carcinoid, and squamous cell carcinoma of which the adenocarcinoma is by far the most common colonic malignancy (Bird et al., 1985). Colonic adenocarcinomas exhibit structural evidence of crypts as demonstrated by glandular characters (Willis, 1967), clearly indicating development from the mucosa. Colon cancer in this dissertation will refer to adenocarcinoma of the colon.

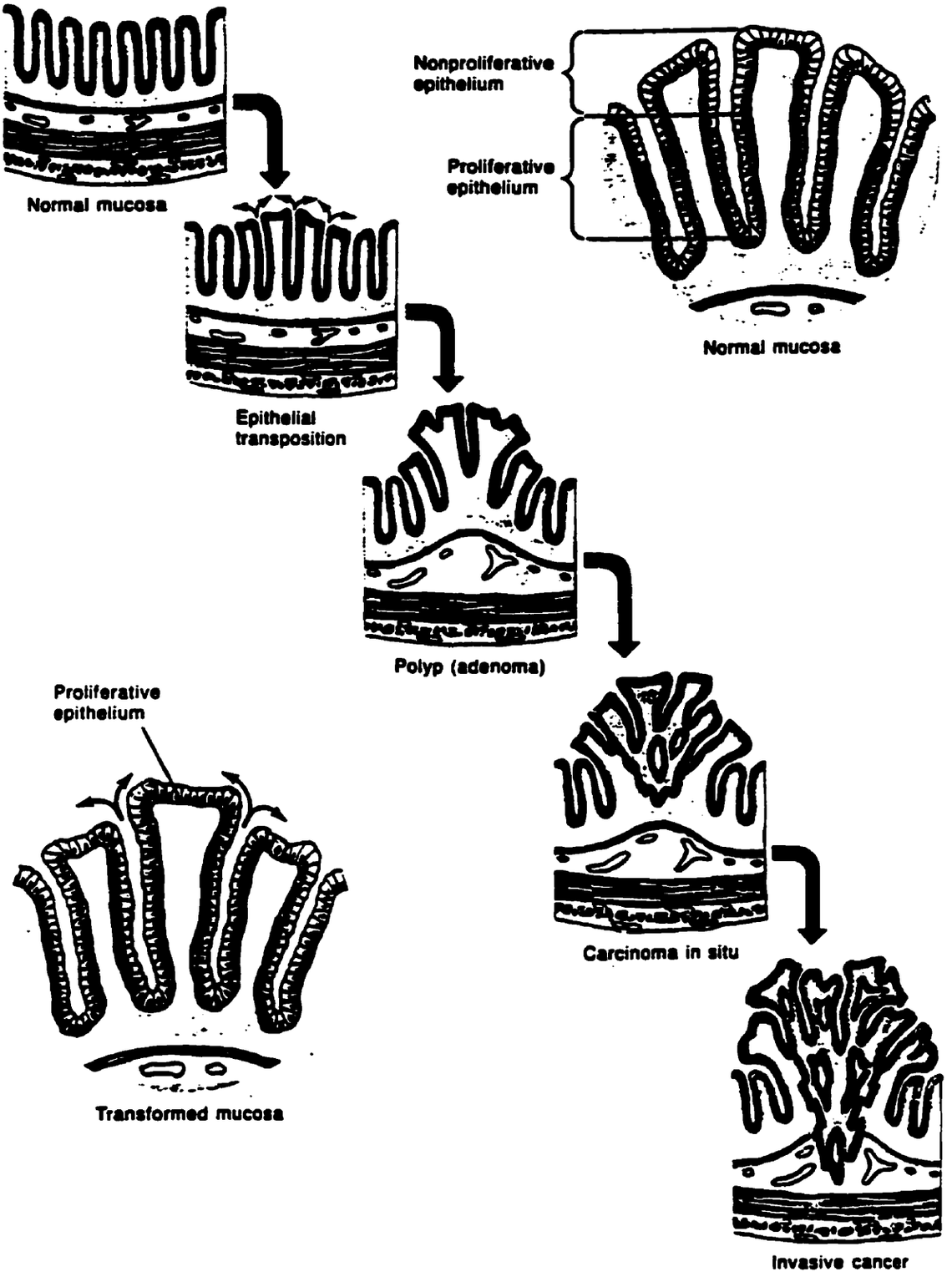
4. Histogenesis of Colon Cancer

> ... a proper understanding of the histogenesis
> of colon cancer is crucial to its prevention ...
> Chang, 1985

Evidence indicates that colonic neoplasms arise from a single crypt (Bussey, 1975; Chang, 1978). At present the stepwise 'polyp cancer' sequence which implies that all carcinomas arise from preexisting polyps is well accepted (O'Brien et al., 1992). In terms of the 'polyp cancer' sequence (Figure 1) two sequential perturbations in the process of epithelial differentiation are crucial for the formation of a neoplastic lesion (Lipkin, 1974). First, the cells migrating toward the luminal surface continue to divide and fail to achieve non-proliferative status. Second, the cells do not respond to signals for apoptosis and exfoliation leading to the retention of abnormally dividing cells in the upper and surface portion of the crypt. The result is the accumulation of atypical cells in the upper part of the crypt to form an early neoplastic lesion (Chang, 1985) and the eventual development of an adenomatous polyp. Adenomas are benign pedunculated or sessile neoplasms with malignant potential exhibiting varying degrees of dysplasia (O'Brien et al., 1992). All adenomas by definition display at least mild dysplasia and about 6% show more severe grades of dysplasia (O'Brien et al., 1992). Dysplasia is categorized into mild, moderate, or severe and is graded according to: (1) increased nuclear size; (2) altered nuclear shape; (3) increased nuclear stain uptake; (4) nuclear pleomorphism (increased variation in size, shape, and stain uptake); (5) increased mitosis; (6) abnormal mitosis; and (7) disordered differentiation (Boone et al., 1991).

Evidence supports the contention that adenomas are precursor lesions of colon cancer. Small areas of adenocarcinoma have been identified in some adenomas (Hamilton, 1992). Adenoma distribution along the colon length has been positively correlated with the distribution of colorectal carcinomas (Hamilton, 1992). Subsequent risk of colorectal carcinoma has been found to be significantly greater in subjects with existing adenomas

Figure 1. The transition from normal mucosa to invasive cancer following the polyp-cancer sequence. The normal mucosa is characterized by active proliferation in the lower two-thirds of the crypt and a non-proliferative crypt in the upper one-third. Expansion of the proliferative compartment towards the colonic lumen is indicative of transformed mucosa. This potentially produces polyp formation and ultimately invasive cancer (taken from Fry et al., 1989).


Normal mucosa
Epithelial transposition
Polyp (adenoma)
Carcinoma in situ
Invasive cancer
Nonproliferative epithelium
Proliferative epithelium
Normal mucosa
Proliferative epithelium
Transformed mucosa

than the general population (Lotfi et al., 1986). Colonoscopic follow-up studies have indicated that the mean age of patients with adenomas is five years earlier than for those with invasive carcinoma suggesting that this may be the lag time in the last step to cancer (Winawer, 1987).

An alternate hypothesis is the *de novo* genesis of carcinomas from flat epithelium without a polyp stage (Maskens & Dujardin-Loitus, 1981a). Carcinomas may develop from both routes (Shamsuddin et al., 1985), however, a great deal of controversy surrounds the *de novo* theory of colon carcinogenesis (Bedenne et al., 1992).

B. Experimental Colon Cancer

1. Epidemiological Studies

Colon cancer incidence varies widely throughout the world. High incidence rates are typical for North America, Western Europe, and New Zealand while the lowest rates are found in most South American countries, Eastern Europe, Asia, and Africa (Armstrong & Doll, 1975; Doll & Peto, 1981; Winawer et al., 1992). Furthermore, the adenoma and colon cancer incidence of immigrants migrating from low to high risk geographic locations matches that of the new resident country (Armstrong and Doll, 1975; Berg & Howel, 1974; Carrol & Khor, 1975; Haenszel & al., 1973). This data suggests that colon cancer is largely an environmental disease and thus the contents that enter the digestive tract, remain undigested and unabsorbed in the small intestine, provide the direct environment to which the colon is exposed. These differences elicited a great deal of interest regarding the dietary practices of these populations. An extensive body of epidemiological research has provided evidence for a role for diet in carcinogenesis and has been the subject of many reviews (Doll, 1996; Doll and Peto, 1981; Jacobs, 1993; Reddy, 1986).

2. Etiological Factors

A variety of dietary constituents have been implicated in the development of colorectal cancer including: excess fat; excess calories; low fiber intake; alcohol; and inadequate intake of vitamins (retinoids, ascorbic acid, αtocopherol) and minerals (calcium and selenium) (Winawer et al., 1992). Although many dietary constituents have been implicated in colon cancer dietary fat intake strongly correlates with disease incidence and mortality (Carrol and Khor, 1975). In general, industrialized societies where fat intakes are high also have high colon cancer mortality rates as compared to non-industrialized societies that have low fat intake coupled with low colon cancer incidence. Several epidemiological studies have linked an increased risk with a high total fat intake (McKeown-Eyssen & Bright-See, 1984; Reddy, 1986; Wynder et al., 1969). However, some controversy surrounds the epidemiological data on the fat-colon cancer connection (Byers & Graham, 1984; Doll, 1996; Marshall, 1986). Agreement in epidemiological studies exists in that if the fat-colon cancer connection is accepted the associated risk is concerned with the total amount consumed (Winawer et al., 1992) as well as the total saturated fat consumed (Doll, 1996).

Experimental data also favors a relationship between colon cancer and fat intake. Studies have demonstrated that colon carcinogenesis is enhanced in high compared to low fat fed animals (Nigro et al., 1975; Reddy et al., 1980; Reddy et al., 1974). However, some controversy exists in experimental studies and this is the subject of review (Newberne & Nauss, 1986). It has been suggested that conflicting findings regarding the promotional effect of fat may be related to the total amount and type of fat consumed (Rao et al., 1993). Several studies have demonstrated that rodent diets containing 20% fat by weight or more from sources such as corn oil, safflower oil, beef fat, or any combination thereof, have a promotional effect on chemically induced colon carcinogenesis (Carroll, 1991; Rao et al., 1993; Reddy, 1986; Reddy & Maeura, 1984).

However, this promotional effect was not observed when the dietary fat was derived from olive oil, fish oil, or coconut oil (Rao et al., 1993; Reddy & Maruyama, 1986).

Although the effect of fat has been well documented, the exact mechanism by which the high fat diet exerts this promotional effect on colon carcinogenesis remains poorly understood. Several mechanisms have been offered to explain the tumor modulating effect of a high fat diet. These include: (1) increased bile acid excretion; (2) increased incorporation of fatty acids into cell membranes; (3) increased synthesis of eicosanoids, namely prostaglandins; (4) increased free radicals generated as a result of fat metabolism; (5) alteration in gut bacteria; and (6) an increase in caloric consumption (Babbs, 1990; Hill, 1990; Rigas et al., 1993; Steinbach et al., 1993; Weinstein, 1991; Winawer et al., 1992).

3. Factors Involved in Animal Investigations

(a) Animal models

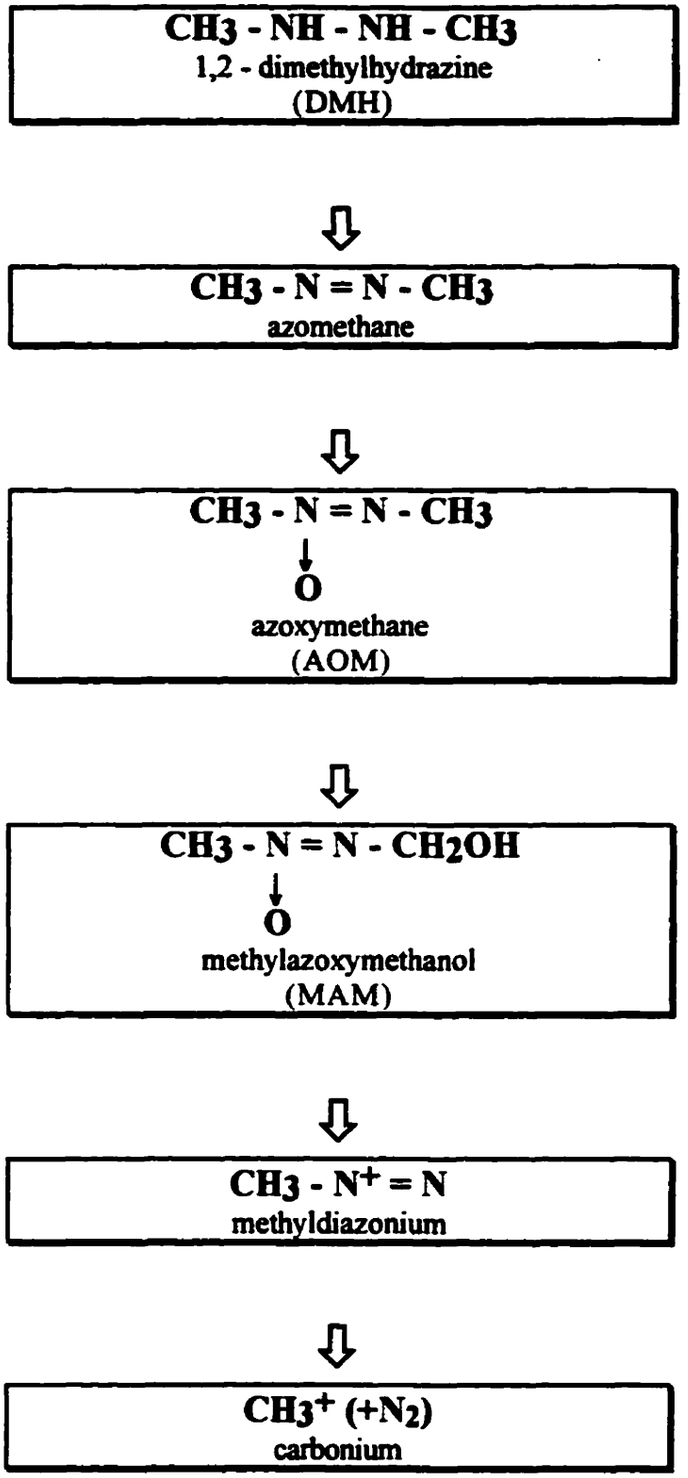
The utilization of animal models has revealed much of what is known today about the stepwise development of colon cancer. In the animal model researchers can specifically induce carcinogenesis at a particular site and observe the modulating effect of an isolated agent. The choice of an appropriate animal model is dependent on the similarities in disease development. 1,2-Dimethylhydrazine (DMH) induced colon cancer in rats was found to duplicate almost every colonic neoplasm of epithelial origin found in humans (Sunter , 1980). In addition, DMH-induced adenocarcinomas were found to have areas of adenomatous epithelium similar to that found in human adenocarcinomas supporting an adenoma-adenocarcinoma sequence in the rat model (Madara et al., 1983). Some similarities between DMH-induced rodent colonic neoplasms and neoplasms of the human colon were compiled by Bird et al. (1985). Neoplastic lesions are found primarily

in the distal colon. Initiated rodent colons or high risk human colons exhibit increased and abnormal cell cycle events. Neoplastic events such as hyperplasia, dysplasia, adenomatous polyps, and carcinomas are found in both rodent and human colons. The initiated rodent colon exhibits both 'adenoma-adenocarcinoma' sequence and *de novo* origination of cancer. Based on histological information it appears that the rodent colon most closely mimics human intestinal neoplasia (Bird et al., 1985).

(b) Colon carcinogens

Several chemical substances have been shown to have carcinogenic action. Some of the earlier compounds were: polycyclic hydrocarbons such as methylcholanthrene and dibenzanthracene; aromatic amines such as 2-acetyl-aminofluorene and 3,2'-dimethyl-4-aminodiphenyl; and nitroso compounds such as N-methyl-N-nitrosourea (Chang, 1984; Greene et al., 1987; Hamilton, 1989; Sunter, 1980; Weisburger, 1971). The most well accepted and widely used compounds are related to the plant substance cycasin (Sunter, 1980). Oral administration of the plant resulted in large bowel neoplasms, however, the tumor incidence was low and gender differences were apparent with males more susceptible than female rats (Sunter, 1980). Further investigations into the metabolic breakdown of cycasin revealed that methylazoxymethanol (MAM) was the active component (Figure 2). Its administration produced large numbers of small intestinal tumors in addition to the large bowel neoplasms. Administration of DMH, a precursor of MAM, provided a more colon specific response with a high tumor yield (Sunter, 1980). Factors such as the potential risk in DMH injection preparation and the potential for DMH to undergo asymmetrical rearrangement to a hydrazine derivative made azoxymethane (AOM) the preferred agent (Weisburger, 1971). The neoplastic lesions induced by either DMH or AOM are similar in terms of development and morphology

Figure 2. Steps involved in the metabolism of cycasin-related compounds. The metabolic product methyldiazonium is a highly reactive intermediary product and it is thought that the alkylating properties of this substance produce the carcinogenic effects of precursors 1,2-dimethylhydrazine and azoxymethane (adapted from Sunter, 1980).


CH3 - NH - NH - CH3
1,2 - dimethylhydrazine
(DMH)
CH3 - N = N - CH3
azomethane
CH3 - N = N - CH3
O
azoxymethane
(AOM)
CH3 - N = N - CH2OH
O
methylazoxymethanol
(MAM)
CH3 - N+ = N
methyldiazonium
CH3+ (+N2)
carbonium

and are considered effective chemical inducers of colonic neoplasms in the animal model (Bird et al., 1985).

(c) Carcinogen metabolism

Metabolic activity of DMH and AOM is first concentrated in the liver and then the colonic epithelium (Greene et al., 1987; Weisburger, 1971). AOM is converted to MAM by a process of α-hydroxylation (Greene et al., 1987). MAM is unstable in aqueous solution and quickly undergoes further metabolism to methyldiazonium and carbonium which are highly reactive and carcinogenic effects derived from their alkylating properties (Sunter, 1980). The major means of distribution is considered to be via the blood stream to extrahepatic tissue such as the colon or further metabolism in the liver (Greene et al., 1987; Sunter, 1980).

Differences in animal species, strain, gender, and age modulate the effects of carcinogen (Bird et al., 1985). The most susceptible strain of rat to colon specific carcinogens is the Sprague-Dawley (Nauss et al., 1987; Nigro, 1985). In addition, carcinogenic effects have been reported to be stronger in young animals of male gender (Hamilton, 1989).

(d) Carcinogen administration

Administration route is important for organ specificity. DMH administered to rats in drinking water produces liver tumors with multiple lung metastases (Bird et al., 1985). Subcutaneous and intrarectal instillation of the carcinogenic agent produces marked colonic epithelial cell death within 24 hrs, hyperproliferation by 96 hrs, and normal epithelial patterns by seven days post treatment leading to large bowel tumor specificity (Sunter, 1980).

The two protocols in carcinogen administration are the one or two injection versus the multiple injection protocol. The former requires a high carcinogen dose and therefore is suitable for the rat but not the murine model. This is based on the low LD_{50} for DMH (35 mg/ b.w. in mice and 215 mg/ b.w. in rats) in rats as compared to mice (Chang, 1984). The multiples injection protocol can be employed in both the rat and murine model as a lower carcinogen dose repeated over several weeks can be tolerated by both species. The advantage of the latter protocol is the high reliability in tumor induction, shorter temporal proximity between induction and tumor appearance, and increased tumor yield (Chang, 1984). The disadvantage is that the protocol obscures the stages of initiation, promotion, and progression and hence is not a good model for investigating events prior to tumor formation (Farber & Cameron, 1980).

C. Biomarkers in the Study of Colon Carcinogenesis

Colon carcinogenesis studies generally employ tumor outcome and risk markers as study endpoints. The advantage of tumorigenesis studies lies in the fact that the efficacy of the intervention agent is evaluated directly on the disease itself. However, the limitation of this approach is that it does not allow for evaluation during disease development, it requires a great deal of time as a result can be quite costly (Bird et al., 1989). This provided impetus to identify intermediate biomarkers in anticipation of extending our knowledge of the carcinogenic process. Biomarkers can be evaluated after short experimental duration and thus demand less time and money. The problem arises that these biomarkers are not the disease itself and are merely associated with increased risk for disease development (Bird et al., 1989). The validity of these biomarkers are questionable and must be carefully scrutinized under a variety of stringent conditions to verify their predictive value regarding disease development. Recently, it has been

proposed that aberrant crypt foci (ACF) represent early precursor lesions of colon cancer (Bird, 1987) and is the subject of review (Bird, 1995). The advantages of this endpoint encompass both of the former endpoints in that the lesions represent the actual disease itself and can be evaluated early in disease development providing insight into the stepwise process of colon carcinogenesis as well as reducing experimental costs and duration (Bird et al., 1989).

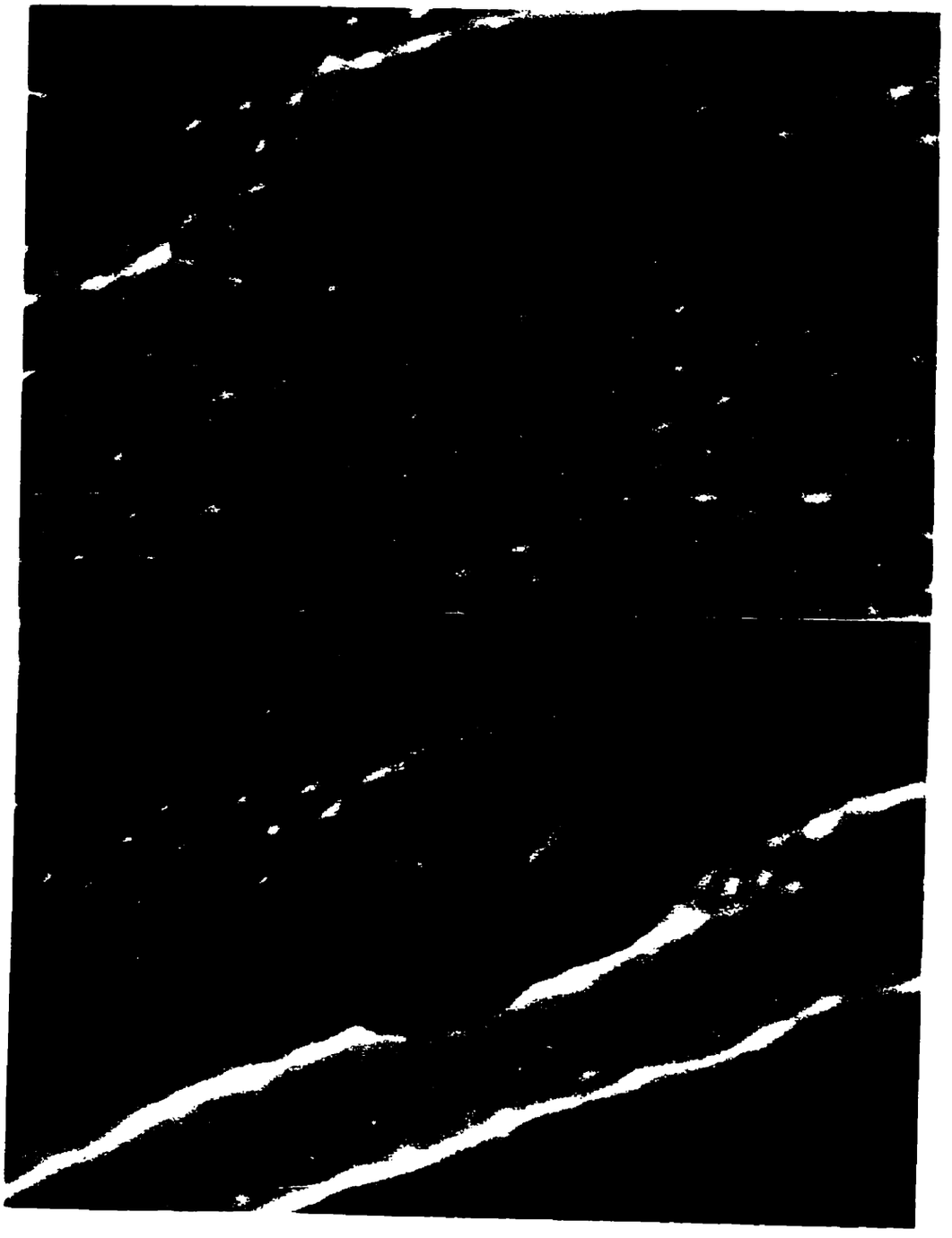
1. Aberrant Crypt Foci

ACF were first identified in carcinogen treated rat colon in 1987 by Bird based on the premise that preneoplastic changes would first occur in single crypts and would be visible topographically (Bird, 1995). These lesions were described as having increased size, thicker epithelial lining, and an increased pericryptal zone (Figure 3) relative to the surrounding normal crypts (Bird, 1987; McLellan & Bird, 1988). ACF have been suggested to be precursor lesions of colon cancer. In general, the cellular, molecular, morphologic, and growth patterns of ACF support this contention (Bird, 1995).

(a) Biological properties of aberrant crypt foci

ACF have been observed two weeks post carcinogen and their number as well as size increased with time (McLellan and Bird, 1988). The induction of ACF in a more resistant murine model exhibited delayed induction of ACF (Bird et al., 1989). ACF are induced specifically with colon carcinogens in a dose dependent manner primarily in the distal colon where tumor are commonly located (McLellan and Bird, 1988; McLellan et al., 1991). ACF formation is inhibited in a murine model highly resistant to carcinogen induced colon tumorigenesis (Rosenberg & Liu, 1995) and by disulfiram, a colon cancer inhibitor (McLellan & Bird, 1991). A tumor promoting high fat diet was shown to promote ACF growth (McLellan and Bird, 1988) and dysplasia was observed in some

Figure 3. Topographical view of aberrant crypt foci (ACF) with various growth features. Note: two ACF (upper panel) consisting of three and two crypts. Each focus is easily distinguishable from surrounding normal crypts by having large crypts with thicker epithelial lining. One focus consisting of six crypts (lower panel). Note: irregular crypt openings.



foci exhibiting enhanced growth characteristics (Bird et al., 1989). Moreover, an ACF remnant has been observed on the surface of an adenoma (Bird, 1995). Aberrations of ACF at the genetic level have been observed in ACF (Stopera & Bird, 1993; Stopera et al., 1992; Vivona et al., 1993). Recently, K-*ras* mutations were observed in 44% and 58% of adenocarcinomas and ACF, respectively (Yamashita et al., 1995). Interestingly, 91% of the mutations observed in adenocarcinomas were a G-T substitution at codon 12 whereas a fraction (33%) of the mutations in ACF were of this variety. These biological properties support the contention that ACF represent precursor lesions of colon cancer.

(b) Aberrant crypt foci in human colonic mucosa

It was suggested that an important factor in validating ACF as part of the stepwise progression of colon cancer would be the identification of ACF in human colonic mucosa (Bird, 1987). ACF were first reported in human colons four years later and found to exhibit many of the same phenotypic characteristics of ACF found in rodent colons (Pretlow et al., 1991). An increased appearance of ACF was reported in patients with colon cancer (Pretlow et al., 1991; Roncucci et al., 1991) or familial adenomatous polyposis (Roncucci et al., 1991) compared to cancer free patients. The similarities between rat and human tumor pathology (Rogers & Gildin, 1975; Ward, 1974) and ACF phenotypic markers (Pretlow et al., 1991) support the use of ACF in the rodent model as an effective tool in understanding human colon carcinogenesis.

(c) Value of ACF as a hallmark of colon cancer

The value of ACF as biomarkers of colon cancer was first demonstrated by their specific induction by colon carcinogens (McLellan and Bird, 1988) and rare appearance in animals exposed to carcinogens targeting other organs (Bird et al., 1989; McLellan and Bird, 1988; Tudek et al., 1989). An important characteristic of a useful biomarker is its

modulation by study agents known to influence disease outcome (Lee et al., 1992) such that phenotypic changes indicate promotion or inhibition (Pitot, 1990). In this regard ACF appear valuable as intermediate biomarkers as their growth and incidence has been shown to be modulated by chemopreventive agents (Bird et al., 1989). The growth and incidence of ACF are quantified by evaluating: (1) the total number of ACF in the colon; (2) the size of the focal lesion; and (3) the number of crypts comprising each focal lesion (McLellan et al., 1991). In several studies the number of ACF increased in response to exposure to tumor promoters (McLellan and Bird, 1988; McLellan et al., 1991; Shivapurkar et al., 1992) and decreased in response to chemopreventive agents (Lam & Zhang, 1991; McLellan and Bird, 1991; Pereira & Khoury, 1991). However, questions regarding validity surfaced with the finding that the total number was drastically reduced in animals exposed to a potent tumor promoter (Bird, 1991; Magnuson & Bird, 1993). Investigations into the predictive value of ACF revealed that crypt multiplicity, namely foci with increased number of crypts, persistently correlated with tumor incidence (Magnuson et al., 1993; Pretlow et al., 1992; Zhang et al., 1992). It was determined that the reduction in ACF was a result of cholic acid promoting a select group of ACF with the highest potential to progress to malignancy (Magnuson et al., 1993). Dilated crypts (large in size), proposed to be less stable than constricted (smaller in size) crypts (Bird and Lafave, 1995), were found to be eliminated by the cholic acid diet suggesting that the constricted crypt phenotype could provide a marker to identify ACF with greater malignant potential (Shirtliff & Bird, 1996). The validation of increased crypt multiplicity as a predictor of tumor outcome in addition to the specificity, growth, morphological, and genotypic features of ACF support the claim that they are preneoplastic lesions of colon cancer (Bird, 1995).

2. Cell Proliferation

The process of cell division is fundamental to growth, however, the loss of normal proliferation is thought to be one of the first steps in colon carcinogenesis. This premise is grounded in the fact that altered cell regeneration is a hallmark feature of cancerous tissue (Farber, 1986; Simanowski et al., 1989) and generally persists until the development of neoplasia (Lipkin, 1974). The current tenet holds that the migration of the proliferative zone towards the lumen ultimately results in the formation of adenoma (Maskens, 1979). Carcinogens generally cause: (1) an upward shift in the proliferative compartment; (2) an increase in the proliferating cell pool; and (3) or a combination of both an upward shift and an increase in proliferating cells (Biasco et al., 1992). The hyperproliferating colon is at risk either due to an increase in the carcinogen's target cell population or due to the decreased time for DNA repair prior to rapid cell turnover (Biasco et al., 1992).

(a) Value of proliferation as a biomarker of colon cancer

Patients with adenomas or adenocarcinomas (Lipkin, 1983; Lipkin & Deschner, 1976; Risio, 1992) as well as those with a hereditary predisposition to colon cancer (Deschner & Maskens, 1975) exhibit abnormal epithelial proliferation. A prolonged increase in the proliferating cell pool, as seen in ulcerative colitis or Crohn's disease, has been associated with an increased and early onset of the disease (Barthold, 1983; Biasco et al., 1992; Petras et al., 1987). Furthermore, the convenience and viability of sample collection make evaluation of cell kinetics on the human colon very attractive as a biomarker.

The glitch in the system is that the predictive value of dietary modification induced changes in the normal appearing carcinogen treated mucosa has not been established (Cameron et al., 1990). Although many studies have used colonic cell

proliferation as a biological endpoint to conclude on the effectiveness of a chemopreventive agent (Bostick et al., 1995; Bostick et al., 1993; Buset et al., 1986; Gregoire et al., 1989; Wargovich et al., 1984; Wargovich et al., 1983; Zhang et al., 1987) many studies that have carried the experiment further have reported that this parameter was not a reliable predictor of tumor outcome (Cameron et al., 1990; Galloway et al., 1987; Glickman et al., 1987; Karkare et al., 1991; Steinbach et al., 1993).

(b) Cell kinetic determinants

The method used to evaluate cell proliferation requires contemplation as each technique explores different aspects of the cell cycle (Biasco et al., 1992). Administration of colchicine to animals prior to termination arrests cells in metaphase allowing for the quantification of mitotic activity after the tissue has been stained. Although several immunohistochemical methods have been established only two protocols have been rigorously tested and well received (Risio, 1992). First, nuclear uptake of pyrimidine analogs of thymidine identify S-phase cells. The most commonly cited of these is 5'-bromo-2'-deoxyuridine (BrdU). BrdU, a thymidine analog with a bromine substitution, is readily taken up by DNA as an alternate to thymidine (Risio, 1992). The second method involves the identification of endogenous antigens relating to proliferation. The most commonly employed are: (1) Nuclear Antigen detected by Ki67 antibody; (2) DNA polymerase alpha; and (3) Proliferating Cell Nuclear Antigen (PCNA/Cyclin) (Risio, 1992). PCNA, which represents the auxiliary protein of DNA polymerase delta, has been well received and commonly used. PCNA is expressed primarily in S-phase cells but also to a lesser extent in cells progressing from G_1 to S phase as well as G_2 and M phase cells (Risio, 1992). This suggests that PCNA identifies cycling cells.

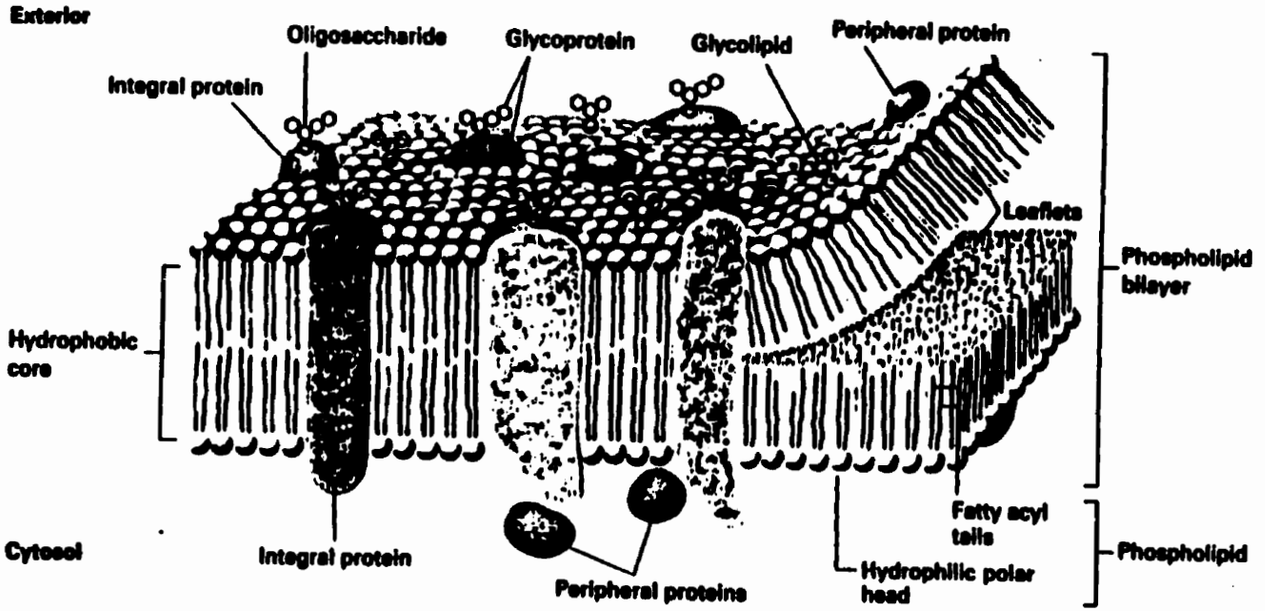
PCNA detected antigens include various naturally occurring cell cycle related proteins (Hall & Woods, 1990). As a result, PCNA labeling indices tend to be greater

than the BrdU labeling indices, and it has been suggested that this may be due to the gradient in PCNA expression that enlarges the window of reactivity (Yamada et al., 1992). Although this method of assessment is in its early stages of development, several studies have reported excellent correlations between BrdU, [^{3}H]thymidine, and PCNA proliferative indices (Bird et al., 1989; Yamada et al., 1992). It has been suggested that PCNA immunohistochemistry may be a superior technique to determine proliferative status as this technique circumvents the use of radioactive ([^{3}H]thymidine) or cytotoxic (colchicine, BrdU) chemicals while still providing spatial and conformational information not available with flow cytometric analysis. The PCNA methodology is therefore ideal in situations where enzymatic and proliferative measurements are to be evaluated on the same specimen.

3. Phospholipid Metabolism

Membranes are composed of both lipid and protein with possible carbohydrate presence. The current model for the architectural organization of virtually all biological membranes is the fluid mosaic model (Figure 4) (Darnell et al., 1990). In this context, phospholipids align their hydrophobic ends together to form a lipid bilayer interspersed with integral and peripheral proteins. Some of the common phospholipids include phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI). Traditionally the phospholipid bilayer was thought to act simply as a barrier and structural matrix for the cell, however, it is now believed that phospholipids themselves participate in cell metabolism (Berridge, 1982). This was first indicated when it was determined that ^{32}P was preferentially incorporated into PI but not other phospholipids when stimulated with acetylcholine (Hokin & Hokin, 1954). Subsequently, numerous studies have demonstrated that changes in membrane lipid composition occur in response to a variety of extracellular messengers (Berridge, 1982). The metabolism of some phospholipids is thought to participate in calcium regulation as

Figure 4. The phospholipid bilayer provides the basic structure for biological membranes. The fluid and dynamic nature of this membrane provides the interface for message transfer while maintaining cellular integrity (taken from Darnell et al., 1990).

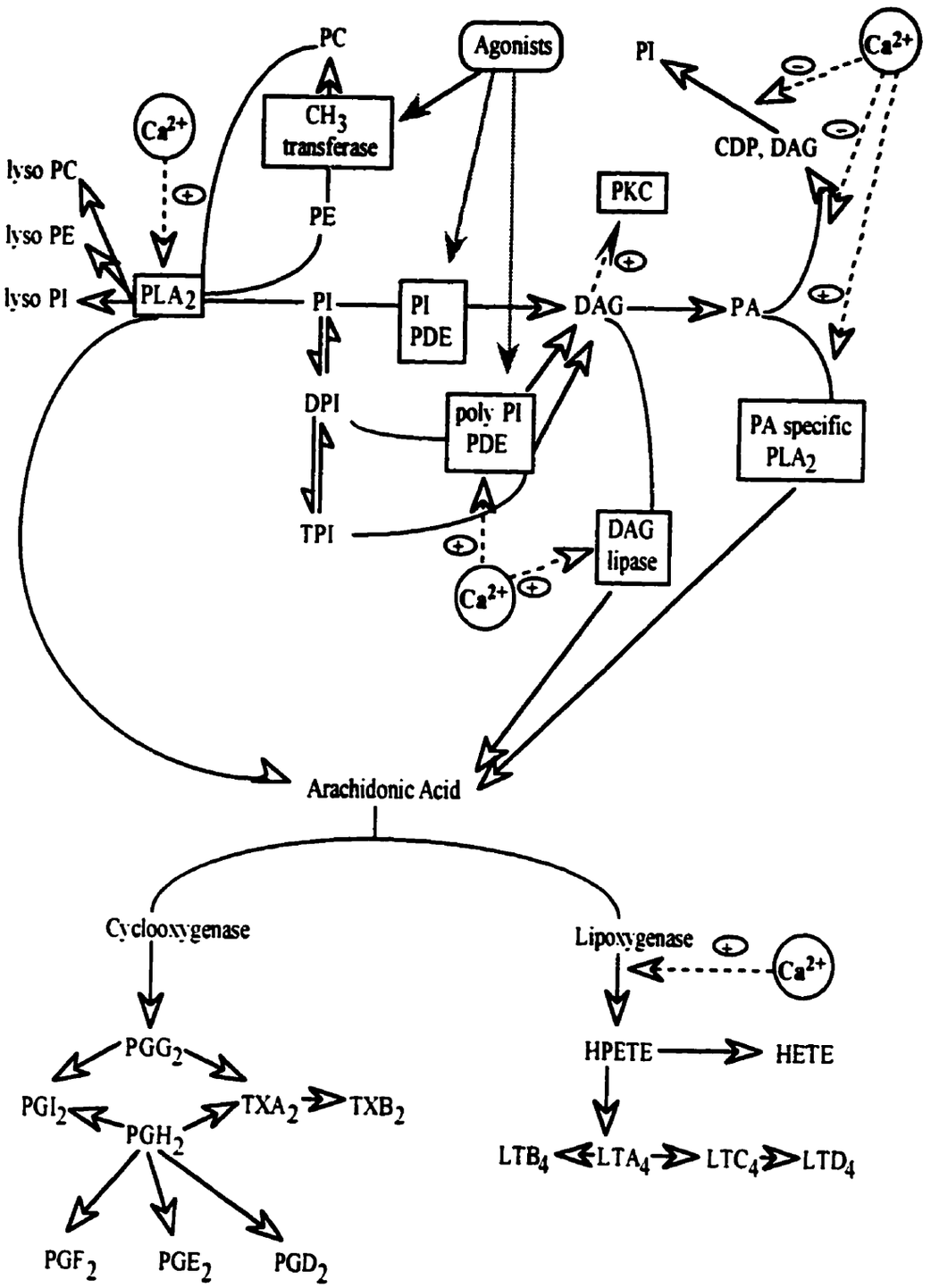

Exterior
Oligosaccharide
Glycoprotein
Glycolipid
Peripheral protein
Integral protein
Leaflets
Phospholipid bilayer
Hydrophobic core
Cytosol
Integral protein
Peripheral proteins
Fatty acyl tails
Hydrophilic polar head
Phospholipid

well as mediating some of the effects of calcium (Berridge, 1982). The phospholipid-calcium relationship is reciprocal such that phospholipids (i.e. 1,4,5-trisphosphate), may stimulate an increase in calcium concentration and conversely calcium may also induce changes in phospholipid metabolism, such as arachidonic acid (AA) release. The primary response associated with calcium signals is the hydrolysis of phospholipid (Berridge, 1982).

(a) The relationship of calcium in phospholipid metabolism

A wide range of receptors, when activated, result in the hydrolysis of PI and the eventual stimulation of calcium as one of a number of intracellular signals. In many situations, such as blood platelets, PI turnover is relatively independent of intracellular calcium (Rittenhouse-Simmons, 1981), however, in some cell types, such as neutrophils or pancreatic islet cells, turnover appears to depend on calcium (Clements et al., 1981; Cockcroft et al., 1980). Many of the agonists that induce hydrolysis of PI also elicit either the influx or internal release of calcium suggesting that these two events may be connected (Michell, 1975; Michell & Kirk, 1981). PI hydrolysis, which leads to the formation of diacylglycerol (DAG) and a phosphoinositide (Nishizuka, 1984), plays an important role in the release of AA. Subsequent metabolism produces a number of highly biologically active metabolites (Figure 5). The three mechanisms of AA release proposed include: (1) cleavage at the phospholipid 2-position by phospholipase A_2 (PLA_2); (2) lipase action on DAG produced by phospholipase C (PLC) induced hydrolysis of PI; and (3) phosphatidic acid (PA)-specific PLA_2 action on PA produced by PLC induced hydrolysis of PI (Bell et al., 1979; Berridge, 1982; Billah et al., 1981). Although the exact steps in the pathway are yet to be delineated it is clear that PLA_2 (Jesse & Franson, 1979), PLC (Feinstein & Sha'afi, 1983) and the DAG lipase (Billah et al., 1980) are dependent on calcium. In addition, the phosphoinositide IP_3 by product of PI hydrolysis

Figure 5. A summary of various points of calcium modulation in phospholipid metabolism. One of the primary actions of calcium is to stimulate the enzymes which mobilize phospholipid hydrolysis thus activating protein kinase C and eventual arachidonic acid release. This is enhanced by the inhibition of phosphatidylinositol resynthesis by calcium. In addition, the synthesis of arachidonic acid metabolites via the 5-lipoxygenase pathway is stimulated by calcium. [Ca^{2+}: calcium ion; CDP,DAG: cytidine diphosphate diacylglycerol; DAG: 1,2-diacylglycerol; DPI: diphosphoinositide; HETE: hydroxyeicosatetraenoate; HPETE: hydroperoxyeicosatetraenoate; LTA_4...D_4: leukotriene A_4....D_4; PC: phosphatidylcholine; PE: phosphatidylethanolamine; PI: phosphatidylinositol; PI-PDE: phosphatidylinositol phosphodiesterase; PA: phosphatidic acid; PLA_2: phospholipase A_2; PGG_2...I_2: prostaglandin G_2...I_2; TXA_2...B_2: thromboxane A_2...B_2; TPI: trisphosphoinositide] (adapted from Berridge, 1982).


PC
Agonists
PI
Ca^{2+}
CH_3 transferase
CDP, DAG
lyso PC
lyso PE
lyso PI
PE
PKC
PLA_2
PI
PI PDE
DAG
PA
DPI
poly PI PDE
PA specific PLA_2
TPI
DAG lipase
Ca^{2+}
Arachidonic Acid
Cyclooxygenase
Lipoxygenase
Ca^{2+}
PGG_2
HPETE
HETE
PGI_2
TXA_2
TXB_2
PGH_2
LTB_4
LTA_4
LTC_4
LTD_4
PGF_2
PGE_2
PGD_2

stimulates an intracellular influx of calcium ions from the endoplasmic reticulum (Darnell et al., 1990) ensuring sufficient calcium for activation. Thus the ensuing conversion of AA to the various active metabolites may then provide a feedback to the calcium signaling system (Berridge, 1982).

(b) Role of phospholipids in the etiology of colon cancer

The link between phospholipid metabolism and carcinogenesis involves the modification of membrane lipid composition by dietary fats which promote tumorigenesis. High fat diets composed of corn oil, safflower oil, beef tallow, or lard promote colon cancer while those with olive or fish oil do not enhance disease outcome (Carroll, 1991; Rao et al., 1993b; Reddy & Maruyama, 1986; Reddy, 1986; Reddy & Maeura, 1984; Reddy et al., 1987). Membrane polar head group composition is altered by high levels of both corn oil and beef tallow (Robblee et al., 1988). Diets high in corn oil, compared to olive oil, alter the fatty acid composition in rat colonic mucosal phospholipids, specifically an increase in linoleic acid and AA (Rao et al., 1993b). Changes in membrane lipid in response to altered type and amount of fat consumed was shown to modify various cell functions (Bruckner et al., 1984; Croft et al., 1985; Lee et al., 1988). Various eicosanoids and PKC (discussed in subsequent sections) are implicated in cancer development and these metabolites are directly influenced by phospholipid metabolism. The key components are AA and DAG, thus events enhancing or inhibiting their formation have a direct bearing on carcinogenesis. The enzymes PLA_2 and PLC are critical regulators of AA release and hence eicosanoid synthesis. In addition, PLC is involved in DAG formation and thus affects PKC dependent signal transduction. Carcinogen treated colonic mucosa and colonic tumors exhibit elevated levels of PLA_2 and PI-specific PLC (Kuratko & Pence, 1995; Rao et al., 1996). Furthermore, these enzymes were enhanced in the colonic mucosa of animals fed a

high corn oil diet compared to those fed low corn oil or high fish oil diets (Rao et al., 1996). In contrast, increased levels of PLA_2s were shown to have a protective effect by altering the cellular microenvironment within intestinal crypts yielding fewer polyps in mice carrying the Apc^{Min} (similar to the FAP-familial adenomatous polyposi seen in humans) mutation (MacPhee et al., 1995). Although a role for phospholipid metabolism is postulated, the exact mechanism has yet to be delineated. However, interesting information regarding the implication that cyclooxygenase-2 (COX-2) plays a key role in tumorigenesis has suggested a potential mechanism for phospholipid involvment (Oshima et al., 1996). It is thought that COX-2 *products* cause tumor promotion and may regulate apoptosis in colonic epithelial cells (Prescott and White, 1996).

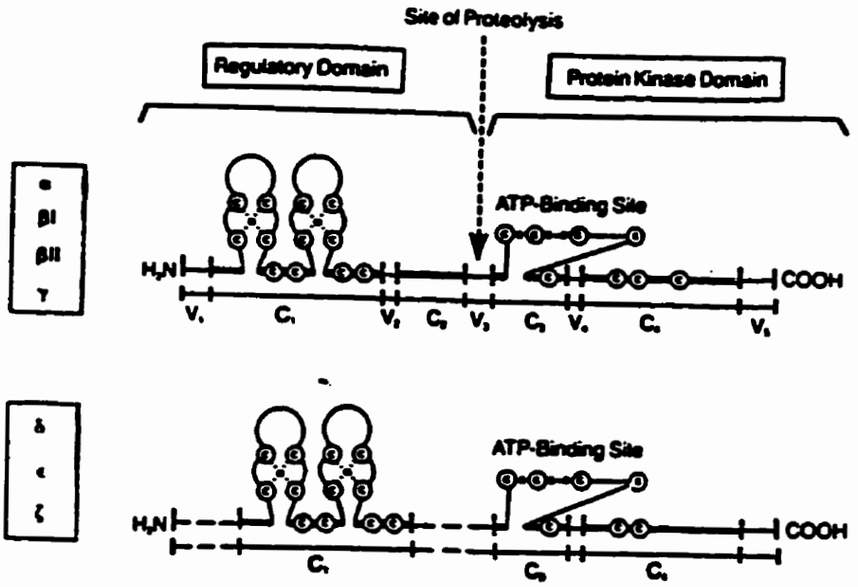
4. Protein Kinase C

The term protein kinase C (PKC) categorizes a group of calcium and phospholipid dependent serine and threonine protein kinases (Housey et al., 1987; Nishizuka, 1988). This family of enzymes, known to exist in various isoforms, is widely distributed in tissues and organs (Nishizuka, 1984). PKC activation results in a variety of cellular responses depending on the cell of origin thus indicating the importance of PKC activity in the biological response to extracellular agents (O'Brian & Ward, 1989). The PKC enzyme consists of a polypeptide chain where the regulatory domain at the N terminus and the catalytic domain at the C terminal region are proteolytically joined by the hinge region (Figure 6a) (Nishizuka, 1988). Proteolysis of the hinge region releases the fully active catalytic fragment suggesting that the binding of the regulatory domain is the de-activating factor (Inoue et al., 1977; Kishimoto et al., 1983). Variations in isozymes appear to involve the regulatory domain where calcium-dependent isozymes such as PKC α, βI, βII, and γ contain both the C_1 and C_2 regions whereas calcium independent isozymes δ,ε, and ζ contain only C_1 (Bell & Burns, 1991; Nishizuka, 1988). The

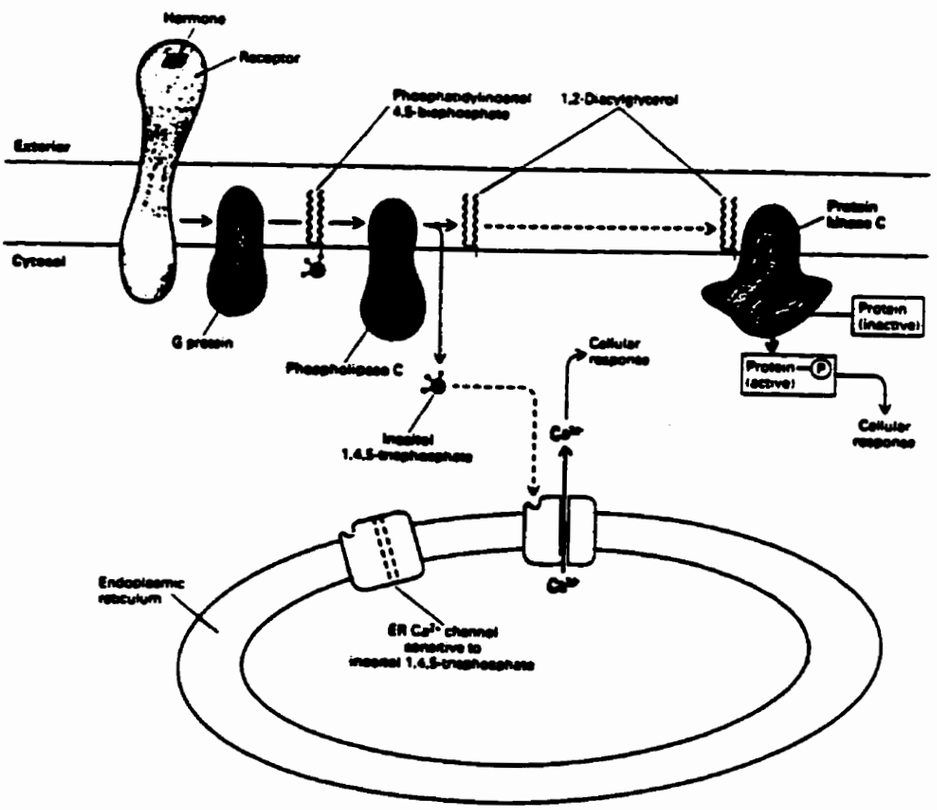
Figure 6a. Common structure of protein kinase C subspecies (taken from Nishizuka, 1989).

Figure 6b. Activation of second messengers, including protein kinase C, signaling pathway. Agonist binding to a receptor triggers activation of a G protein that, in turn, activates phospholipase C. The enzyme cleaves phosphatidylinositol 4,5-bisphosphate producing: (i) inositol 1,4,5-trisphosphate stimulates the release of Ca^{2+} from the endoplasmic reticulum; and (ii) 1,2-diacylglycerol which in conjunction with Ca^{2+} helps to activate protein kinase C (taken from Berridge, 1985).

6a


Site of Proteolysis
Regulatory Domain
Protein Kinase Domain
α
βI
βII
γ
ATP-Binding Site
H2N
COOH
V1
C1
V2
C2
V3
C3
V4
C4
V5
δ
ε
ζ
ATP-Binding Site
H2N
COOH
C1
C2
C4

6b


Hormone
Receptor
Phosphatidylinositol 4,5-bisphosphate
1,2-Diacylglycerol
Exterior
Cytosol
Protein kinase C
Protein (inactive)
Protein (active)
P
G protein
Phospholipase C
Cellular response
Inositol 1,4,5-trisphosphate
Ca2+
Cellular response
Endoplasmic reticulum
ER Ca2+ channel sensitive to inositol 1,4,5-trisphosphate
Ca2+

catalytic domain of the PKC isozymes remains constant and is composed of conserved regions C_3 and C_4 with the ATP binding site.

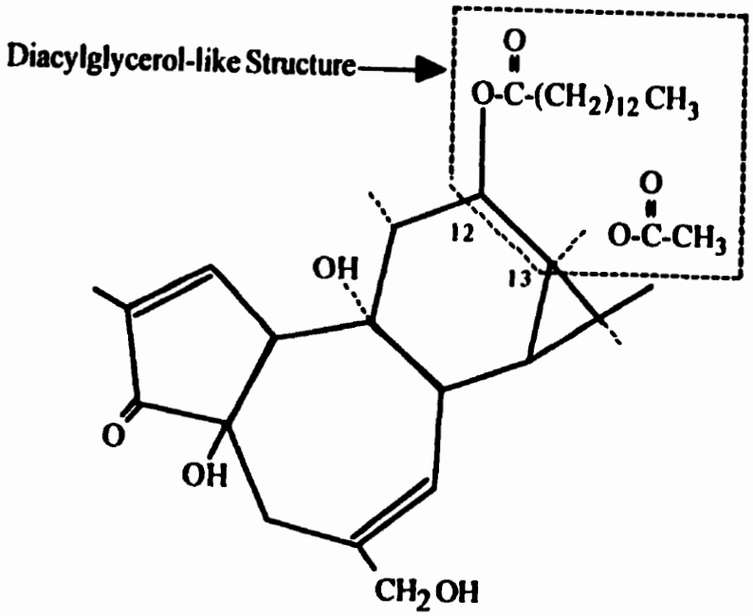
(a) The interrelationship between calcium and protein kinase C

Transmission of information from extracellular signals across the cell membrane is achieved in a large part through a cascade of events leading to the degradation of various membrane phospholipids (Nishizuka, 1992). Research supports the participation of PKC in this signal transduction network (Figure 6b) (Nishizuka, 1992). PKC exists as an inactive cytoplasmic protein, however, upon exposure to an influx of calcium ions the enzyme migrates and binds to the cytoplasmic facing plasma membrane awaiting activation by DAG (Darnell et al., 1990). Unsaturated fatty acids have also been shown to induce translocation (Diaz-Guerra et al., 1991). A large number of extracellular signals such as hormones, neurotransmitters, and some growth factors can induce inositol phospholipid turnover producing DAG and a phosphoinositide (Nishizuka, 1984) indicating an important role for PKC in cell metabolism (O'Brian and Ward, 1989). Additionally, hydrolysis of other phospholipids such as phosphatidylcholine (PC) produces DAG at a later stage in the cellular response (Nishizuka, 1992). PKC activation efficacy varies depending on the fatty acid composition of DAG (Mori et al., 1982). Generally, DAG posses a stearoyl and an arachidonyl residue at position 1 and 2, respectively (Michell, 1975). It is thought that IP_3 may participate through its stimulation of Ca^{2+} release from the endoplasmic reticulum (Berridge, 1987; Farese, 1988). It has been demonstrated that PKC activation and Ca^{2+} mobilization act synergistically to elicit a full physiologic response (Nishizuka, 1989). PKC activation results in a variety of cellular responses depending the cell of origin (Darnell et al., 1990).

(b) Role of protein kinase C in the etiology of colon cancer

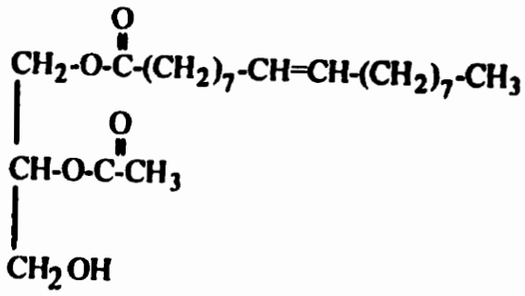
A role for PKC in carcinogenesis was targeted when it was determined that phorbol ester tumor promoters activate PKC (Castagna et al., 1982). These lipid soluble tumor promoters traverse the plasma membrane unaided and play a part in the transition from normal to malignant status (Darnell et al., 1990). The binding of the phorbol ester to the C1 PKC region is dependent on phospholipid (Sumida et al., 1993). The cysteine rich regions act similarly to zinc fingers which function to bind the PL (Sumida et al., 1993). The steriochemistry of phorbol esters is somewhat similar to that of DAG (Figure 7), which presumably is how they act as a substitute to activate PKC (Nishizuka, 1984). The mechanism of action attributed to the phorbol esters is an enhanced affinity for Ca^{2+} resulting in full activation with a negligible change in Ca^{2+} concentration (Yamanishi, 1983). Much of the support for PKC as a mitigating factor in colon carcinogenesis comes from bile acid studies (O'Brian and Ward, 1989). Tumor promoting bile acids have been shown to enhance PKC activity two fold (Fitzer et al., 1987). The mechanism of action by which bile acids stimulate PKC may include detergent effects (Fitzer et al., 1987) or stimulation of phospholipase C catalyzed DAG production (Craven et al., 1987; Takenawa & Nagai, 1981). Down regulation of PKC activity has been reported in animal tumors (Craven & DeRubertis, 1992; Wali et al., 1991) and human colonic carcinomas (Guillem et al., 1987; Kopp et al., 1991) however, other researchers have reported no change (Hashimoto et al., 1989; McGarrity & Peiffer, 1994). It is suggested that down regulation of PKC activity is one of the events that occurs *during* cancer development (O'Brian and Ward, 1989). The primary function attributed to bile acids in carcinogenesis is the enhancement of colonic mucosal proliferation. Thus the ameliorating effect of calcium on bile acid induced proliferation may be mediated through PKC activation. To this end, Pence et al. (1995) reported that at 15 weeks of bile acid promoted colon carcinogenesis high calcium (2.0%) reduced PKC activity, however, at 5 and 30 weeks

Figure 7. Depiction of the structural similarity between phorbol ester and diacylglycerol (taken from Nishizuka, 1989).



12-O-tetradecanoylphorbol-13-acetate

Phorbol Ester



1-Oleoyl-2-acetyl-glycerol

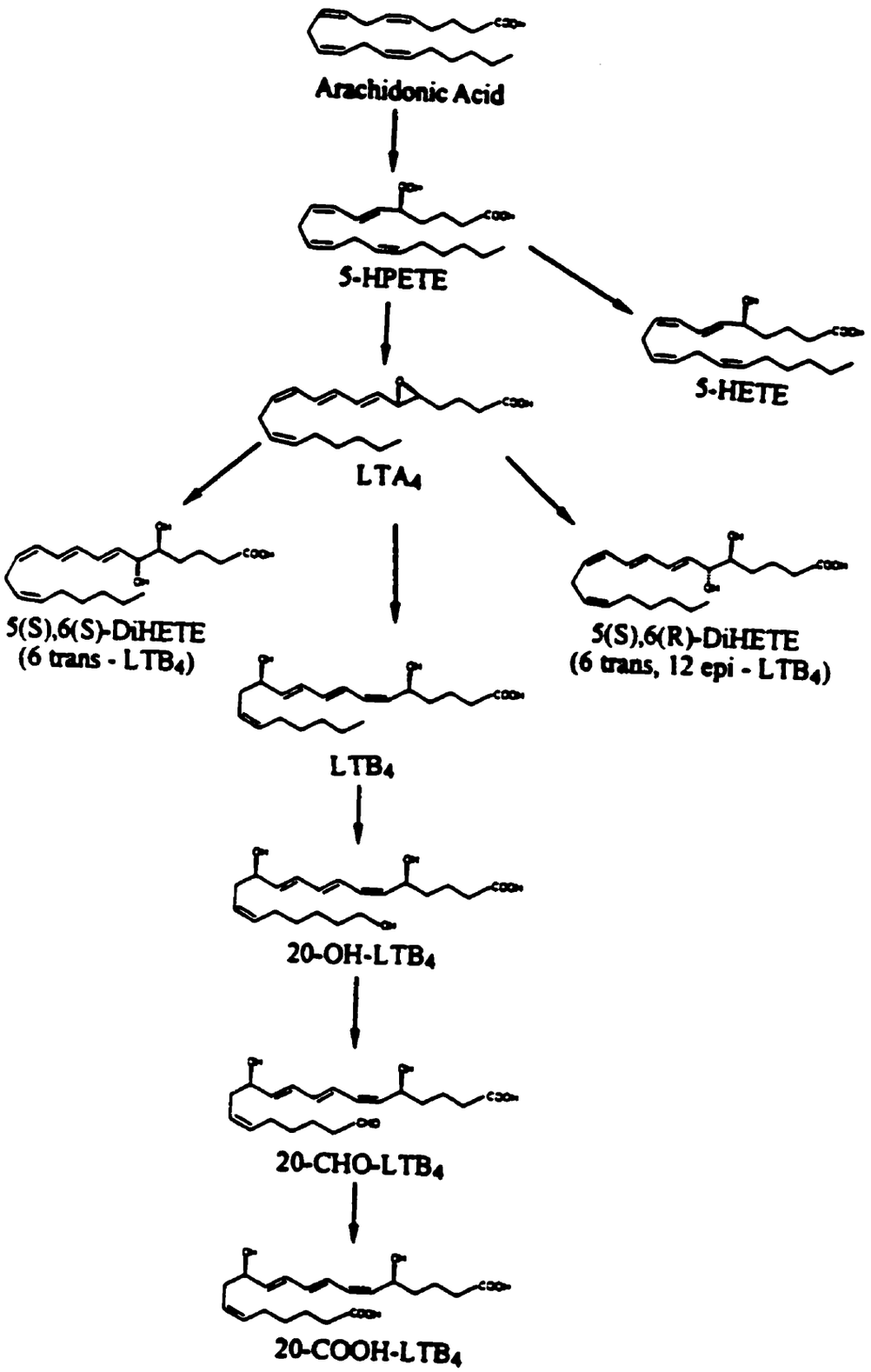
Permeable Diacylglycerol

there were no significant effects of this diet. Although a substantial body of evidence has linked PKC activity to colon cancer development further clarification of this enzyme as a hallmark of colon cancer is required.

5. Leukotrienes

Eicosanoids represent a subclass of unsaturated (C_{20}) fatty acids categorized by the degree of unsaturation (Mayes, 1988). AA and some other C_{20} fatty acids are the precursor metabolites for this group of physiologically and pharmacologically active substances composed of prostanoids and leukotrienes (LTs) synthesized via the cyclooxygenase and lipoxygenase pathways, respectively (Mayes, 1988). The direction of AA metabolism is dependent on cell type and stimulus (Serhan et al., 1982). For example, stimulated neutrophils favor the lipoxygenase pathway with leukotriene B_4 (LTB_4) as their main LT product (Feinstein and Sha'afi, 1983). AA is generally donated from plasma membrane phospholipids at the 2-position (Mayes, 1988). Leukotrienes are characterized by one or two oxygen substituents and three conjugated double bonds (Mead et al., 1986). AA, the primary LT precursor, may be metabolized by three different lipoxygenases which insert oxygen at the 5, 12, or 15 position (Mayes, 1988). Only AA metabolized by 5-lipoxygenase produces LTs (Figure 8) while the other lipoxygenases form hydroxyeicosatetranoate (HETE) products such as 12-HETE and 15-HETE (Feinstein and Sha'afi, 1983). The first LT formed is LTA_4, however due to its instability it is quickly converted into LTB_4 or LTs C_4, D_4, and E_4 (Mead et al., 1986). The trans isomers 6-trans-LTB_4 and 6-trans-12-epi-LTB_4 are sometimes produced during the formation of LTB_4 (figure 9) (Feinstein and Sha'afi, 1983). In addition, ω-oxidation of LTB_4 produces 20-OH-LTB_4 and 20-COOH-LTB_4 and may represent the initial steps in degradation (Mead et al., 1986). The functionality of LTB_4 is extremely sensitive to its natural configuration and modifications result in a 50 to 100-fold loss of activity (Lewis et

Figure 8. Arachidonic acid metabolites produced via the 5-lipoxygenase pathway, highlighting the synthesis of leukotriene B_4 (LTB_4). The metabolites 6 trans-LTB_4 and 6 trans, 12 epi-LTB_4 represent trans isomers with reduced activity. The metabolites 20-OH-LTB_4 and 20-COOH-LTB_4 represent degradation products with reduced functionality. [DiHETE: dihydroxyeicosatetraenoate; HETE: hydroxyeicosatetraenoate; HPETE: hydroperoxyeicosatetraenoate; LTA_4...D_4: leukotriene A_4....D_4] (adapted from Mayes, 1988).


Arachidonic Acid
5-HPETE
5-HETE
LTA4
5(S),6(S)-DiHETE
(6 trans - LTB4)
5(S),6(R)-DiHETE
(6 trans, 12 epi - LTB4)
LTB4
20-OH-LTB4
20-CHO-LTB4
20-COOH-LTB4

al., 1981; Naccache et al., 1982). The conversion of LTB_4 to its ω-oxidation products inhibits functional activity and the ability to increase Ca^{2+} uptake (Naccache et al., 1982). LT's, such as LTB_4, act to increase vascular permeability, stimulate the attraction and activation of leukocytes, and induce aggregatory, chemotactic, and degranulation responses (Feinstein and Sha'afi, 1983; Mayes, 1988). This suggests that LTs may be important regulators in diseases with an inflammatory component (Mayes, 1988). Further, LTs easily migrate from the cell of origin and stimulate other tissues (Dahlen et al., 1980).

(a) The interrelationship between calcium and leukotriene synthesis

The circular relationship of calcium in this process is predominantly related to the release of AA and the 5-lipoxygenase enzyme. Calcium is involved in the enzymatic release of AA from membrane phospholipids (described previously in section C. 3. a) and reciprocally changes in calcium membrane permeability or influx from internal pools are mediated by second metabolites of AA. The lipoxygenase pathway is stimulated by calcium at the 5-lipoxygenase step and resultant LTB_4 can mediate calcium mobilization (Serhan et al., 1982).

(b) Role of the lipoxygenase pathway in the etiology of colon cancer

Eicosanoids are implicated in carcinogenesis through a potential role in tumor promotion, invasiveness, and metastatic spread (Karmali, 1987; Levine, 1988). Tumor promoters, both TPA and non-TPA, stimulate both the cyclooxygenase and lipoxygenase pathways indicating that the mechanism of action is mediated through the activation of AA metabolism (Ohuchi et al., 1987). The human leukemia cell line HL-60 was shown to be stimulated by lipoxygenase products (Miller et al., 1989). A human gastric cancer cell line was shown to produce growth stimulatory autocoids by directing AA metabolism

through the 5-lipoxygenase pathway (Shimakura & Boland, 1992). Carcinogen treated rat colon was found to contain increased HETE compared to normal counterparts and colons treated with a chemopreventive agent exhibited reduced HETE levels (Rao et al., 1993a). It has been suggested that the contribution of eicosanoids to colon carcinogenesis is through the stimulation of mucosal proliferation (Rigas et al., 1993). In point of fact, bile salts which increase colonic mucosal proliferation also have been shown to stimulate the synthesis of LTB_4 in an intestinal epithelial cell line (Dias et al., 1994). Research has demonstrated that it is possible to influence the type of eicosanoid synthesized by varying PUFA intake and thus, provides the opportunity to influence disease by dietary manipulation (Mayes, 1988).

Research in LT production involves the stimulation of cells with calcium ionophores. Two such agents include A23187 and thapsigargin. A23187 is an antibiotic and divalent cation ionophore (Mead et al., 1986). It is believed that the production of AA derivatives by A23187 is mediated through an increase in cytoplasmic calcium and the activation of AA release by phospholipase (Mead et al., 1986). Thapsagargin, a cell permeable non-TPA tumor promoter, induces the release of intracellular calcium without the hydrolysis of inositol phospholipids (Ohuchi et al., 1987).

D. Calcium and Cancer

1. Calcium Metabolism

While 99% of the body's calcium is stored in the skeletal system, the remaining 1% distributed in extra- and intracellular fluids is essential to the function of many tissues and thus vital to survival (Arnaud & Sanchez, 1990; Avioli, 1988; Hunt & Groff, 1990). Calcium is more than simply a membrane trigger but rather functions as an internal coordinator integrating cellular activities (Williams, 1976). The pivotal characteristic to

the effectiveness of calcium as modulator is the striking gradient in Ca^{2+} concentration (10 000:1) between the extracellular and cytoplasmic fluid (Barritt, 1992). Maintenance of this gradient is attributable to the low permeability of membranes, high affinity of regulatory Ca^{2+}-binding proteins, and Ca^{2+} transporters (Barritt, 1992; Hunt and Groff, 1990). Within the cell the main Ca^{2+} stores include the endoplasmic or sarcoplasmic reticulum and the mitochondria (Barritt, 1992). Thus, calcium influx produces a dramatic change in concentration which sets off a cascade of events and is quickly removed to correct the concentration gradient making it an effective coordinating system. The calcium ion regulates a number of important physiologic and biochemical processes. These include: neuromuscular excitability; blood coagulation; secretory processes; membrane integrity; plasma membrane transport; enzyme reaction; release of hormones and neurotransmitters; and proper concentrations for bone mineralization (Arnaud and Sanchez, 1990; Hunt and Groff, 1990). Plasma calcium is distributed in three major fractions: complexed, protein bound, and ionized (Bringhurst, 1989). The fraction of calcium that is complexed to organic and inorganic acids is small (~8-14%), ultrafiltrable and plays a relatively minor role as a reservoir for ionized calcium (Arnaud and Sanchez, 1990; Avioli, 1988). The protein bound fraction (~40%) is biologically inert, however, approximately 80% of calcium is bound to albumin and 20% is bound to globulin forming an important reservoir of ionized calcium (Arnaud and Sanchez, 1990; Avioli, 1988). The ionized form (Ca^{2+}) is the only biologically active species and constitutes 46-50% of the total calcium (Avioli, 1988).

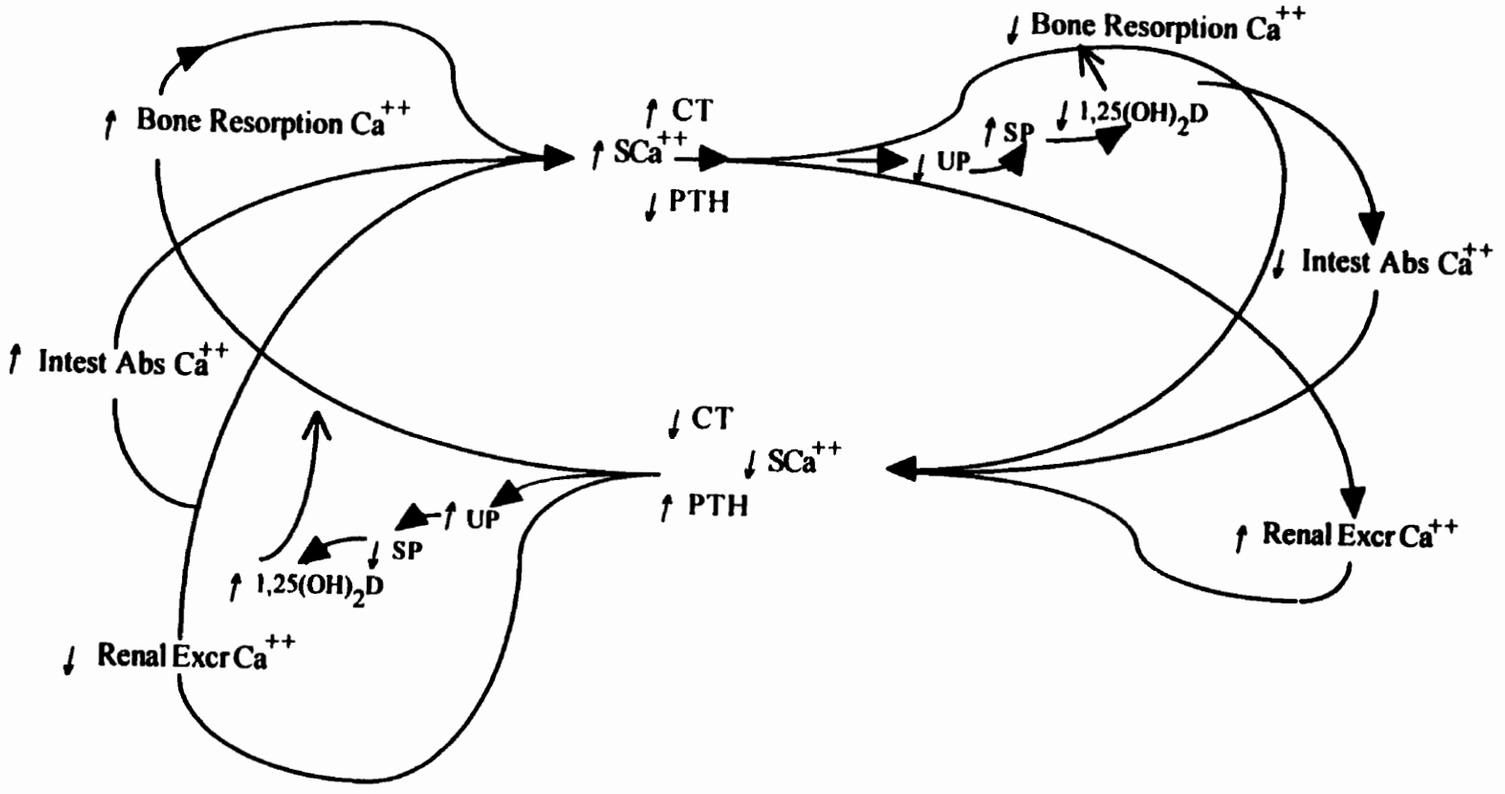
The amount of dietary calcium required to maintain metabolic balance, which is defined by dietary intake versus urinary and fecal excretion, varies with three basic parameters (Bringhurst, 1989). Requirement is dependent on: physiologic need for the mineral; the ability of the intestine to absorb the mineral; and the ability of the kidneys to conserve the mineral (Bringhurst, 1989; MacManus et al., 1982). Any dietary deficiency

or excess is accommodated to maintain the specific plasma concentration. Average absorption efficiency is in the rage of 25-35% of ingested calcium (Heaney & Recker, 1986) and sources such as dairy, cereal, or vegetable yield calcium with similar efficiency (Heaney et al., 1989). Dietary deprivation of calcium will result in adaptive alterations in the production and secretion of the calciotropic hormones that will in turn minimize negative balance of the ion. The endocrine system that regulates this homeostasis is complex and mediated by the polypeptide hormones calcitonin (CT) and parathyroid hormone (PTH) in conjunction with the steroid hormone $1,25(OH)_2D_3$ (Arnaud, 1978; Aurbach et al., 1985). The relationship is depicted in figure 9. The overlapping feedback loops represent the target organs and the controlling components; serum calcium, PTH, CT, and $1,25(OH)_2D_3$. In brief, detected low levels of serum calcium stimulate an increase in PTH secretion with a concomitant decrease in CT. Decreased bone resorption, decreased intestinal absorption, and increased renal excretion of calcium results in a counterbalance in serum values resulting in a slight decrease in serum calcium. These events occur within milliseconds and are constantly repeated to balance plasma calcium at physiological concentrations with minimal oscillation.

2. Calcium and Colon Carcinogenesis

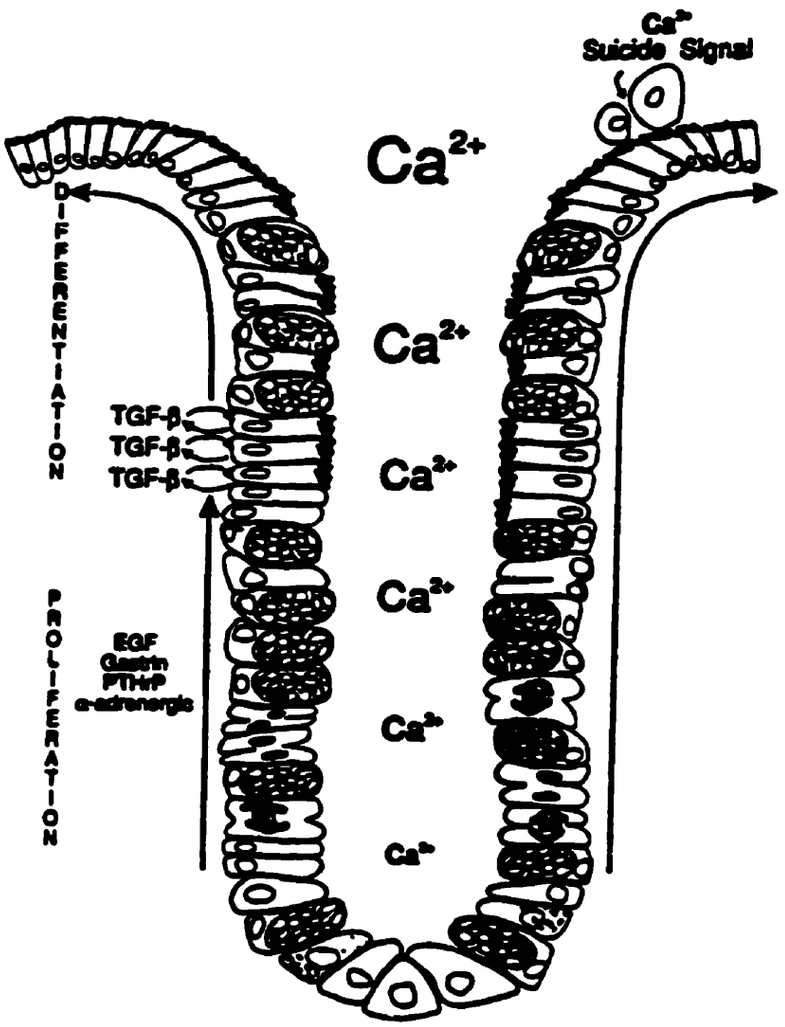
Architecturally, the colonic mucosa is composed of finger-like projections which are lined with epithelial cells (described previously). The positioning of epithelial cells within these crypts is critical to the stage of cell development. Colonic epithelial cells move through five developmental stages while migrating up the crypt toward the lumen (Chang, 1985). This migration involves a switch in cell status from proliferative to differentiated for which external calcium concentration is paramount (Whitfield, 1992). Colonic cells in media containing 0.1mM calcium proliferate optimally, however, at concentrations of 0.8 to 2.0mM proliferation is halted (Buset et al., 1986). Whitfield

Figure 9. Diagrammatic representation of the homeostatic regulation of serum calcium. Depending on the serum levels of ionic calcium, parathyroid hormone, and calcitonin the response from bone, intestine, and kidney tissues act to maintain ideal calcium concentration. [$1,25(OH)_2D$: 1,25-dihydroxycholecalciferol; CT: calcitonin; PTH: parathyroid hormone; SCa: serum calcium; SP: serum phosphorus; UP: urinary phosphorus] (taken from Arnaud, 1978).


↓ Bone Resorption Ca++
↑ CT
↑ SCa++
↓ PTH
↑ SP
↓ 1,25(OH)2D
↓ UP
↓ Intest Abs Ca++
↑ Bone Resorption Ca++
↑ Intest Abs Ca++
↓ CT
↓ SCa++
↑ PTH
↑ UP
↓ SP
↑ 1,25(OH)2D
↑ Renal Excr Ca++
↓ Renal Excr Ca++

(1991) proposes that a sulfomucin-determined cryptal calcium gradient controls the developmental progression of epithelial colonocytes (Figure 10). In this context, calcium concentration is relatively low at the base of the crypt increasing toward the lumen. Thus proliferation, while promoted in the basal regions, is stopped as the cells move up the crypt and differentiation is triggered. It is further proposed that as cells migrate to the mucosal plain changes in external calcium induce programmed suicide and eventual sloughing off into the fecal stream (Whitfield, 1992). As cells undergo transformation they become unresponsive to external signaling (Pitot, 1993). At the adenoma stage cells fail to respond, respond sporadically, or respond incompletely to calcium concentrations which inhibit proliferation and induce differentiation of normal cells (Whitfield, 1992). Moreover, these higher levels of calcium, while inhibiting normal cell proliferation, actually enhance the proliferative activity of adenoma cells (Buset et al., 1986; Whitfield, 1992). In this way calcium may selectively enhance the growth of transformed cells that are not inhibited by the high extracellular concentration of calcium. These cells migrate toward the mucosal plain uninhibited and unresponsive, piling up and presumably forming adenomas (Whitfield, 1992). Key to this process of malignant transformation are modifications at the cell membrane level (Hoelzl-Wallach, 1968; Montagnier & Torpier, 1976; Smith & Kenyon, 1973). An increase in extracellular calcium of tumor cells (Anghileri et al., 1976; Anghileri et al., 1977) suggests that cell membrane impairment is a phenomena of carcinogenesis (Anghileri, 1982). Perturbation in cell membrane integrity disrupts the delicate balance of extra- and intra- cellular calcium leading to a rapid influx of calcium ions (Anghileri, 1982). This imbalance leads to either cell death by necrosis or dedifferentiation of damaged cells (Anghileri, 1982; Gold & Greeman, 1965). The latter results in cells becoming more primitive and autonomous with each successive generation, characteristic of neoplastic transformation (Anghileri, 1982).

Figure 10. A proposed calcium gradient model to control the transition from proliferation to differentiation in the normal colonic epithelium. The concentration of calcium is lowest at the bottom of the crypt increasing towards the lumen. Proliferation is thought to be suppressed and differentiation stimulated at a specific calcium concentration threshold (taken from Whitfield, 1991).


Ca2+
Suicide Signal
Ca2+
DIFFERENTIATION
Ca2+
TGF-β
TGF-β
TGF-β
Ca2+
Ca2+
PROLIFERATION
EGF
Gastrin
PTHrP
α-adrenergic
Ca2+
Ca2+

3. Epidemiology

Epidemiological studies have indicated that environmental factors play a critical role in colon cancer risk (Schottenfeld & Winawer, 1982). Among the environmental factors diet appears to be most strongly implicated in this risk (Potter, 1992; Willett & MacMahon, 1984) and is thus a rich area for research. Food frequency questionnaires used in a hospital-based case-control study indicated that a larger percentage of cancer patients report that "they never drank milk" compared to non-cancer controls (Stocks & Karn, 1933). Initial research identified Seventh-Day Adventists (SDA) at low risk for developing colon cancer (Phillips, 1975). This population abstain from alcohol, tea, coffee, cigarettes and are predominantly lacto-ovo vegetarians. A positive correlation was drawn for increased milk consumption and a reduced risk of colon cancer compared to controls. In a 19-year prospective study Garland et al. (1985) followed 1,954 middle aged men for an association between dietary intake of both calcium and vitamin D with respect to the risk of colorectal cancer. Diet histories analyzed by means of a food composition table indicated that calcium intake was lower in men who developed colorectal cancer. A case-control study in Utah revealed that the lower colon cancer mortality for this state was accompanied by a higher calcium intake compared to the national average (Slattery et al., 1988). A prospective study of 7,472 men using 24 hour diet recall together with cases cited in the cancer registry identified a decreased colon cancer risk with increasing calcium intake (Stemmermann et al., 1990). However, not all epidemiological studies have found this inverse association. Higginson (1966) found no statistically significant difference in calcium intake between 340 colorectal cancer patients and 1,020 control patients. A case-control study in Japan indicated that milk was not correlated with incidence of large bowel cancer (Wynder et al., 1969). A case-control study carried out in Greece by Manousos et al. (1983) found no difference in calcium intake between patients with colon cancer and controls. In a case-control study of

Japanese-American men the association between calcium and colon cancer risk was found to be not significant (Heilbrun et al., 1986). While no direct relationship can be drawn from these studies the proposed protection factor derived from dietary calcium is likely dependent on the presence and intensity of other risk factors along with general dietary intake and genetic predisposition.

4. Experimental Animal Investigations

Experimental investigations into the role of calcium as a modulator of colon carcinogenesis have yielded variable results (Table 1). These studies appear to differ from each other with respect to experimental protocol as well as dietary composition (Table 2). These factors may, in part, account for the disparity in reported effects of calcium on biological parameters.

(a) Cell proliferation studies

Calcium as a potential chemopreventive agent gained popularity when it was reported that the hyperproliferation associated with the initial stages of cholic acid-promoted tumorigenesis was ameliorated by calcium in the murine model (Wargovich et al., 1983). Calcium supplementation significantly reduced the number of tritiated labeled cells and mitotic figures per crypt. Furthermore, calcium reduced the mitogenic effect of intrarectal fatty acid installation (Wargovich et al., 1984). At this point it was hypothesized that dietary fats promoted colon cancer by increasing the levels of free fatty acids and bile acids and that calcium ameliorated this effect by forming insoluble calcium soaps (Newmark et al., 1984). It was of great interest to find that increased dietary calcium modified the toxicity of orally intubated beef tallow or cholic acid (Bird, 1986) and the chronic feeding of cholic acid (Bird et al., 1986) as measured by the reduced number of mitotic figures and tritiated labeled cells. Ornithine decarboxylase (ODC), a

Table 1: Various results of experimental investigations on calcium and its role in colon carcinogenesis		
Source	Animal Model	Experimental Effect of Calcium
CaPhos	C57BL/6J mouse	• decreased mitogenesis induced by cholic acid (5% CO)(Bird et al., 1986)
	F344 rat	• increased TI and number of tumors per tumor bearing animal (5% CO) (McSherry et al., 1989)
CaCarb	C57BL/6J mouse	• decreased bile acid induced proliferation (5% CO) (Wargovich et al., 1983)
	F344 rat	• no effect on TI (5% MF) (Nelson et al., 1987)
	F344 rat	• decreased AOM induced ODC and Tyr-K activity (4.5% MF) (Arlow et al., 1989)
	F344 rat	• decreased TI with increasing Ca (23.5% CO) (Skrypec & Bursey, 1988)
	F344 rat	• no effect on TI -decreased adenomas (5% CO - 0.5% CA) (Pence et al., 1995)
	SD rat	• low (vs adequate) Ca diet increased LI after 16, 20, & 28 wks (3.5% CO) (Reshef et al., 1990)
CaLact	C57BL/6J mouse	• decreased fatty acid induced proliferation (5% CO) (Wargovich et al., 1984)
	F344 rat	• TI lower in 0.32% Ca compared to 0.04% Ca (20% CO) (Wargovich et al., 1990)
		• no effect on TI (5% CO) (Wargovich et al., 1990)
		• ACF lower in 0.32% Ca versus 0.04% Ca (5% CO) (Weisburger et al., 1994)
		• no effect on ACF- 0.32% versus 0.04% Ca (23.5% CO) (Weisburger et al., 1994)
	SD rat	• low (vs adequate) Ca diet increased LI after 16, 20, & 28 wk (3.5% CO) (Reshef et al., 1990)
CaGluco	F344 rat	• decreased TI (20% CO) (Pence & Buddingh, 1988)
		• no effect on TI (5% CO) (Pence & Buddingh, 1988)
	F344 rat	• no effect on TI in full colon, high Ca lower TI in distal colon (5% CO) (Karkare et al., 1991)
		• no effect on LI (5% CO) (Karkare et al., 1991)

ACF: aberrant crypt foci; CA: cholic acid; Ca: calcium; CaCarb: calcium carbonate; CaGluco: calcium gluconate; CaLact: calcium lactate; CaPhos: calcium phosphate; CO: corn oil; LI: labeling index; MF: mixture of poly and saturated fats; ODC: ornithine decarboxylase; TI: tumor incidence; Tyr-K: tyrosine kinase

Table 2: Various experimental protocols used in calcium investigations.

	Wargovich et al., 1983, 1984	Bird et al., 1985	Nelson et al., 1987	Pence and Buddingh, 1988	Skyrpec & Bursey 1988	Arlow et al., 1989
Source	CaCarb	CaPhos	CaCarb	CaGluco	CaCarb	CaCarb
Calcium Treatment	Acute	Chronic (2 wk)	Chronic	Chronic (30 wks)	Chronic (28 wks)	Acute (5 days)
Concentration (w/w)	gavage bolus 0 and 300-500 mg/kg	0.1, 0.5, and 1.0% (diet)	0.5%, 0.75% and 37 g milk powder per kg dry diet	0.5 and 1.0% (diet)	0.25, 0.5, and 1.5% (diet)	0.8 and 1.3% (diet)
Proximity of calcium to carcinogen	N/A	N/A	Concurrent	2 wk prior	2 wk prior	Concurrent
Chemical carcinogen	N/A	N/A	DMH 20 mg/kg	DMH 30 mg/kg i.p.	AOM 20 mg/kg s.c.	AOM 20 mg/kg s.c.
Number of carcinogen treatments	N/A	N/A	6	10	2	1
Parameters measured	Cell production S-Phase cells	Mitotic activity S-phase cells	Tumor Incidence	Tumor Incidence	Tumor Incidence	ODC activity Tyr-K activity

ACF: aberrant crypt foci; AOM: azoxymethane; CaCarb: calcium carbonate; CaGluco: calcium gluconate; CaLact: calcium lactate; CaPhos: calcium phosphate; DMH: 1,2-dimethylhydrazine; i.r.: intra rectal perfusion; i.p.: intraperitoneal injection; MNNG; N-methyl-N-nitro-N-nitrosoguanidine; MNU: N-methyl-N-nitrosourea; NMU: N-nitrosomethylurea; ODC: ornithine decarboxylase; s.c.: subcutaneous injection; Tyr-K: tyrosine kinase.

Table 2: Various experimental protocols used in calcium investigations. (continued)						
	McSherry et al., 1989	Reshef et al., 1990	Wargovich et al., 1990	Karkare et al., 1991	Weisburger et al., 1994	Pence et al., 1995
Source	CaPhos	CaCarb & CaLact	CaLact	CaGluco	CaLact	CaCarb
Calcium Treatment	Chronic (28 wk)	Chronic (20-40 wk)	Chronic (24 wks)	Chronic (32 wks)	Chronic (9 wks)	Chronic (38 & 13 wks)
Concentration (w/w)	0.5 and 1.6% (diet)	0.05 and 0.7% (drinking water)	0.04 and 0.32% (diet)	0.2, 0.5, 1.0 and 2.0% (diet)	0.04 and 0.32% (diet)	0.5 and 2.0% (diet)
Proximity of calcium to carcinogen	Concurrent & 2 wk prior	8 wk prior	1 wk delay	2 wk delay	1 wk prior	1 and 38 wk delay
Chemical carcinogen	MNU 2 mg/dose i.r.	MNNG 1.5 mg/ dose	AOM 8 mg/kg s.c.	DMH 200 mg/kg s.c.	NMU 2.0 mg/rat i.r.	AOM 12 mg/kg s.c.
Number of carcinogen treatments	2	8 to 28	8	1	4	2
Parameters measured	Tumor Incidence	S-phase cells	Tumor Incidence	Tumor Incidence S-Phase cells	ACF	Tumor Incidence PKC, ODC

ACF: aberrant crypt foci; AOM: azoxymethane; CaCarb: calcium carbonate; CaGluco: calcium gluconate; CaLact: calcium lactate; CaPhos: calcium phosphate; DMH: 1,2-dimethylhydrazine; i.r.: intra rectal perfusion; i.p.: intraperitoneal injection; MNNG; N-methyl-N-nitro-N-nitrosoguanidine; MNU: N-methyl-N-nitrosourea; NMU: N-nitrosomethylurea; ODC: ornithine decarboxylase; s.c.: subcutaneous injection; Tyr-K: tyrosine kinase.

marker of proliferation, was suppressed in the colonic mucosa of high calcium fed animals (Arlow et al., 1989). Calcium was also found to ameliorate the *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) induced hyperproliferation of the colonic epithelium in the presence of a low fat diet (Reshef et al., 1990). Karkare et al. (1991) reported that increasing levels of calcium (double and four times the recommended levels) decreased the number of tritiated labeled cells.

The inhibitory effect of calcium on proliferation has been demonstrated in tissues other than colon. An increase of calcium in mouse epidermal cell culture medium was found to result in the cessation of proliferation and the induction terminal differentiation (Hennings et al., 1980). In addition, high calcium intake was found to reduce the high fat induced hyperproliferation of murine mammary epithelium (Jacobson et al., 1989; Zhang et al., 1987).

(b) Tumorigenesis studies

Several studies have found calcium to significantly alter the risk of colon cancer development. Calcium supplementation in the drinking water of rats following small-bowel resection resulted in the development of fewer tumors compared to controls (Appleton et al., 1987). Pence and Buddingh (1988) investigated an interrelationship between calcium and vitamin D in the presence of a high fat diet and found that supplemental dietary calcium resulted in a decreased tumor incidence. A reduction in tumor incidence was also found in animals consuming three times the recommended level of calcium in a high fat diet (Skrypec & Bursey, 1988). In an attempt to modulate human nutrient density levels of calcium Wargovich et al. (1990) found that rats fed low calcium developed significantly more tumors in the high but not the low fat diet. Recently, it was demonstrated that calcium fed for 38 weeks immediately post carcinogen and then discontinued decreased ($p \leq 0.05$) intestinal tumor incidence but significance was lost when

isolated to the colon (Pence et al., 1995). Interestingly, calcium introduced after 38 weeks and fed for the remainder of the study actually increased ($p \leq 0.05$) tumor burden compared to controls.

Conversely, some studies have failed to demonstrate a protective effect for supplemental calcium. In one study DMH-initiated rats supplemented with either milk or calcium yielded similar tumor incidence to those on rat chow (Nelson et al., 1987). Calcium was found to have no significant effect on tumor incidence of DMH-initiated rats (Kaup et al., 1989). Moreover, animals consuming both the high fat and high calcium diets exhibited the highest tumor incidence. McSherry et al. (1989) reported no effect of calcium on tumor incidence and found that animals consuming calcium had a higher tumor incidence and number of tumors per animal compared to controls. Karkare et al. (1991) found no protective effect on tumor incidence from supplemental calcium either double of four times the recommended level fed in a low fat milieu. Pence et al. (1995) found that supplemental calcium fed for the before or after 38 weeks in the presence of cholic acid did not alter colonic tumor incidence.

5. Clinical Studies

The hypothesis that dietary calcium supplementation could also be protective in humans followed the finding that calcium suppressed hyperproliferation in the rodent model. Lipkin and Newmark (1985) observed that asymptomatic subjects at high risk for colon cancer after prolonged supplemental calcium intake exhibited a reduction in the colonic epithelial labeling index. Similarly, subjects with first degree relatives with colon cancer or those previously diagnosed with adenomas exhibited a significantly reduced labeling index after three months of calcium supplementation (Rozen et al., 1989). Patients diagnosed with adenomas also responded favorably to calcium supplementation (Barsoum et al., 1992; Wargovich et al., 1992). In contrast, several researchers have found

no beneficial effect of calcium on rectal proliferation in: high risk patients having first degree relatives with colon cancer (Gregoire et al., 1989); those with familial adenomatous polyposis (Stern et al., 1990); or those with sporadic adenomas (Bostick et al., 1995; Bostick et al., 1993). Results from clinical investigations are ambiguous and do not provide a clear role for calcium in the etiology of human colon cancer. Moreover, the validity of this marker to predict the decreased likelihood to develop colonic tumors is questionable (Zimmerman, 1993). Further research is clearly needed to fully understand the role of calcium in colon carcinogenesis.

Section III

MATERIALS AND METHODS

Chapter 3. MATERIALS AND METHODS

A. Animals

Female and male weanling Sprague-Dawley rats were obtained from Campus Breeding, Department of Animal Care, University of Manitoba, Winnipeg, Canada. Animals were housed 2-3 per wire cage with a 12/12 h (light/dark) cycle and given laboratory chow and water *ad libitum* until initiation of each experiment. Temperature and humidity were controlled at 22°C and 50%, respectively. All animals were cared for in accordance with the National Research Council's Guide for the Use and Care of Experimental Animals.

B. Diets

Semi-purified diets were formulated based on the composition of the AIN-76 diet (American Institute of Nutrition, 1977; 1980). The control diet contained: 500 g dextrose; 200 g vitamin-free casein; 150 g cornstarch; 50 g corn oil; 50 g cellulose; 35 g AIN-76 mineral mix; 10 g AIN-76 vitamin mix; 3 g DL-methionine; and 2 g choline bitartrate. A more detailed list of ingredients is found in Appendix I. All diet ingredients except corn oil (Mazola), beef tallow (Maple Leaf), calcium salts (Sigma Chemical Co., St. Louis, MO, U.S.A.), cornstarch, and dextrose were from the United States Biochemical Corp. Diet composition of each experiment is detailed in Appendix I.

C. Carcinogen

AOM (Sigma) in sterile saline was injected s.c. at a dose of 15 - 20 mg/kg body weight as described in each study. The 1 or 2 injection protocol was implemented in all studies as described in each chapter. Control animals were given sterile saline.

D. Identification and Assessment of ACF

Rats were killed by CO_2 asphyxiation. Colons were removed, flushed with cold phosphate-buffered saline (PBS), slit longitudinally and fixed flat on filter paper in either 10% neutral buffered formalin or 70% ethanol. After fixation, colons were stained for 15-20 min in 0.2% methylene blue (Sigma) dissolved in PBS. ACF were identified by viewing the colons, mucosal side up, with a light microscope at a magnification of 10X following the protocol established by Bird (1987). The criteria used to identify ACF from normal crypts using this method have been previously described (Bird et al., 1989). These include: (i) increased size; (ii) thicker epithelial cell lining; (iii) increased pericryptal zone relative to normal crypts; and (iv) elongated luminal opening. Visualization and quantification of the number, size, distribution, and multiplicity of ACF were determined for the entire length of the colon. To determine size, an ocular grid was used to measure the approximate area occupied by the ACF as viewed at 100X magnification. To determine distribution, the number of ACF in every 2 cm, starting from the rectal end, was recorded and in some case organized into three sections. The rectal end represented the first 4 cm from the rectal end, the mid region was the next 4 cm, and the cecal end was the remaining 4 cm from the mid region. To determine crypt multiplicity, the number of aberrant crypts (AC) in each focus (AC/focus) was recorded.

E. Preparation of Tissue for Histological Analysis

Metaphase Arrest Cells. Animals were injected with colchicine (1 mg/ kg body weight, Sigma) 2 h prior to killing by CO_2 asphyxiation. Longitudinal sections from three 2 cm segments colon from the rectal, mid, and cecal regions were processed for histology. Tissue sections were embedded in paraffin wax, and 5 μm thick sections were stained with hematoxylin and eosin for microscope viewing. For determination of mitotic index, ten well-oriented crypts were evaluated in which the base, lumen, and top of the

crypts could be seen displaying a U-shaped configuration. The number and the position of the positively (nuclear staining) identified cells in each crypt column were recorded in terms of serial position counting upwards from position 1 at the base of the crypt up to the mouth of the crypt. The index was calculated as the number of positive cells per crypt divided by the total number of cells per crypt (crypt height) multiplied by 100.

PCNA Labeled Cells. Labeling was determined on colonic tissue fixed in 70% ethanol embedded in paraffin wax, and 5 μm thick sections were processed for PCNA immunohistochemistry. This technique employs the unlabeled antibody bridge method using the Universal Peroxidase kit from Signet (ID Labs Inc., London, Canada) similar to the method described by Richter et al. (1992). Tissue sections were deparaffinized and then flooded with normal goat serum and incubated for 20 min to block nonspecific binding. The anti-PCNA monoclonal antibody (Dimension Laboratories, Inc., Missauga, Canada) diluted with antibody diluting buffer (1:40) was applied to tissue sections, and the slides were subsequently incubated for 1 h. Each tissue section was then sequentially incubated with anti-mouse IgG (antibody bridge) and mouse IgG peroxidase (labeling agent). The peroxidase reaction was initiated by immersing the slides in 0.06% 3,3'-diaminobenzidine tetrahydrochloride (Sigma) in PBS to which 0.03% H_2O_2 had been added immediately prior to use. Finally, the slides were lightly counterstained with hematoxylin, dehydrated, and mounted with Permount (Fisher Scientific, Ottawa, Canada). All incubations were carried out in a humidified chamber at room temperature, and between incubations slides were extensively washed with PBS. The PCNA labeling index was determined by identifying positive (nuclear staining) cells in ten well oriented crypts as described for metaphase arrest cells.

BrdU Labeled Cells. Animals were injected with BrdU (30 mg/ kg body weight, Sigma) 1 h prior to killing by CO_2 asphyxiation. BrdU immunohistochemistry was performed as described for PCNA immunohistochemistry with the additional step of

DNA digestion by 2N HCl treatment for 1 h following rehydration. The anti-BrdU monoclonal antibody (Becton-Dickson, San Jose,CA, U.S.A.) 1:40 dilution in antibody diluting buffer was applied to tissues for 1 h. The BrdU labeling index was determined by identifying positive (nuclear staining) cells in ten well oriented crypts as described for metaphase arrest cells.

F. Analysis of Colonic Lipids

Lipids were extracted as described by Folch et al. (1957) using chloroform/methanol (2:1, vol/vol). Phospholipids were separated by thin-layer chromatography using Silica Gel Merck 60 (BDH Inc, Toronto, Canada) precoated plates. All major phospholipids were clearly separated following development of the plates in chloroform/methanol/acetic acid/water (50:37.5:3:1.5, by vol). Lipid bands were visualized with 2'7'-dichlorofluorescein and identified by comparison of their migration rate with those of standard phospholipids (Serdary Research Laboratories Inc., Canada). Bands corresponding to PC, PE, PS, PI and sphingomylein were scraped from the plates and the lipid fractions were directly transmethylated with 6% sulfuric acid in ethanol for 2 h at 80°C in the presence of heptadecanaoic acid (17:0) as internal standard. Following incubation, the reaction mixture was cooled and diluted with water (1 mL) and petroleum ether (2 mL). The upper phase containing the fatty acid methyl esters was removed, dried under pure nitrogen, and reconstituted in small volumes of hexane. Reconstituted fatty acid methyl esters were analyzed by gas-liquid chromatography (Varian Starr 3400; Varian Instruments, Palo Alto, CA, U.S.A.) using a fused Omega Wax capillary column (30 X 0.25 μm) held at 160°C for 1 min, then ramped to 220°C at 2°C per min, and then held for 20 min using helium as the carrier gas.

Section IV

VARIABLES AFFECTING CALCIUM INVESTIGATIONS

Chapter 4.

THE EFFECT OF ALTERED DIETARY PHOSPHORUS ON COLONIC CELL PROLIFERATION AND THE INDUCTION AND GROWTH OF ABERRANT CRYPT FOCI

1. Introduction

It is postulated that dietary calcium acts as a chemopreventive agent in colon carcinogenesis (Newmark et al., 1984). If calcium exerts an effect in the development of colon cancer then it is prudent to investigate influencing factors. Many experimental variables influence the biological responses measured at the tissue level. Before commencing studies on calcium there were two important issues requiring clarification: (1) what level of phosphorus to use and (2) which calcium salt to use when making a high calcium diet. Presently there is a paucity of information regarding the role of elemental phosphate on colon carcinogenesis. While the effect of calcium has been investigated in both animal and human studies, the effect of phosphate has hardly been studied *in vivo* (Grovers & Van der Meer, 1993). Considerable controversy exists on the effect of the counter ion in calcium supplements such as the phosphate component (Lupton et al., 1995). It has been proposed that the ratio of calcium to phosphorus should be maintained at 1 (Bird et al., 1986). The main objective of this dissertation was to assess the effect of calcium on colonic cell proliferation and in colon carcinogenesis in defined conditions. Therefore experiments were designed to establish whether the phosphorus level was an important variable in modulating biological responses in the colon. The hypothesis that changing dietary phosphorus would modulate biological processes in the colonic epithelium was tested by evaluating the response of ACF and proliferative indices to low (0.1%) or normal (0.5%) dietary phosphorus.

2. Material and Methods

Animals. Female Sprague Dawley rats approximately five to six weeks old were acclimatized for one week prior to initiating experimental procedures.

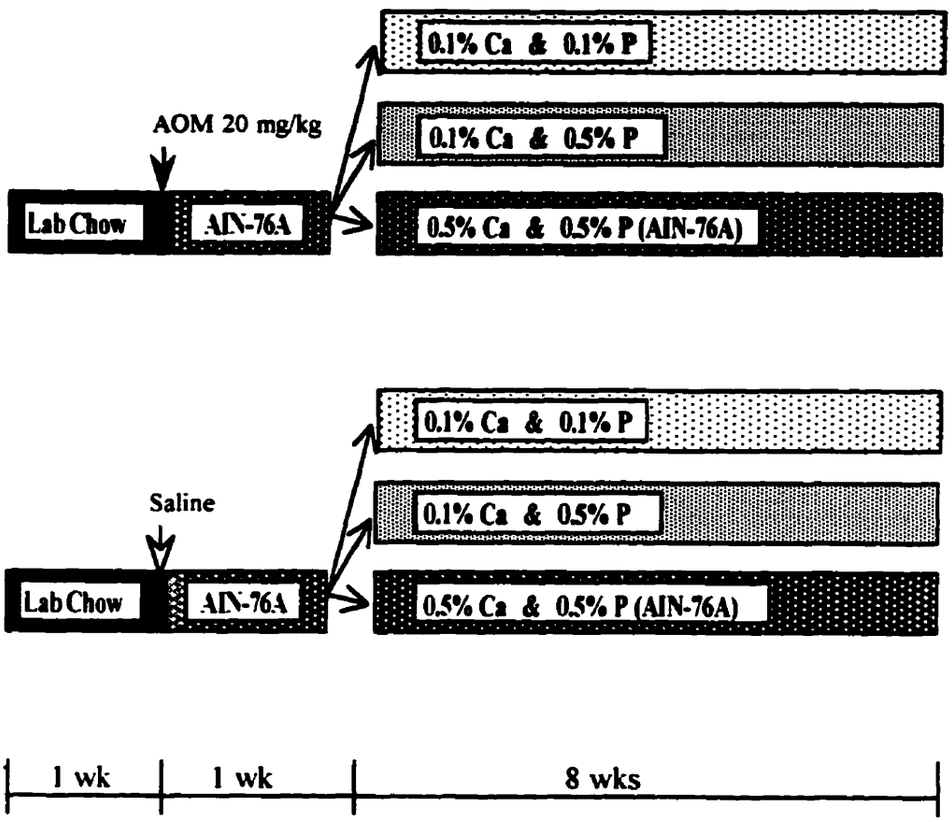
Experimental Diets. The formulated diets were based on the AIN-76A diet (American Institute of Nutrition, 1977; 1980) with the exception of $CaHPO_4$. To achieve the 0.1% Ca diet, 0.4% $NaPO_4$ was added to 0.1% $CaHPO_4$ to maintain the phosphate level in the diet mix. All diets are itemized in Appendix I.

Study Design. Animals were injected with a single dose of either saline (AOM-) or azoxymethane (AOM+) 20 mg/kg and fed the AIN-76A control diet for one week. Animals were then randomized into five dietary groups (8 rats/group/injection protocol) and fed the experimental diets for eight weeks (Figure 11). Two and one hour prior to termination by carbon dioxide asphyxiation animals received an i.p. injection of colchicine 1 mg/kg body weight or 5'-bromo-2'-deoxyuridine (BrdU) 30 mg/kg body weight. Colons were removed immediately after termination and flushed with phosphate buffered saline (PBS), slit open from cecum to anus, and fixed in 70% ethanol. Colons were assessed for aberrant crypt foci (ACF) and proliferative indices.

Quantification of aberrant crypt foci and cell proliferation measurements. The number, distribution, and multiplicity of ACF were determined for the entire length of the colon as described previously. The number of mitotic figures, BrdU labeled cells, and PCNA labeled cells as well as corresponding crypt height were determined as described previously.

Statistical Analysis. Statistical analysis of the data was performed using the analysis of variance (ANOVA) as carried out by the SAS statistical software for microcomputers. Duncan's Multiple Range test was used to separate treatment means when the ANOVA indicated significant differences. Differences were regarded as significant if $p<0.05$. Correlational analysis between the variables was performed using

Figure 11: Schematic representation of the study design used in tdetermining the effect of altered levels of dietary phosphorus.


AOM 20 mg/kg
Lab Chow
AIN-76A
0.1% Ca & 0.1% P
0.1% Ca & 0.5% P
0.5% Ca & 0.5% P (AIN-76A)
Saline
Lab Chow
AIN-76A
0.1% Ca & 0.1% P
0.1% Ca & 0.5% P
0.5% Ca & 0.5% P (AIN-76A)
1 wk
1 wk
8 wks

the Univariate Procedure as carried out by the SAS statistical software for microcomputers. Data which conformed to the normal distribution was analyzed using Pearson's Correlation Coefficient, Spearman's Correlation Coefficient was used to analyze data that did not conform to the normal distribution. Correlational plots were visualized to exclude the possibility of non linear relationships. A correlation was regarded as significant if $p<0.05$.

3. Results

Body weight. Weight gain (Table 3) was similar for all levels of dietary calcium in both the AOM- and AOM+ treated animals.

Characteristics of ACF. Altering the level of phosphorus in the diet significantly altered the growth and development of ACF (Table 4). Animals fed the low calcium diet exhibited an increased total ACF population regardless of the dietary phosphorus level (Table 4). The effect of the calcium:phosphorus ratio was apparent in the multiplicity of ACF where overall multiplicity, AC/focus, was increased when only dietary calcium was reduced. Feeding animals both a low calcium and phosphorus diet resulted in a significantly lower overall multiplicity (Table 4). This was due to the significant increase in foci with only one crypt, which was double that found in animals receiving 0.5% phosphorus regardless of dietary calcium levels. Decreasing the level of dietary calcium, but maintaining phosphorus levels comparable to the control diet, increased the number of lesions with advanced growth features, $AC \geq 3$. This effect was lost when phosphorus levels were reduced to match dietary calcium levels (Table 4).

The majority of the total ACF population were found in the mid region of the colon in both the control diet and the low calcium with 0.5% phosphorus ($p \leq 0.05$). This pattern was no longer significant when the diet was both low in calcium and phosphorus. The dietary protocols modulated the various regions of the colon in a similar fashion as

Table 3 : Effect of Altered Calcium and Phosphorus Ratios on Weight Gain of Sprague Dawley Rats Treated With or Without Carcinogen.[a]

	Wk 0	Wk 2	Wk 4	Wk 6	Wk 8
AOM+					
0.1% Ca : 0.1% P	101.9 ± 6.2	161.1 ± 6.4	190.4 ± 7.6	212.5 ± 7.5	229.3 ± 8.0
0.1% Ca : 0.5% P	96.1 ± 5.9	154.1 ± 5.3	185.8 ± 6.0	201.9 ± 6.2	226.0 ± 6.7
0.5% Ca : 0.5% P	97.5 ± 4.2	160.8 ± 5.7	197.5 ± 7.4	220.5 ± 8.9	239.4 ± 9.5
AOM-					
0.1% Ca : 0.1% P	106.2 ± 4.4	160.0 ± 9.6	219.8 ± 6.6	233.2 ± 14.4	258.8 ± 13.9
0.1% Ca : 0.5% P	105.6 ± 2.6	162.8 ± 3.1	206.8 ± 3.5	233.8 ± 3.5	261.5 ± 3.1
0.5% Ca : 0.5% P	95.7 ± 4.2	154.0 ± 3.5	196.0 ± 5.5	231.8 ± 6.0	259.0 ± 7.3

[a] Values represent means (grams) ± S.E.

Table 4 : Effect of Altered Calcium and Phosphorus Ratios on the Growth and Modulation of Aberrant Crypt Foci with Various Growth Features in Sprague Dawley Rats Treated with or without Carcinogen. [a]

	Total [b]	AC/focus [c]	AC 1 [d]	AC 2 [d]	AC≥3 [d]
0.1% Ca : 0.1% P	254.9 ± 16.6 [e]	2.4 ± 0.4 [f]	62.5 ± 7.5 [e]	99.3 ± 7.2 [e]	96.0 ± 12.9
0.1% Ca : 0.5% P	232.8 ± 16.8 [e]	3.0 ± 0.6 [e]	34.6 ± 6.6 [f]	77.8 ± 10.3	119.1 ± 14.3 [e]
0.5% Ca : 0.5% P	179.9 ± 9.7 [f]	2.7 ± 0.3	32.0 ± 4.7 [f]	68.5 ± 4.7 [f]	79.4 ± 5.0 [f]

[a] Values represent means ± S.E.

[b] Total: Number of ACF per colon.

[c] AC/focus: Number of aberrant crypts per focal lesion.

[d] AC1...AC≥3: Number of foci with 1...≥3 aberrant crypts.

[e-f] : Means within a column with different superscripts differ at $p<0.05$.

was observed for the entire colon (Table 5). In the rectal and mid regions, the total ACF population and AC≥3 were elevated in low calcium fed animals regardless of dietary phosphorus (Table 5). Foci with only one crypt were significantly elevated in low calcium low phosphorus fed animals (Table 5). These trends, while evident, were not significant in the caecal end of the rat colon.

Cell proliferation measurements. Proliferation was measured by identifying cells undergoing mitosis (Table 6), cells labeled with BrdU (Table 7) and those expressing PCNA (Table 8).

The mitotic index was reduced in the AOM+ treated animals fed the low calcium diet regardless of phosphorus content, reaching significance in the mid colonic region (Table 6). Although AOM+ treated animals responded to dietary manipulation the mitotic index of AOM- treated animals demonstrated no consistent pattern of modulation (Table 6). The proliferative zone was unaffected in either AOM- or AOM+ treated colons.

The labeling index decreased significantly in response to a reduction in dietary calcium in both the rectal and mid region of the AOM- treated rat colons when the phosphorus level was maintained at the 0.5% level (Table 7). This trend was also evident in the rectal end of AOM+ treated colons. However, when the phosphorus level was reduced to match the percent composition of dietary calcium a very different effect was observed. The labeling index increased in both the rectal and mid colonic regions in rats fed a low calcium:phosphorus ratio diet compared to the control diet regardless of carcinogen administration (Table 7). The proliferative zone was not altered in any consistent manner by the dietary protocols, however, the zone was consistently greater in the mid region compared to the respective rectal end values (Table 7).

Table 5 : Effect of Altered Calcium and Phosphorus Ratios on the Growth of Aberrant Crypt Foci with Various Growth Features along the Length of the Colon. [a]

	Total [b]	AC 1 [c]	AC 2 [c]	AC≥3 [c]
Rectal End				
0.1% Ca : 0.1% P	87.4 ± 11.0 [d]	21.3 ± 3.8 [d]	34.5 ± 6.0 [d]	31.7 ± 5.5 [d]
0.1% Ca : 0.5% P	71.4 ± 6.5	9.8 ± 1.4 [e]	26.4 ± 4.3	35.1 ± 4.8 [d]
0.5% Ca : 0.5% P	54.8 ± 4.9 [e]	6.8 ± 1.2 [e]	23.7 ± 2.4 [e]	24.1 ± 2.7 [e]
Mid Region				
0.1% Ca : 0.1% P	103.5 ± 10.1 [d]	25.6 ± 3.3 [d]	39.6 ± 4.4 [d]	38.3 ± 5.9
0.1% Ca : 0.5% P	98.1 ± 10.1 [d]	12.6 ± 2.8 [e]	30.9 ± 4.7	54.7 ± 8.2 [d]
0.5% Ca : 0.5% P	72.4 ± 4.7 [e]	12.1 ± 2.4 [e]	25.7 ± 2.1 [e]	34.5 ± 3.4 [e]
Cecal End				
0.1% Ca : 0.1% P	65.5 ± 5.1	15.4 ± 2.0	25.0 ± 2.9	24.2 ± 3.4
0.1% Ca : 0.5% P	61.7 ± 9.3	10.9 ± 2.0	21.0 ± 3.9	29.9 ± 6.6
0.5% Ca : 0.5% P	54.1 ± 4.5	13.6 ± 2.3	19.6 ± 2.5	20.9 ± 3.5

[a] Values represent means ± S.E. See text for detailed description of segmental colonic divisions.

[b] Total: Number of ACF per colon.

[c] AC1...AC≥3: Number of foci with 1...≥3 aberrant crypts.

[d-e] : Means within a column with different superscripts differ at $p<0.05$.

Table 6 : Effect of Altered Calcium and Phosphorus Ratios on the Mitotic Activity of the Rat Colonic Epithelium [a]

	Rectal End		Mid Region	
	MI [b]	PZ [c]	MI [b]	PZ [c]
AOM+				
0.1% Ca : 0.1% P	5.9 ± 0.6	11.8 ± 1.5	6.9 ± 0.8 [e]	12.8 ± 0.6
0.1% Ca : 0.5% P	5.7 ± 0.9	10.1 ± 0.6	7.4 ± 1.6 [e]	14.3 ± 0.8
0.5% Ca : 0.5% P	7.0 ± 2.6	12.1 ± 0.5	12.0 ± 0.6 [d]	14.2 ± 0.7
AOM-				
0.1% Ca : 0.1% P	9.3 ± 1.1	12.4 ± 1.3	8.7 ± 0.6	12.4 ± 0.8
0.1% Ca : 0.5% P	7.8 ± 1.1	11.7 ± 1.1	9.1 ± 0.6	13.7 ± 1.6
0.5% Ca : 0.5% P	7.5 ± 0.5	10.8 ± 0.7	8.5 ± 0.9	13.0 ± 1.4

[a] Values represent means ± S.E. See text for detailed description of segmental colonic divisions.

[b] MI: Mitotic index - the number of mitotic figures per crypt divided by twice the number of cells per mid axial crypt then multiplied by 100.

[c] PZ: Proliferative zone - the highest position of the mitotic cells per crypt per group.

[d-e] : Means within a column with different superscripts differ at $p<0.05$.

Table 7 : Effect of Altered Calcium and Phosphorus Ratios on BrdU Labeling in the Rat Colonic Epithelium.[a]

	Rectal End				Mid Region			
	LI[b]		PZ[c]		LI[b]		PZ[c]	
AOM+								
0.1% Ca : 0.1% P	8.5 ±	0.6[d]	11.3 ±	1.0	10.1 ±	1.7[d]	16.4 ±	0.7
0.1% Ca : 0.5% P	6.3 ±	0.6[e]	11.4 ±	0.5	8.1 ±	1.2	13.8 ±	0.8
0.5% Ca : 0.5% P	7.2 ±	0.7	12.3 ±	0.5	6.9 ±	1.0[e]	14.4 ±	1.4
AOM-								
0.1% Ca : 0.1% P	8.3 ±	0.5[d]	10.2 ±	0.4	10.2 ±	0.7[d]	16.6 ±	1.4[d]
0.1% Ca : 0.5% P	3.3 ±	0.7[f]	8.5 ±	0.5	5.8 ±	0.8[f]	12.4 ±	0.7[e]
0.5% Ca : 0.5% P	5.9 ±	0.4[e]	9.8 ±	0.9	9.1 ±	0.4[e]	16.0 ±	1.0

[a] Values represent means ± S.E. See text for detailed description of segmental colonic divisions.

[b] LI: BrdU Labeling index - the number of labeled cells per crypt divided by twice the number of cells per mid axial crypt then multiplied by 100.

[c] PZ: Proliferative zone - highest position of BrdU labeled cells per crypt per group.

[d-f] : Means within a column with different superscripts differ at $p<0.05$.

PCNA labeling revealed no consistent proliferative pattern in either the rectal or mid region. The proliferative zone was greatest in the control diet reaching significance in the mid colonic region (Table 8).

Comparative analysis of biological endpoints. A positive correlation was not found between ACF with advanced growth features and any of the three proliferative indices examined in either the mid or the rectal colonic regions (Table 9). A significant, though weak, relationship was identified between BrdU and PCNA labeling in the mid region, however, this relationship was not evident in the rectal colonic tissue. Similarly, a positive though weak correlation was demonstrated in the AOM- treated rectal crypts but was not found in corresponding mid region tissue. Overall there were no consistent predictors of dietary modulation on the disease process by proliferative cell measurements.

4. Discussion

The purpose of the present study was to determine whether alterations of dietary phosphorus could influence biomarkers of colon carcinogenesis or the disease process itself. It was observed that phosphorus itself was able to exert an effect on the growth and modulation of aberrant crypt foci. Altering the level of dietary phosphate elicited a varied modulating response in various ACF parameters, the total number of ACF were unchanged whereas focal multiplicity was significantly elevated in response to dietary phosphorus. Lesions with advanced growth features demonstrated modulation by phosphorus and further analysis indicated that ACF in the mid region were the most amenable to the effect of altered dietary phosphate. Additionally, a change in dietary phosphate impacted on the proliferation indices measured in the rat colonic epithelium, however, these changes were inconsistent. Correlational analysis demonstrated that proliferative patterns could not predict the response of ACF to the altered dietary milieu.

Table 8 : Effect of Altered Calcium and Phosphorus Ratios on PCNA Labeling in the Rat Colonic Epithelium.[a]

	Rectal End		Mid Region	
	LI[b]	PZ[c]	LI[b]	PZ[c]
AOM+				
0.1% Ca : 0.1% P	17.5 ± 1.9	15.8 ± 1.0	16.3 ± 1.4	15.4 ± 1.0[e]
0.1% Ca : 0.5% P	14.0 ± 2.5	15.4 ● 0.6	15.3 ● 1.0	17.0 ● 0.6
0.5% Ca : 0.5% P	17.5 ± 1.0	16.9 ± 1.5	14.6 ● 1.4	20.8 ± 1.4[d]

[a] Values represent means ● S.E. See text for detailed description of segmental colonic divisions.

[b] LI: PCNA Labeling index - the number of labeled cells per crypt divided by twice the number of cells per mid axial crypt then multiplied by 100.

[c] PZ: Proliferative zone - the highest position of the mitotic cells per crypt per group.

[d-e] : Means within a column with different superscripts differ at $p<0.05$.

Table 9: Correlational Analysis Between the Various Biological Endpoints.

Biological Parameter		R Value	P Value
Mid Region [a]			
AOM+			
	AC≥3 [a] * MI [d]	-0.3588	0.09
	AC≥3 [a] * BrdU LI [d]	0.0392	0.86
	AC≥3 [a] * PCNA LI [e]	0.0118	0.96
	BrdU LI [d] * PCNA LI [e]	0.4114	0.01
	BrdU LI [d] * MI [d]	0.0298	0.23
	PCNA LI [e] * MI [d]	0.0298	0.88
AOM-			
	BrdU LI [d] * MI [d]	-0.2526	0.23
Rectal End [a]			
AOM+			
	AC≥3 [b] * MI [c]	0.1747	0.41
	AC≥3 [a] * BrdU LI [d]	0.1366	0.52
	AC≥3 [a] * PCNA LI [e]	-0.2022	0.34
	BrdU LI [d] * PCNA LI [e]	0.1599	0.37
	BrdU LI [d] * MI [d]	0.0896	0.36
	PCNA LI [e] * MI [d]	0.0856	0.63
AOM-			
	BrdU LI [d] * MI [d]	0.4406	0.03

[a] See text for detailed description of colonic segmental divisions.

[b] AC≥4: Number of foci with ≥4 aberrant crypts.

[c] MI: mitotic index - the number of mitotic figures per crypt divided by twice the number of cells per mid axial crypt then multiplied by 100.

[d] BrdU LI: labeling index - the number of BrdU labeled cells per crypt divided by twice the number of cells per mid axial crypt then multiplied by 100.

[e] PCNA LI: labeling index - the number of PCNA labeled cells per crypt divided by twice the number of cells per mid axial crypt then multiplied by 100.

These findings indicate that indeed dietary phosphate can and does exert a separate effect on the development of colon carcinogenesis.

Epidemiological evidence indicates that among the cancers influenced by dietary composition are colon and mammary cancer (Jensen, 1986), both of which show a strong positive correlation with dietary fat. Carroll et al. (1991) reported that rats consuming a low calcium diet in the presence of high dietary phosphate developed significantly more mammary tumors than animals consuming both low or high dietary calcium:phosphate. In the present study it was observed that ACF with advanced growth features, which have been shown to correlate with tumor incidence (Magnuson et al., 1993), were also elevated in animals consuming the low calcium and control phosphate (0.1%Ca & 0.5%P) diet. These findings suggest that altered dietary phosphate modulates the disease process and perhaps colonic tumor outcome.

Cats et al. (1993) found that cytolytic activity was not affected by supplemental calcium triphosphate and suggested that interference of phosphate inhibited calcium's protective effect. Hu et al. (1989) reported that phosphate itself altered proliferative indices measured on the mouse colonic epithelium. This is consistent with the current findings that proliferative patterns were affected by dietary phosphate. In non-carcinogen treated animals the colons of animals receiving low calcium control phosphate (0.1%Ca & 0.5%P) demonstrated a significantly lower BrdU LI in both rectal and mid regions compared to the low calcium low phosphate (0.1%Ca & 0.1%P) and control (0.5%Ca & 0.5%P) diets.

A lumenward shift of the main proliferative compartment of the colonic crypt, referred to as a phase 2 defect, has been described in the early phase of carcinogenesis (Deschner & Maskens, 1982; Lipkin, 1974). It has been suggested that this phase 2 defect may be a more reliable marker of malignant development compared with hyperproliferation alone (Risio et al., 1991; Sunter, 1986). In the present study, the mid

region BrdU PZ was significantly smaller in -AOM normal dietary phosphate (0.1%Ca & 0.5%P) fed animals compared to those fed the low phosphate (0.1%Ca & 0.1%P) diet.

Feeding calcium (0.5%) reduced hyperproliferation and hyperplasia in animals fed the high-risk (low calcium) Western-style diet (Richter et al., 1995). Nobre-Leitao et al. (1995) found that animals on low calcium (0.05%) compared to normal calcium (0.5%) diets demonstrated an upward shift in their proliferative compartment, phase 2 defect, indicating an increased risk for malignant transformation. In the present study, while phosphate was held constant, AOM$^-$ low calcium (0.1%) fed animals exhibited lower BrdU LI and PZ than those fed the control (0.5%) calcium diets. No significant changes were observed for mitotic activity or PCNA LI. These findings are in conflict with the aforementioned authors, however, aberrant crypts with advanced growth features were significantly greater in the low compared to normal calcium fed animals. Other researchers have found decreased proliferative indices with diets that promote eventual tumor growth (Galloway et al., 1987; Klurfeld et al., 1987; Richter et al., 1992).

The results of this study demonstrate that the growth and development of ACF are amenable to changes in phosphate and/or calcium as early as 8 weeks dietary intervention. To our knowledge this is the first time dietary phosphate has been shown to alter the disease process as measured by ACF. As a result, to minimize possible confounding effects of dietary modulation, elemental phosphorus should be held constant in all experimental diets and maintained at levels set out by the AIN-76A recommendations for a rodent control diet.

Chapter 5.

A COMPARATIVE STUDY OF VARIOUS CALCIUM SALTS ON COLONIC CELL PROLIFERATION AND THE INDUCTION AND GROWTH OF ABERRANT CRYPT FOCI

1. Introduction

The proposal that high calcium nutriture may reduce the risk of colon cancer has received a great deal of attention during the last decade. The role of calcium in colon carcinogenesis intensified with the observations that treatment of oral calcium on a group of patients at high risk for colon cancer resulted in a return of hyperproliferative colonic mucosa to a near normal state (Lipkin and Newmark, 1985). In fact the theory of calcium chemoprevention in colon cancer has been based on studies that have employed colonic epithelial proliferative status as the biological endpoint. Supplemental calcium has been shown to suppress proliferative activity of colonic crypts in the rodent model (Arlow et al., 1989; Rozen et al., 1989; Wargovich et al., 1984), however, long term studies in animal models have reported both suppressed (Pence & Buddingh, 1988; Skrypec & Bursey, 1988; Wargovich et al., 1990) and enhanced tumor outcome (McSherry et al., 1989) in response to increased dietary calcium. These studies appear to differ from each other with respect to experimental protocol as well as dietary composition. Several researchers have questioned whether the form of calcium used might influence its role in colon carcinogenesis and to date this issue has not been addressed (Karkare et al., 1991; Wargovich et al., 1990; Lupton et al., 1995). It is suggested that different calcium salts may exert different effects on the disease development. The main objective of the this study was to compare various calcium salts for their ability to modulate colonic cell proliferation and growth of aberrant crypt foci. It was hypothesized that calcium salts that rendered a colon more quiescent than the control group with respect to proliferation

and growth of ACF would identify a form of calcium salt(s) with higher biological activity. It was important to compare the calcium salts at the same level, therefore 0.5% by weight of the diet was chosen as it is the level generally present in a defined experimental diet for rodents.

2. Material and Methods

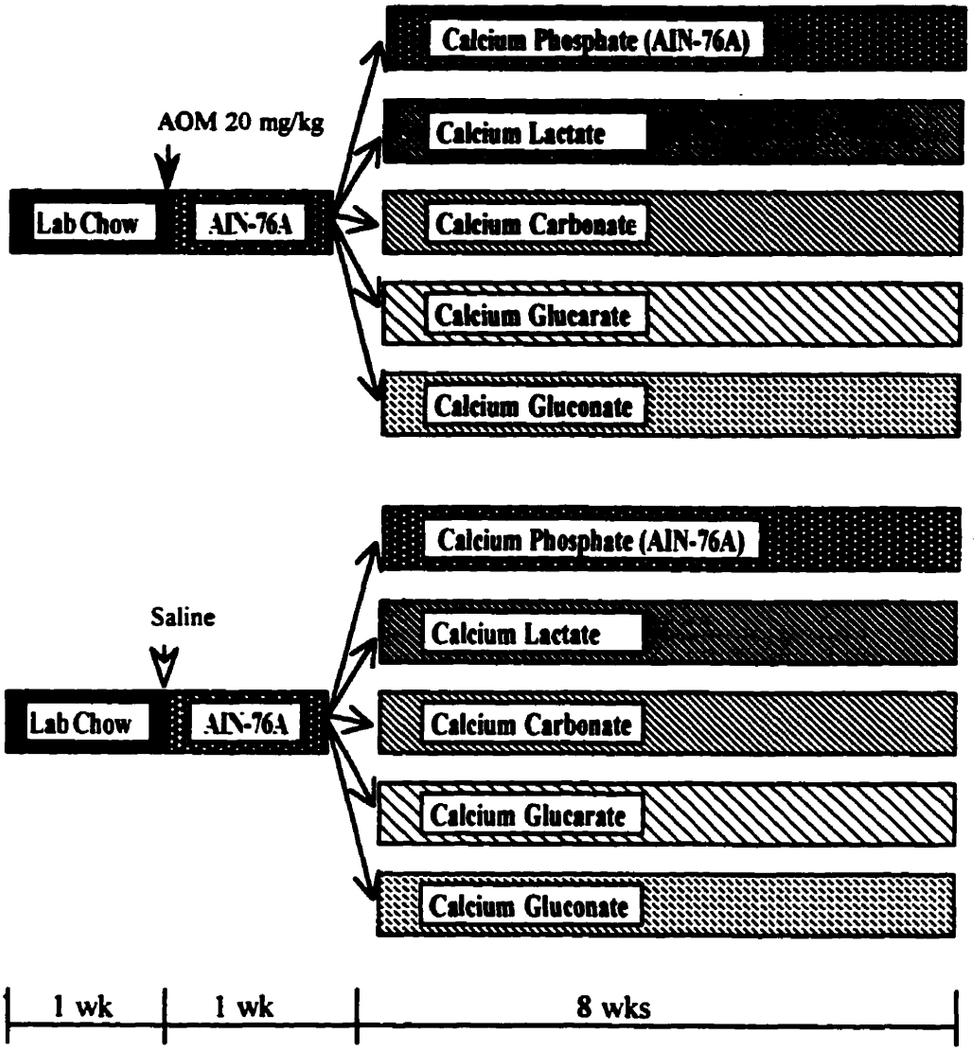
Animals. Female Sprague Dawley rats approximately five to six weeks old were acclimatized for one week prior to initiating experimental procedures.

Experimental Diets. The formulated diets were based on the AIN-76A diet (American Institute of Nutrition, 1977; 1980) with the exception of $CaHPO_4$. Calcium (Ca) was added in the form of phosphate (CaPhos, AIN-76A control), carbonate (CaCarb), lactate (CaLact), gluconate (CaGluco), or glucarate (CaGluca). In the latter four groups NaH_2PO_4 was added to balance phosphate levels in the diet. All diets are itemized in Appendix I.

Study Design. Animals were injected with a single dose of either saline (AOM-) or azoxymethane (AOM+) 20 mg/kg and fed the AIN-76A control diet for one week. Animals were then randomized into five dietary groups (8 rats/group/injection protocol) and fed the experimental diets for eight weeks (Figure 12). Two or one hour prior to termination by carbon dioxide asphyxiation animals received an i.p. injection of colchicine 1 mg/kg body weight or 5'-bromo-2'-deoxyuridine (BrdU) 30 mg/kg body weight, respectively. Colons were removed immediately after termination and flushed with phosphate buffered saline (PBS), slit open from cecum to anus, and fixed in 70% ethanol. Colons were assessed for aberrant crypt foci (ACF) and proliferative indices.

Quantification of aberrant crypt foci and cell proliferation measurements. The number, distribution, and multiplicity of ACF were determined for the entire length of the colon as described previously. The number of mitotic figures, BrdU labeled cells, and

Figure 12: **Schematic representation of the study design used in the study of various calcium salts.**


AOM 20 mg/kg
Lab Chow
AIN-76A
Calcium Phosphate (AIN-76A)
Calcium Lactate
Calcium Carbonate
Calcium Glucarate
Calcium Gluconate
Saline
Lab Chow
AIN-76A
Calcium Phosphate (AIN-76A)
Calcium Lactate
Calcium Carbonate
Calcium Glucarate
Calcium Gluconate
1 wk
1 wk
8 wks

PCNA labeled cells as well as corresponding crypt height were determined as described previously.

Statistical Analysis. Statistical analysis of the data was performed using the analysis of variance (ANOVA) as carried out by the SAS statistical software for microcomputers. Duncan's Multiple Range test was used to separate treatment means when the ANOVA indicated significant differences. Differences were regarded as significant if $p<0.05$.

3. Results

Body weight. Weight gain of experimental animals was not statistically different (Table 10) initially or over the eight week dietary protocol in either the AOM- or AOM+ treated animals.

Characteristics of ACF. The modulation of ACF in the rat colon by various calcium salts was similar (Table 11). The total number of ACF in the colon were similar in all groups although the total value was lower in the animals receiving CaCarb, CaGluca, and CaGluco. ACF exhibiting advanced growth features, classified as those with four or more aberrant crypts per focus ($AC \geq 4$), were also similar among the various calcium salt groups. The distribution of ACF throughout the colon in response to the modulation of the various calcium salts is shown in Table 12. The majority of ACF were found in the mid region of the rat colons ($p \leq 0.05$). ACF in all regions of the colon were modulated in a similar fashion in response to altered calcium salt exposure, however, the mid colonic region of CaCarb fed animals displayed the fewest focal lesions. The distribution of lesions with advanced growth features ($AC \geq 4$) among various diet groups suggested that ACF growth responded similarly to all calcium salts.

Cell proliferation measurements. Proliferation was measured by identifying cells undergoing mitosis (Table 13) and cells labeled with BrdU (Table 14). Rectal

Table 10: Effect of Various Calcium Salts on Weight Gain of Sprague Dawley Rats Treated With or Without Carcinogen. [a]

	Wk 0	Wk 2	Wk 4	Wk 6	Wk 8
AOM+					
CaPhos	97.5 ± 4.2	160.8 ± 5.7	197.5 ± 7.4	220.5 ± 8.9	239.4 ± 9.5
CaLact	108.2 ± 5.2	168.8 ± 6.2	201.4 ± 5.0	227.0 ± 6.2	245.3 ± 7.7
CaCarb	99.8 ± 4.6	158.0 ± 3.0	193.9 ± 4.4	212.6 ± 5.1	232.4 ± 5.7
CaGluca	98.7 ± 6.5	160.8 ± 5.4	195.3 ± 4.4	217.3 ± 5.1	238.1 ± 5.0
CaGluco	97.6 ± 4.9	151.8 ± 4.2	188.6 ± 3.7	208.7 ± 4.3	232.7 ± 3.7
AOM-					
CaPhos	95.7 ± 4.2	154.0 ± 3.5	196.0 ± 5.5	231.8 ± 6.0	259.0 ± 7.3
CaLact	103.0 ± 4.8	159.2 ± 10.3	208.5 ± 14.5	230.8 ± 15.7	252.7 ± 12.9
CaCarb	98.0 ± 2.8	150.7 ± 4.3	191.7 ± 7.9	224.2 ± 8.2	250.3 ± 10.6
CaGluca	95.8 ± 7.3	147.7 ± 4.2	200.2 ± 5.2	228.5 ± 6.8	253.2 ± 7.8
CaGluco	100.3 ± 6.3	155.8 ± 9.4	205.5 ± 13.4	239.7 ± 15.7	264.7 ± 15.9

[a] Values represent means (grams) ± S.E.

Table 11: Effect of Various Sources of Calcium on the Modulation of Aberrant Crypt Foci in the Rat Colon. [a]

	Total [b]	AC 1 [c]	AC 2 [c]	AC 3 [c]	AC≥4 [c]	AC/focus [d]
CaPhos	179.9 ± 9.7	32.0 ± 5.0	68.5 ± 4.7	41.0 ± 2.1	38.4 ± 3.7	2.7 ± 0.3
CaLact	164.5 ± 13.9	30.3 ± 5.1	67.7 ± 9.9	39.4 ± 3.4	27.1 ± 5.2	2.6 ± 0.6
CaCarb	155.8 ± 9.2	27.7 ± 4.4	58.7 ± 4.0	39.4 ± 3.9	30.0 ± 6.0	2.6 ± 0.4
CaGluca	152.9 ± 7.6	23.1 ± 4.4	60.2 ± 4.6	39.8 ± 1.7	29.8 ± 5.5	2.7 ± 0.4
CaGluco	155.9 ± 9.8	20.0 ± 1.3	53.8 ± 4.7	44.8 ± 2.9	37.3 ± 6.7	2.8 ± 0.5

[a] Values represent means ± S.E.

[b] Total: Number of ACF per colon.

[c] AC 1...AC≥4: Number of foci with 1...4≥ aberrant crypts.

[d] AC/focus: Number of aberrant crypts per focal lesion.

Table 12: Modulation of Foci with Various Growth Features by Various Calcium Salts along the Length of the Colon. [a]

	Total [b]	AC 1 [c]	AC 2 [c]	AC 3 [c]	AC≥4 [c]
Rectal End					
CaPhos	54.7 ± 4.9	6.9 ● 1.2	23.8 ● 2.4	14.4 ● 2.0	9.8 ● 1.0
CaLact	41.5 ± 4.6	5.4 ± 1.4	21.3 ± 4.2	8.1 ● 1.5	6.7 ± 1.9
CaCarb	47.8 ● 5.8	7.3 ● 1.5	19.1 ● 1.7	13.1 ± 2.9	8.2 ± 2.1
CaGluca	50.1 ● 2.6	4.9 ● 1.4	21.4 ● 1.5	13.9 ± 1.4	9.9 ± 2.0
CaGluco	44.8 ● 3.3	3.0 ± 0.7	16.6 ± 1.8	13.6 ± 2.0	11.7 ± 2.2
Mid Region					
CaPhos	71.0 ± 5.0	11.6 ± 2.6	24.9 ± 2.2	17.1 ± 1.9	17.4 ± 2.3
CaLact	63.1 ± 7.1	10.4 ± 2.6	25.3 ± 4.3	15.1 ± 2.4	12.4 ± 3.7
CaCarb	59.6 ± 5.9	10.8 ● 2.8	22.4 ± 3.2	14.2 ± 1.5	12.1 ± 2.9
CaGluca	61.4 ± 7.6	10.1 ± 2.7	23.3 ± 2.9	14.1 ± 2.2	13.9 ± 2.1
CaGluco	62.2 ± 7.3	7.9 ± 1.4	20.8 ± 2.4	16.7 ± 1.6	16.9 ± 4.0
Cecal End					
CaPhos	54.1 ± 4.5	13.6 ± 2.3	19.6 ± 2.5	9.6 ± 1.6	11.3 ± 2.5
CaLact	57.0 ± 7.7	14.6 ± 2.2	22.0 ± 3.6	13.0 ± 2.2	7.7 ● 1.7
CaCarb	48.2 ± 6.6	10.6 ● 1.9	17.1 ± 3.1	12.1 ● 1.7	8.4 ± 2.2
CaGluca	40.0 ± 4.7	7.1 ± 1.5	15.0 ± 2.6	10.2 ± 1.3	7.7 ± 1.6
CaGluco	49.0 ± 4.5	9.1 ± 1.5	16.7 ± 2.4	14.6 ± 1.3	8.7 ± 1.4

[a] Values represent means ± S.E. See text for detailed description of segmental colonic divisions.

[b] Total: Number of ACF per colon.

[c] AC 1...AC≥4: Number of foci with 1...≥4 aberrant crypts.

Table 13: Modulating Effect of Various Sources of Calcium on the Mitotic Activity of the Rat Colonic Epithelium. [a]

	Rectal End		Mid Region	
	MI [b]	PZ [c]	MI [b]	PZ [c]
AOM+				
CaPhos	7.0 ± 2.6	12.1 ± 0.5	12.0 ± 0.6 [d]	14.2 ± 0.7 [d]
CaLact	6.2 ± 0.6	9.3 ± 1.2	6.6 ± 1.1 [e]	10.6 ± 0.7
CaCarb	4.6 ± 1.1	9.5 ± 0.6	3.3 ± 0.5 [e]	9.7 ± 1.5 [e]
CaGluca	7.2 ± 1.6	10.6 ± 1.7	4.5 ± 0.9 [e]	11.9 ± 2.2
CaGluco	4.9 ± 1.2	10.5 ± 2.4	9.9 ± 1.3 [d]	12.3 ± 0.4
AOM-				
CaPhos	7.5 ± 0.5	10.7 ± 0.6 [e]	8.5 ± 0.9	13.0 ± 1.4
CaLact	8.0 ± 0.8	11.3 ± 1.1 [e]	11.0 ± 2.4 [d]	13.3 ± 1.3
CaCarb	9.6 ± 1.3 [d]	11.3 ± 1.5 [e]	9.4 ± 0.7	12.7 ± 1.4
CaGluca	6.5 ± 0.9 [e]	9.8 ± 0.7 [e]	9.4 ± 0.9	14.7 ± 1.1 [d]
CaGluco	9.1 ± 0.7	17.4 ± 0.1 [d]	6.1 ± 0.9 [e]	9.7 ± 0.8 [e]

[a] Values represent means ± S.E. See text for detailed description of segmental colonic divisions.

[b] MI: Mitotic index - the number of mitotic figures per crypt divided by twice the number of cells per mid axial crypt then multiplied by 100.

[c] PZ: Proliferative zone - the highest position of the mitotic cells per crypt per group.

[de] : Means within a column with different superscripts differ at $p \leq 0.05$.

Table 14: Modulating Effect of Various Sources of Calcium on the BrdU Labeling in the Rat Colonic Epithelium. [a]

	Rectal End		Mid Region	
	LI [b]	PZ [c]	LI [b]	PZ [c]
AOM+				
CaPhos	7.2 ± 0.7 [d]	12.3 ● 0.5	6.9 ● 1.0	14.4 ± 1.4
CaLact	6.7 ± 0.3 [de]	11.5 ± 1.4	5.2 ± 0.4	13.8 ± 1.0
CaCarb	4.8 ± 0.6 [e]	10.3 ● 0.9 [e]	6.4 ± 0.7	12.8 ± 1.4
CaGluca	8.2 ± 0.9 [d]	12.3 ● 0.7	7.9 ± 0.8	16.2 ± 0.3
CaGluco	7.7 ± 0.8 [d]	14.2 ● 1.1 [d]	8.0 ± 0.8	15.8 ± 1.1
AOM-				
CaPhos	5.9 ● 0.4 [f]	9.8 ± 0.9 [e]	9.0 ● 0.4 [d]	10.7 ± 0.6 [d]
CaLact	8.4 ● 1.1	9.4 ● 1.8 [e]	9.0 ± 0.7 [d]	11.3 ± 1.1 [d]
CaCarb	9.3 ± 1.2 [de]	10.7 ● 0.8 [e]	10.9 ± 0.8 [d]	11.3 ± 1.5
CaGluca	7.0 ± 1.0 [ef]	9.2 ± 1.0 [e]	8.5 ● 1.0 [d]	9.8 ± 0.7 [e]
CaGluco	9.6 ● 0.3 [d]	14.2 ± 0.1 [d]	5.8 ± 0.8 [e]	17.4 ± 0.1 [d]

[a] Values represent means ± S.E. See text for detailed description of segmental colonic divisions.

[b] LI: BrdU Labeling index - the number of labeled cells per crypt divided by twice the number of cells per mid axial crypt then multiplied by 100.

[c] PZ: Proliferative zone - the highest position of the labeled cells per crypt per group.

[d-f]: Means within a column with different superscripts differ at $p \leq 0.05$.

proliferation in AOM+ treated animals ranged from 4.6 to 7.2 for the mitotic index (MI) and 9.3 to 12.1 for the proliferative zone (PZ). In the mid region AOM+ treated animals ranged from 3.3 to 12.0 for the MI and 9.7 to 14.2 for the PZ with CaPhos (control) exhibiting the highest values and CaCarb the lowest values in both parameters. In the AOM- treated animals rectal proliferation ranged from 6.5 to 9.6 for the MI and 9.8 to 17.4 for the PZ. While CaCarb fed animals displayed an elevated MI the PZ of CaGluco fed animals was significantly greater than all other groups. Mid region AOM- treated animals exhibited a range of 6.0 to 11.0 for the MI and 9.7 to 14.7 for the PZ where animals fed CaGluco demonstrated the lowest activity in both parameters.

BrdU labeled rectal proliferation in AOM+ animals ranged from 4.8 to 7.7 for the labeling index (LI) and 10.3 to 14.2 for the PZ. Animals consuming the CaCarb diet displayed the lowest values in both parameters. Proliferation in the mid region of AOM+ treated animals ranged from 5.2 to 8.0 for the LI and 12.8 to 16.2 for the PZ. The rectal proliferation in AOM- treated animals ranged from 5.9 to 9.6 for LI and 9.2 to 14.2 for the PZ with CaGluco fed animals exhibiting the highest values in both parameters. Proliferation in the mid region of AOM- treated animals ranged from 5.8 to 10.9 for the LI and 9.8 to 17.4 for the PZ. The LI was lowest for animals fed the CaGluco diet, however, the PZ was also the largest for this group.

The overall effect of carcinogen treatment on the proliferative indices was evaluated in both the rectal and mid colonic epithelium (Table 15). The MI and BrdU LI were consistently greater in the non-carcinogen treated colonic mucosa compared to the respective carcinogen treated colonic rectal and mid region mucosa. Similarly, the rectal end indices were lower than mid region values for both MI and BrdU LI in both the carcinogen and non-carcinogen tissues.

Table 15: Effect of Carcinogen Treatment on Proliferative Indices in the Rat Colonic Epithelium. [a]

	Rectal End	Mid Region
MI [b]		
AOM+	6.0 ± 0.5 [d]	7.3 ± 1.6
AOM-	8.1 ± 0.6 [e]	8.9 ± 0.8
BrdU [c]		
AOM+	6.7 ± 0.5	6.9 ± 0.5
AOM-	8.0 ± 0.7	8.6 ± 0.8

[a] Values represent means ± S.E. See text for detailed description of segmental colonic divisions.

[b] MI: Mitotic index - the number of mitotic figures per crypt divided by twice the number of cells per mid axial crypt then multiplied by 100.

[c] LI: BrdU labeling index - the number of BrdU labeled cells per crypt divided by twice the number of cells per mid axial crypt then multiplied by 100.

[d-e] : Means within a column with different superscripts differ at $p \leq 0.05$.

4. Discussion

The purpose of the present study was to determine whether alterations in the anionic component of calcium salts could modulate the disease process. It was observed that the various calcium salts investigated exerted similar effects on the induction and growth parameters of the ACF. The effect of various calcium salts on epithelial cellular kinetic indices was variable and lacked any identifiable trend. Therefore, the primary findings of this study suggest that varying the form of dietary calcium among those investigated would not alter the effect of calcium in the early stages of colon carcinogenesis.

Crypt multiplicity, lesions with advanced growth characteristics and not total number of ACF provide an early and persistent predictor of resulting tumor incidence (Magnuson et al., 1993). Zhang et al. (1992) and Pretlow et al. (1992) also found crypt multiplicity to be correlated with tumor incidence. Therefore, the present finding that modulation of either total number of ACF or AC≥4 in either the full colon or in distinct segments of the colon were unchanged by varying the source of calcium indicates that the source of dietary calcium does not influence the early stages in the stepwise development of colon cancer.

A reduction in colonic tumor incidence as a result of increased dietary calcium compared to the control has been reported for CaCarb, CaGluco, and CaGluca (Pence et al., 1988; Skrypec et al., 1988; Wargovich et al., 1990). Paradoxically, it has also been reported that dietary calcium from these various sources increased or did not affect tumor incidence (Karkare et al., 1991; Nelson et al., 1987; Pence et al., 1988; Wargovich et al., 1990). It has been suggested that a clear cut inhibitory effect of calcium is present only in a high fat diet (Karkare et al., 1991). This suggests that the potential effect of dietary calcium can not be attributed to any one particular form of calcium and is consistent with our findings.

Gover et al. (1994) reported a similar antiproliferative effect of calcium from calcium carbonate, calcium phosphate, and milk mineral. In a study looking at calcium phosphate, casein, calcium lactate, and a 50/50 combination of phosphate and carbonate, the authors found that calcium phosphate fed animals demonstrated a lower LI in the proximal but not distal colon (Lupton et al., 1995). Alternatively, Cats et al. (1993) reported that calcium phosphate was not a suitable calcium compound for dietary intervention as it interfered with the protective effect of calcium on the cytolytic activity of fecal water. Generally in the present investigation, the proliferative kinetics of AOM-treated colonic epithelium was not indicative of a change in AOM+ treated mucosa and vice versa, therefore the differences among various proliferative indices was not attributable to any one calcium salt. Furthermore, a common trend between the rectal and mid region colonic epithelial proliferative indices in either S-phase detected (BrdU labeled) cells or the number of mitotic cells was lacking. In contrast, we demonstrated that the mitotic index and BrdU labeling index were consistently higher in AOM- treated mucosa in both the rectal and mid colonic regions. In addition, the rectal region exhibited an overall lower proliferation than in the mid region of the colon, most notably in the AOM-treated colons.

Dwivedi et al. (1989) examined the effect of CaGluca versus CaGluco and observed that colonic tumor incidence was reduced only in the CaGluca fed animals, thus the authors concluded that the effect was a result of the glucarate and not the dietary calcium component. Our results did not demonstrate a similar inhibition by CaGluca, however, subtle differences exist between these two studies. In the present study the semi-synthetic diet based on the AIN-76A composition provides dietary calcium at 0.5% w/w, diets were introduced 1 week after carcinogen administration, and parameters were evaluated at a very early time point. By contrast, Dwivedi et al. (1989) used a chow diet containing 1.10% calcium w/w with the addition of dietary calcium and as such compared

diets of approximately 1.5% calcium w/w employing tumor incidence as the biological endpoint. CaGluco was introduced prior to carcinogen administration and compared to CaGluca pre- and post- carcinogen administration. While CaGluca feeding resulted in similar tumor outcome regardless of introduction it is not evident whether CaGluco would yield similar patterns. Finally, it is plausible that the glucarate component may exert its effect later in the development of colon cancer and thus the effect was not observed in this investigation. Additionally, dietary calcium at levels three times the recommended for rodent intake may modulate the disease process differently than at lower levels.

The results of this study demonstrate that the various calcium complexes investigated exerted similar effects on the induction and growth parameters of the preneoplastic lesions, ACF. Although much has been speculated on the relative efficacy of various calcium salts this is the first study to systematically evaluate these dietary components on biomarkers and early disease endpoints of colon cancer. Colonic epithelial proliferative patterns of the animals fed various calcium salts were inconsistent and lacked any identifiable trend in the colons of animals injected with or without AOM. In conclusion, the anionic component of the calcium salt did not alter the effect of calcium on the early stages of colon carcinogenesis. However, considering the controversy with CaGluca and CaGluco, the fact that the addition of CaPhos changes both the calcium and phosphate levels, and that colons of CaCarb fed animals were among the most quiescent future studies will include CaCarb when calcium levels in the diet are to be increased.

Section V

MODULATION BY CALCIUM IN THE EARLY STAGES OF COLON CARCINOGENESIS

Chapter 6.

MODULATION IN THE INDUCTION AND GROWTH OF ABERRANT CRYPT FOCI BY DIETARY CALCIUM IN THE PRESENCE OF LOW OR HIGH FAT

1. Introduction

In the last decade a great deal of attention has been given to the role of calcium in colon carcinogenesis. The potential importance of calcium in colon carcinogenesis was further accenuated by the observations of Lipkin and Newmark (1985) that oral administration of calcium to a group of patients at high risk for colon cancer resulted in a return of hyperproliferative colonic mucosa to a near normal state. Many epidemiological studies have supported an inverse relationship between dietary calcium intake and the incidence of colon cancer, however, others have been unable to demonstrate this inverse relationship. In several studies it has been reported that elevated levels of dietary calcium suppress epithelial proliferative activity (Lipkin & Newmark, 1985; Arlow et al., 1989; Rozen et al., 1989). The most intriguing nutrient effect is the reported ability to inhibit colon tumor incidence (Pence & Buddingh, 1988; Wargovich et al., 1990), however, subsequent studies have been unable to substantiate this effect (Karkare et al., 1991; McSherry et al., 1989). It has been suggested that calcium's chemopreventive action is effective only in the presence of a high fat diet (Karkare et al., 1991).

Many different protocols have been employed making it difficult to elucidate the influence that calcium may exert in colon carcinogenesis. A thorough investigation of calcium in the stepwise development of colon cancer is needed to determine if possible chemopreventive properties are prevalent. The main purpose of the present study was to systematically examine the effect of varying doses of calcium on the progression of

colon cancer. In the present study, the hypothesis that supplemental calcium may impede the disease process in a dose dependent manner was tested by examining the modulating effect of varying levels of dietary calcium in both a low-fat and high-fat diet on the growth and development of ACF, precursor lesions of colon cancer.

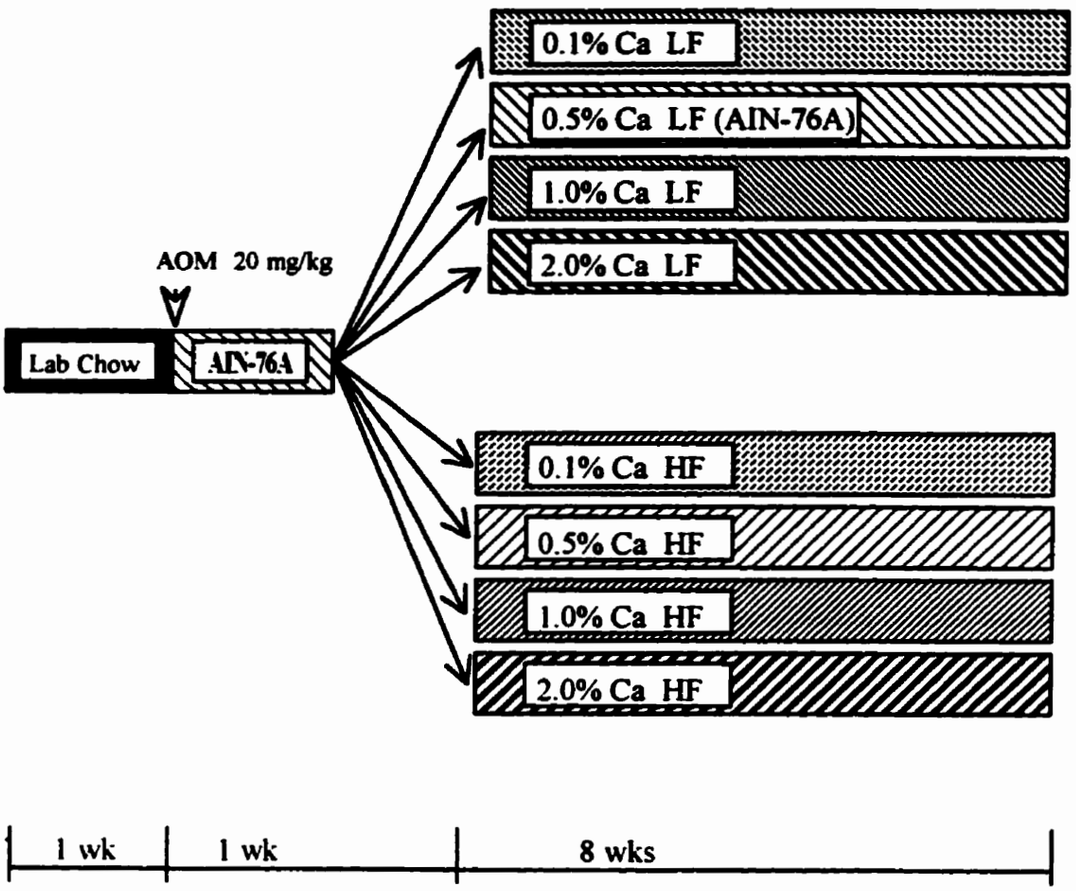
2. Material and Methods

Animals. Female Sprague Dawley rats approximately five to six weeks old were acclimatized for one week prior to initiating experimental procedures.

Experimental Diets. The formulated diets were based on the AIN-76A diet (American Institute of Nutrition, 1977; 1980) with the exception of $CaHPO_4$. For the high-fat diet, an additional 15% fat by weight was added as beef tallow at the expense of carbohydrate. The nutrients per kilocalorie density of the diets were identical except for fat and carbohydrate. Calcium (Ca) levels were altered by adding $CaCO_3$ to the base $CaHPO_4$ in the mineral mix to maintain the phosphate level in the diet at control levels. To achieve the low calcium diet (0.1% Ca), 0.4% $NaHPO_4$ was added to 0.1% $CaHPO_4$ to maintain the phosphate level in the diet mix. All diets are itemized in Appendix I.

Study Design. Animals were injected with a single dose of azoxymethane (20 mg/kg) and fed the AIN-76A control diet for one week. Animals were then randomized into eight dietary groups (8 rats/group) and fed the experimental diets for eight weeks (Figure 13). Two and one hour prior to termination by carbon dioxide asphyxiation animals received an i.p. injection of colchicine 1 mg/kg body weight or 5'-bromo-2'-deoxyuridine (BrdU) 30 mg/kg body weight. Colons were removed immediately after termination and flushed with phosphate buffered saline (PBS), slit open from cecum to anus, and fixed in 70% ethanol. Colons were assessed for aberrant crypt foci (ACF) and proliferative indices.

Figure 13: Schematic representation of the study design used in the study of varying levels of calcium on the induction and growth of aberrant crypt foci in rats treated with azoxymethane.


0.1% Ca LF
0.5% Ca LF (AIN-76A)
1.0% Ca LF
2.0% Ca LF
AOM 20 mg/kg
Lab Chow
AIN-76A
0.1% Ca HF
0.5% Ca HF
1.0% Ca HF
2.0% Ca HF
1 wk
1 wk
8 wks

Quantification of aberrant crypt foci and cell proliferation measurements. The number, distribution, and multiplicity of ACF were determined for the entire length of the colon as described previously. The number of mitotic figures and BrdU labeled cells as well as corresponding crypt height were determined as described previously.

Statistical Analysis. Two-way analysis of variance (ANOVA) was used to analyze the effect of dietary calcium and fat, which looks at the main effects of these nutrient in addition to the interactions between them on experimental variables, as carried out by the SAS statistical software for microcomputers. Duncan's Multiple Range test was used to separate treatment means when the ANOVA indicated significant differences. Differences were regarded as significant if $p<0.05$. Correlational analysis between the variables was performed using the Univariate Procedure as carried out by the SAS statistical software for microcomputers. Data which conformed to the normal distribution was analyzed using Pearson's Correlation Coefficient, Spearman's Correlation Coefficient was used to analyze data that did not conform to the normal distribution. Correlational plots were visualized to exclude the possibility of non linear relationships. A correlation was regarded as significant if $p<0.05$.

3. Results

Body weights. Animal body weights (Table 16) were similar initially and for the following four weeks. Analysis of main effects revealed that by week six and eight dietary fat exerted a significant effect on body weight.

Characteristics of ACF. The effect of altered dietary calcium and fat on the total number of ACF and various growth features is shown in Table 17. A dose response relationship between the level of calcium and ACF growth features was not found. Analysis of main effects revealed that the parameters significantly affected were crypt multiplicity (No. AC/Focus) and ACF with advanced growth features ($AC \geq 4$). It

Table 16: The Effect of Varying Dietary Calcium in the Presence or Absence of High Fat on Weight Gain of Sprague Dawley Rats Treated with Azoxymethane. [a]

	Wk 0	Wk 2	Wk 4	Wk 6	Wk 8
5% Low Fat					
0.1% Ca	127.1 ± 4.8	174.3 ± 5.9	206.5 ± 6.7	220.3 ± 3.8 [c]	237.5 ± 4.5 [c]
0.5% Ca	125.3 ± 3.2	176.8 ± 4.6	206.4 ± 6.3	231.6 ± 4.8	243.4 ± 5.9
1.0% Ca	120.6 ± 3.8	173.5 ± 6.4	208.8 ± 7.7	239.3 ± 8.7	247.6 ± 9.3
2.0% Ca	125.0 ± 6.5	170.9 ± 8.2	211.0 ± 11.9	236.3 ± 12.8	252.8 ± 13.4
20% High Fat					
0.1% Ca	128.0 ± 4.4	180.3 ± 5.1	214.4 ± 6.0	235.0 ± 7.1	246.3 ± 7.7
0.5% Ca	120.3 ± 4.1	179.3 ± 7.1	223.8 ± 8.9	254.2 ± 11.6 [b]	273.1 ± 13.9 [b]
1.0% Ca	188.5 ± 5.3	178.4 ± 5.2	217.1 ± 5.8	241.8 ± 7.6	265.1 ± 9.8
2.0% Ca	199.6 ± 3.5	176.6 ± 5.6	218.4 ± 7.8	246.5 ± 9.8	267.1 ± 11.6
Main Effects [d]					
Fat	p = 0.3728	p = 0.2612	p = 0.0707	p = 0.0483	p = 0.0175
Ca	p = 0.3750	p = 0.8944	p = 0.9332	p = 0.2848	p = 0.2574
Fat * Ca	p = 0.8945	p = 0.9947	p = 0.9071	p = 0.7135	p = 0.7660

[a] Values represent means (grams) ± S.E.

[b-c] : Means within a column with different superscripts differ at $p \leq 0.05$.

[d] Main Effects: Two Way Anova p values.

Table 17: The Effect of Varying Dietary Calcium in the Presence or Absence of High Fat and Fat Ratios on the Induction and Growth of Aberrant Crypt Foci with Various Growth Features in the Azoxymethane induced Rat Colon. [a]

	Total [b]	AC/Focus [c]	AC 1 [d]	AC 2 [d]	AC 3 [d]	AC ≥4 [d]
5% Low Fat						
0.1% Ca	161.1 ± 13.5	2.7 ± 0.1	26.1 ± 4.9	60.6 ± 4.4	39.3 ± 3.7	35.1 ± 2.8
0.5% Ca	170.7 ± 20.7	2.5 ± 0.1	30.0 ± 3.5	68.3 ± 7.9	43.7 ± 6.3	28.7 ± 6.5 [fg]
1.0% Ca	173.9 ± 14.1	2.7 ± 0.1 [e]	27.4 ± 3.7	62.8 ± 6.3	44.0 ± 4.0	39.8 ± 3.7 [ef]
2.0% Ca	170.9 ± 20.1	2.3 ± 0.1 [f]	38.4 ± 4.2	73.1 ± 6.4	37.6 ± 6.8	21.8 ± 6.6 [g]
20% High Fat						
0.1% Ca	192.6 ± 20.2	2.4 ± 0.1	42.3 ± 6.1	77.6 ± 10.0	40.3 ± 4.7	32.5 ± 6.0
0.5% Ca	180.3 ± 22.1	2.7 ± 0.1 [e]	29.6 ± 5.9	67.6 ● 10.4	43.4 ± 6.7	40.0 ± 5.4 [ef]
1.0% Ca	168.3 ± 22.6	2.4 ± 0.2	41.0 ± 7.8	63.8 ± 10.9	36.8 ± 6.6	26.8 ± 6.0 [fg]
2.0% Ca	204.4 ± 8.0	2.7 ± 0.1 [e]	34.1 ± 6.3	70.3 ± 4.2	52.8 ± 2.9	47.3 ± 6.6 [e]
Main Effects [h]						
Fat	p = 0.1850	p = 0.8353	p = 0.1135	p = 0.5112	p = 0.5596	p = 0.2038
Ca	p = 0.8189	p = 0.8636	p = 0.7089	p = 0.7416	p = 0.7000	p = 0.9958
Fat * Ca	p = 0.6532	p = 0.0022	p = 0.1756	p = 0.5643	p = 0.2060	p = 0.0062

[a] Values represent means ± S.E.

[b] Total: Number of ACF per colon.

[c] AC/focus: Number of aberrant crypts per focal lesion.

[d] AC 1...AC≥4: Number of foci with 1...≥4 aberrant crypts.

[e-g] : Means within a column with different superscripts differ at p≤0.05.

[h] Main Effects: Two Way Anova p values.

was observed that the relative effectiveness of dietary calcium on these parameters was dependent on the level of fat in the diet (Fat*Ca interaction), as seen in Figure 14. A similarity in the response of ACF growth was observed in animals fed the 0.1%Ca or 1.0%Ca as well as those fed the 0.5%Ca or 2.0%Ca diets. Interestingly, the different calcium levels appeared to have the opposite effect in the low versus the high fat environment. Among the low fat fed animals those receiving 0.5%Ca or 2.0%Ca yielded lower AC≥4 values than in the 0.1%Ca and 1.0%Ca groups. Conversely, when high fat diets were provided to the animals AC≥4 values were lower in 0.1%Ca and 1.0%Ca compared to 0.5%Ca and 2.0%Ca groups. These results reflected a significant interaction between the level of fat and calcium in the dietary milieu.

The effect of altered calcium and fat on the distribution of AC≥4 and multiplicity throughout the colon is shown in Table 18. Regional differences were noted with respect to distribution of ACF along the length of the colon. The mid region of the colon had a higher number of ACF than the rectal or cecal regions (results not shown). A similar distribution pattern was noted for AC≥4 regardless of the level of Ca. The number of AC≥4 was modulated similarly by the level of fat and calcium in both the rectal and cecal regions. Among the low fat fed animals those fed 2.0%Ca exhibited lower values in the rectal and cecal region compared to the other levels of calcium. The mid colonic region of high fat fed animals appeared to be more resistant to modulation by dietary calcium than those fed low fat diets.

Cell proliferation measurements. Mucosal epithelial proliferation was measured by identifying cells in metaphase arrest (Table 19) and cells in S-phase (Table 20). The main modulating factor in both rectal and mid region mitotic index (MI) was dietary calcium. The effect on MI was in the order of 0.1%Ca < 0.5%Ca > 1.0%Ca or 2.0%Ca in both regions. Overall, the metaphase cell associated proliferative zone was not significantly altered by dietary manipulation. A significant interaction was observed

Figure 14: Effect of a dose dependent increase in calcium in the presence or absence of high fat on the number of aberrant crypts with advanced growth features in the colons of Sprague Dawley rats treated with azoxymethane. Means with different superscripts differ at $p<0.05$.

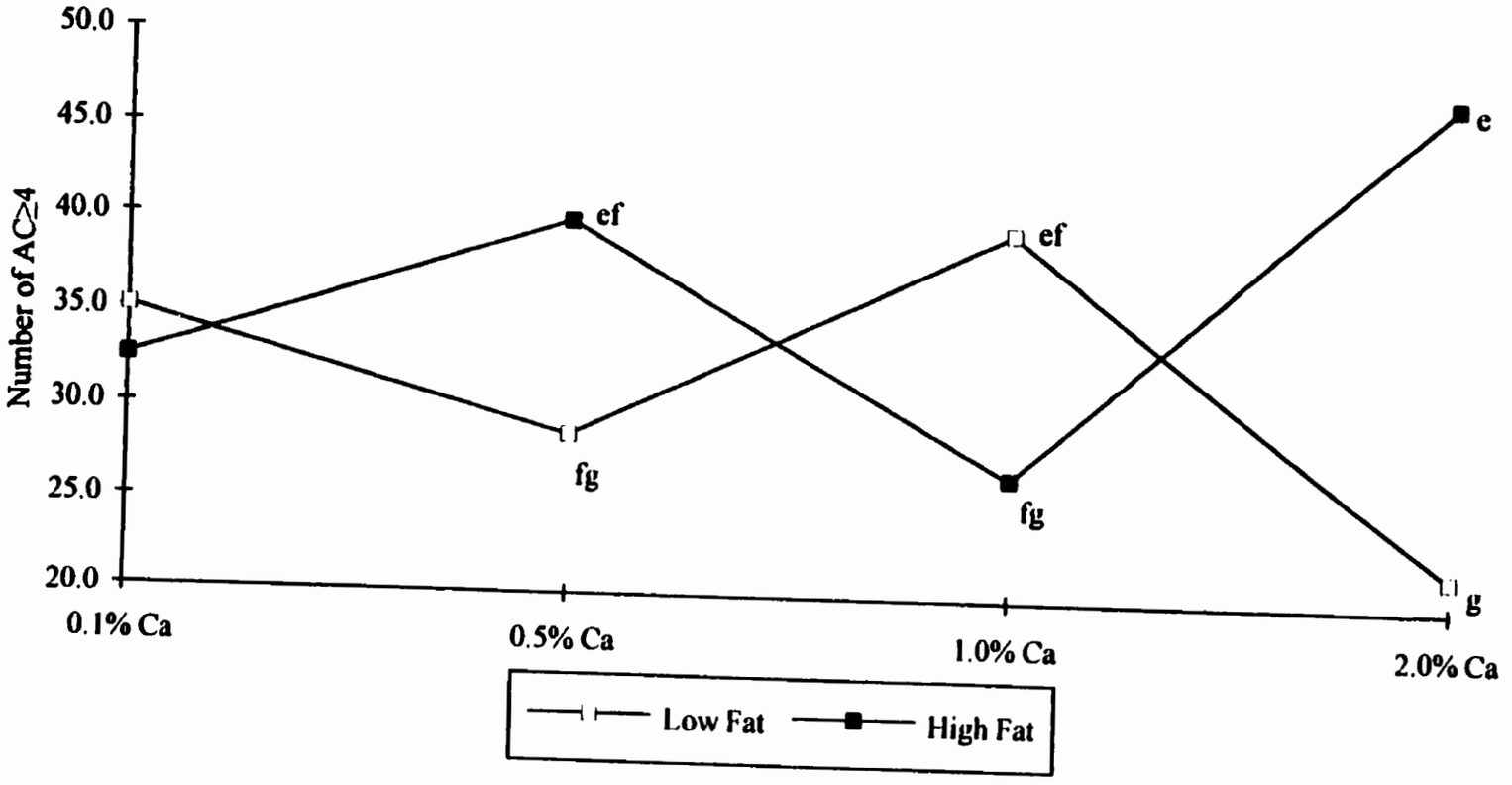

50.0
45.0
40.0
35.0
30.0
25.0
20.0
Number of AC≥4
ef
ef
e
fg
fg
g
0.1% Ca
0.5% Ca
1.0% Ca
2.0% Ca
Low Fat
High Fat

Table 18: The Effect of Varying Dietary Calcium in the Presence or Absence of High Fat on the Regional Appearance of Aberrant Crypt Foci and Foci with Advanced Growth Features in Rat Colon Treated with Azoxymethane. [a]

	Rectal End		Mid Region		Cecal End	
	AC≥4 [b]	AC/Focus [c]	AC≥4 [b]	AC/Focus [c]	AC≥4 [b]	AC/Focus [c]
5% Low Fat						
0.1% Ca	7.8 ± 1.6 [e]	2.6 ± 0.1	16.8 ± 3.1	2.8 ± 0.0 [de]	10.3 ± 1.8	2.6 ± 0.1
0.5% Ca	9.7 ± 2.7	2.6 ± 0.1	11.4 ± 3.5	2.7 ± 0.1	7.4 ± 2.4	2.3 ± 0.1
1.0% Ca	7.1 ± 1.1 [e]	2.7 ± 0.1	18.7 ± 1.8	2.9 ± 0.1 [d]	14.0 ± 2.9 [d]	2.7 ± 0.1 [d]
2.0% Ca	6.6 ± 1.8 [e]	2.3 ± 0.1	10.5 ± 3.8	2.3 ± 0.1 [f]	4.6 ± 1.6 [e]	2.2 ± 0.1 [e]
20% High Fat						
0.1% Ca	7.8 ± 2.0 [e]	2.5 ± 0.2	18.0 ± 4.2	2.5 ± 0.2	8.8 ± 2.6	2.3 ± 0.1
0.5% Ca	11.8 ± 2.2	2.8 ± 0.2	17.1 ± 2.9	2.9 ± 0.2 [d]	11.0 ± 2.6	2.5 ± 0.1
1.0% Ca	7.4 ± 1.9 [e]	2.3 ± 0.1	14.1 ± 3.6	2.4 ± 0.2 [ef]	5.2 ± 1.7 [e]	2.3 ± 0.2
2.0% Ca	14.7 ± 2.9 [d]	2.4 ± 0.4	18.6 ± 3.3	2.8 ± 0.1 [de]	13.8 ± 3.0 [d]	2.6 ± 0.2
Main Effects [g]						
Fat	p = 0.0773	p = 0.9269	p = 0.3809	p = 0.8939	p = 0.8989	p = 0.8343
Ca	p = 0.1921	p = 0.4359	p = 0.8572	p = 0.2416	p = 0.9964	p = 0.5804
Fat * Ca	p = 0.1750	p = 0.1184	p = 0.1788	p = 0.0043	p = 0.0027	p = 0.0143

[a] Values represent means ± S.E. See text for detailed description of segmental colonic divisions.

[b] AC ≥4: Number of foci with ≥4 aberrant crypts

[c] AC/focus: Number of aberrant crypts per focal lesion.

[d-f] : Means within a column with different superscripts differ at p≤0.05.

[g] Main Effects: Two Way Anova p values.

Table 19: The Effect of Varying Dietary Calcium in the Presence or Absence of High Fat on Mitotic Activity of Rat Colon Treated with Azoxymethane . [a]

	Rectal End		Mid Region	
	MI [b]	PZ [c]	MI [b]	PZ [c]
5% Low Fat				
0.1% Ca	2.6 ± 0.5 [e]	7.3 ± 0.8 [e]	4.5 ± 0.4	8.6 ± 0.8
0.5% Ca	5.4 ± 1.2	11.1 ± 1.1	5.6 ± 1.1 [de]	12.8 ± 0.6
1.0% Ca	4.0 ± 0.8 [e]	11.6 ± 1.1	2.9 ± 0.6 [f]	8.0 ± 2.3 [e]
2.0% Ca	3.8 ± 0.7 [e]	9.3 ± 1.4	4.2 ± 0.5	11.9 ± 0.5
20% High Fat				
0.1% Ca	3.6 ± 0.4 [e]	9.9 ± 1.0	4.4 ± 0.7	12.1 ± 0.8
0.5% Ca	7.4 ● 1.6 [d]	13.6 ± 0.3 [d]	6.0 ● 0.9 [d]	14.9 ● 1.5 [d]
1.0% Ca	4.7 ± 0.7	10.6 ● 1.8	3.4 ± 0.6 [ef]	11.7 ± 2.3
2.0% Ca	4.9 ± 1.4	10.2 ● 2.6	5.0 ● 0.6	14.0 ± 1.6 [d]
Main Effects [g]				
Fat	p = 0.0966	p = 0.2465	p = 0.4478	p = 0.0732
Ca	p = 0.0213	p = 0.0920	p = 0.0135	p = 0.0712
Fat • Ca	p = 0.9263	p = 0.5802	p = 0.9415	p = 0.6737

[a] Values represent means ± S.E. See text for detailed description of segmental

[b] colonic divisions.

MI: Mitotic index - the number of mitotic figures per crypt divided by twice the number of cells per mid axial crypt then multiplied by 100.

[c] PZ: Proliferative zone - the highest position of the mitotic cells /crypt /group.

[d-f] : Means within a column with different superscripts differ at $p \leq 0.05$.

[g] Main Effects: Two Way Anova p values.

Table 20: The Effect of Varying Dietary Calcium in the Presence or Absence of High Fat on BrdU Labeling in Rat Colon Treated with Azoxymethane. [a]

	Rectal End		Mid Region	
	LI [b]	PZ [c]	LI [b]	PZ [c]
5% Low Fat				
0.1% Ca	5.9 ± 0.9 [d]	8.6 ± 0.8 [d]	7.2 ● 1.1 [d]	12.4 ± 1.1 [d]
0.5% Ca	3.1 ± 0.6 [f]	6.3 ± 1.1 [d]	3.8 ± 0.5 [ef]	9.6 ± 1.0
1.0% Ca	3.3 ± 0.5 [f]	6.9 ± 1.1 [d]	3.8 ± 0.9 [ef]	10.5 ± 2.6
2.0% Ca	4.0 ± 0.2 [ef]	7.3 ± 1.1 [d]	2.7 ± 0.6 [f]	6.1 ± 1.3 [e]
20% High Fat				
0.1% Ca	5.5 ± 0.8 [de]	9.2 ± 1.0 [d]	6.3 ± 1.2 [de]	12.9 ± 1.8 [d]
0.5% Ca	3.7 ± 0.7 [ef]	8.7 ● 1.1 [d]	4.9 ± 0.9	10.2 ● 0.8
1.0% Ca	3.6 ± 0.5 [ef]	9.4 ● 1.4 [d]	3.1 ± 0.9 [f]	8.7 ± 2.1
2.0% Ca	1.0 ● 0.3 [g]	2.4 ± 0.7 [e]	2.6 ± 0.4 [f]	8.7 ± 0.6
Main Effects [h]				
Fat	p = 0.1728	p = 0.8514	p = 0.7968	p = 0.6642
Ca	p = 0.0002	p = 0.0049	p = 0.0004	p = 0.0244
Fat * Ca	p = 0.0294	p = 0.0053	p = 0.6762	p = 0.5684

[a] Values represent means ● S.E. See text for detailed description of segmental colonic divisions.

[b] LI: BrdU labeling index - the number of labeled cells per crypt divided by twice the number of cells per mid axial crypt then multiplied by 100.

[c] PZ: Proliferative zone - the highest position of the labeled cells /crypt /group.

[d-g] : Means within a column with different superscripts differ at $p \leq 0.05$.

[h] Main Effects: Two Way Anova p values.

Table 21: Correlational Analysis between the Various Biological Endpoints.

Biological Parameter	R Value	P Value
Full Colon Length		
AC≥4 [a] * LI [b] –M [c]	0.2025	0.8275
AC≥4 [a] * LI [b] –R [c]	-0.4299	0.2878
AC≥4 [a] * MI [d] –M [c]	0.1339	0.7107
AC≥4 [a] * MI [d] –R [c]	0.2485	0.5529
Mid Region [c]		
AC≥4 [a] * LI [b]	0.2835	0.4962
AC≥4 [a] MI [d]	-0.1628	0.7001
LI [b] * MI [d]	0.1183	0.7803
Rectal End [c]		
AC≥4 [a] * LI [b]	-0.3713	0.3652
AC≥4 [a] MI [d]	0.5629	0.1463
LI [b] * MI [d]	-0.4888	0.2193

[a] AC≥4: Number of foci with ≥4 aberrant crypts.

[b] LI: BrdU labeling index - the number of labelled cells per crypt divided by twice the number of cells per mid axial crypt then multiplied by 100.

[c] See text for detailed description of colonic segmental divisions:

[d] MI: Mitotic index - the number of mitotic figures per crypt divided by twice the number of cells per mid axial crypt then multiplied by 100.

between dietary calcium and fat with respect to the modulation of the rectal end BrdU labeling index (LI) and PZ. A dose related reduction in the BrdU LI in the rectal and mid regions was noted with increasing levels of calcium. However, in the mid colonic region dietary calcium was the critical modulator of cells engaged in S-phase as well as the size of the proliferative zone occupied by the S-phase cells.

Comparative analysis of biological endpoints. Correlation comparisons were performed between the various biological parameters examined and are shown in Table 21. A significant correlation was not found between ACF with advanced growth features and the colonic cell kinetic indices measured.

4. Discussion

The purpose of the present study was to systematically examine the potential chemopreventive effect of supplemental calcium and determine if this effect was evident in both a low and high fat diet or solely in the latter. It was observed that a significant nutrient interaction exists in the modulation of ACF which display advanced growth characteristics. The addition of 2.0% calcium in the presence of a high fat diet significantly promoted ACF with four or more crypts, however, in the presence of a low fat diet their growth was suppressed. The 2.0%Ca diet significantly reduced the labeling index in the presence of a high fat diet, however, this decrease was not demonstrated in the mitotic index. Although a consistent trend for the reduction of the mitotic index by 1.0%Ca compared to the 0.5%Ca groups in both low and high fat diets was observed, this decrease was not demonstrated for either labeling index or lesions with advanced growth characteristics. As a result no significant correlations were evident among the various parameters. The primary findings of this study suggest that a dose dependent increase in calcium does not provide a distinct chemopreventive effect in the rat model of colon carcinogenesis. Moreover, these findings suggest that the effects of supplemental

calcium are complex, related to other dietary factors, and have the potential for both benefit and harm.

The relationship between dietary calcium and fat is intricate and the response of various biological endpoints to these nutrients is dependent on their level in the diet. Wargovich et al. (1990) found that in the presence of a high fat the addition of calcium (0.32%Ca) lowered tumor incidence compared to the low calcium (0.04%Ca) group, however, in the presence of a low fat diet this response was reversed. In the study of Skrypec and Bursey (1988) the incidence of neoplastic lesions decreased as dietary calcium (0.25%, 0.5%, & 1.5%Ca) increased in the presence of a high fat diet. Pence and Buddingh (1988) reported that rats fed a high calcium (1.0%Ca) diet in the presence of a high fat had a lower tumor incidence, incidence of adenocarcinomas, total tumor frequency, and adenocarcinoma frequency compared to those fed the normal calcium (0.5%Ca) diet. Conversely, animals fed the low fat and high calcium diet (1.0%Ca) exhibited elevated values in all the aforementioned parameters as compared to the normal calcium (0.5%Ca) fed animals. This is consistent with the findings of the present study where the number of AC≥4 was lower in rats consuming the high fat 1.0%Ca diet but greater in those fed the low fat 1.0%Ca diet compared to their respective control (0.5%Ca) groups. Karkare et al. (1991) reported that tumor incidence was not statistically affected by the dose dependent increase in calcium in the presence of a low fat diet, however, the lowest tumor incidence was observed in the high calcium (2.0%Ca) fed animals. Similarly, in the present study the high calcium (2.0%Ca) low fat fed animals exhibited the lowest AC≥4.

Currently, the dogma that abnormal cell proliferation is an early biological alteration within the carcinogenesis process was investigated. Several researchers have reported decreased proliferative activity of colonic crypts in response to supplemental calcium (Arlow et al., 1989; Rozen et al., 1989; Wargovich et al., 1984). In the present

study the labeling index and the size of the proliferating compartment were consistently suppressed in animals consuming the high calcium (2.0%Ca) high fat diet. The mitotic index revealed decreased proliferation in animals fed the 1.0%Ca diet in both the rectal and mid region but this difference was lost in the mid region of animals fed the 2.0%Ca diet. This disparity of proliferative status agreement between the rectal and mid regions has been noted by other researchers (Karkare et al., 1991; Steinbach et al., 1993). In addition, it was observed that the proliferative status of the colonic epithelium as measured by the mitotic activity did not reflect the response observed for the BrdU LI. This indicates that cell production rate and S-phase cell number were differentially altered by the dietary protocol in the same colon. Another possibility is that the assessment of these parameters at one time point may not reflect overall proliferative status of the colonic mucosa. Robblee et al. (1989) demonstrated that an increase in LI, as determined by autoradiography was not always accompanied by a similar change in the mitotic activity. Furthermore, the authors felt that their findings cast doubt on the validity of either LI and/or MI as risk markers for colon tumorigenesis.

Steinbach et al. (1993) measured proliferative indices at early (10 and 21 wks) and late (34 wks) time points and found that no significant differences between proliferative indices were observed at the early time point and only at 34 weeks did differences become significant at which time tumors were also detected. Similarly, Thurnherr et al. (1973) found that early (6 wk) evaluation of proliferative status showed no significant increase even though 90% of colons developed tumors at 12 weeks. Karkare et al. (1991) found that the response of cell kinetic indices to a dose dependent increase in calcium did not correlate with tumor incidence in either the distal or proximal colon. Steinbach et al. (1993) reported that while animals fed a high fat diet exhibited significantly fewer labeled cells the eventual tumor outcome of this group was significantly greater than for animals receiving a low fat diet. This is similar to the

discrepancy in the present study where animals receiving the high calcium (2.0%Ca) high fat diet exhibited the lowers BrdU LI and yet harbored an elevated number of AC≥4 which have been positively correlated with tumor outcome (Magnuson et al., 1993; Pretlow et al., 1992; Zhang et al., 1992). These findings and those of others question the tenet that assessment of cell proliferative status provides an early biological endpoint in measuring the risk for colon tumorigenesis and suggest that further evaluation of the predictive ability of colonic proliferative status be assessed.

The results of this study demonstrate that modulation of the number and growth features of ACF depends on the level of calcium and fat in the diet and that the rectal regions of the colons differ from proximal in response to these dietary components. While the interaction between calcium and fat is complex with regard to the growth response ACF the results of the biomarker BrdU LI indicate a clear reduction in proliferative status with higher levels of calcium in both low and high fat diets. Although ACF have been correlated with tumor outcome the predictive value of cell proliferation biomarkers remains unclear and as a result a distinct chemopreventive effect of a high calcium diet in the rat model of colon carcinogenesis was not demonstrated. The value of this finding is that it questions the validity of the purported benefit of high dietary calcium. Much of the current belief in the chemopreventive nature of calcium is based on its ablity to decrease the proliferative status of the colonic epithelium. This has propelled funding of clinical human trials which evaluate rectal colonic proliferation as a measure of effectiveness. Clearly more investigation is required to understand the biological impact of high dietary calcium in colon carcinogenesis. As parameters were quantified at an early time point and it would be valuable to further evaluate intervention with dietary calcium during the later stages of the disease process.

Chapter 7.

THE EFFECT OF DIETARY CALCIUM ON PROTEIN KINASE C ACTIVITY IN THE COLONIC MUCOSA OF SPRAGUE DAWLEY RATS FED LOW OR HIGH FAT

1. Introduction

Several studies have suggested that diets high in total fats are positively correlated with increased mortality from colon cancer (Carrol & Khor, 1975; Carroll, 1991; Wynder et al., 1983). It has been shown that calcium can ameliorate colonic hyperproliferation in the rodent model (Bird et al., 1986; Wargovich et al., 1984). Possible mechanisms that have been put forth include the "calcium-soap hypothesis" (Newmark et al., 1984) and/or the induction of terminal differentiation (Hennings et al., 1980; McGrath & Soule, 1984; Whitfield, 1992). It has been suggested that a disorder of signal transduction pathways may be an integral step in the multistage development of colon cancer (Weinstein, 1990). Protein kinase C (PKC), a signal transduction component, has been identified as a cellular receptor for the phorbol ester tumor promoters and activity of this enzyme is increased by these substances (Blumberg, 1988). PKC associated with the inner surface of the plasma membrane, activated by diacylglycerol and calcium, is believed to be the active enzyme fraction (O'Brian & Ward, 1989).

To date the role of dietary calcium with respect to the activity of this enzyme has not been investigated in the whole animal model. Additionally, although fat has been implicated as a promoter of colon cancer little is actually known about the exact mechanism by which it exerts its effect. Therefore the first step in understanding the pathobiological significance of this enzyme is to examine the effect in the normal colonic epithelium. In the present study, the hypothesis that supplemental calcium and fat may alter signal transduction pathways was tested by examining the modulating effect of

varying levels of dietary calcium in both a low and high fat diet on the colonic epithelium PKC activity and the corresponding relationship to proliferative indices.

2. Material and Methods

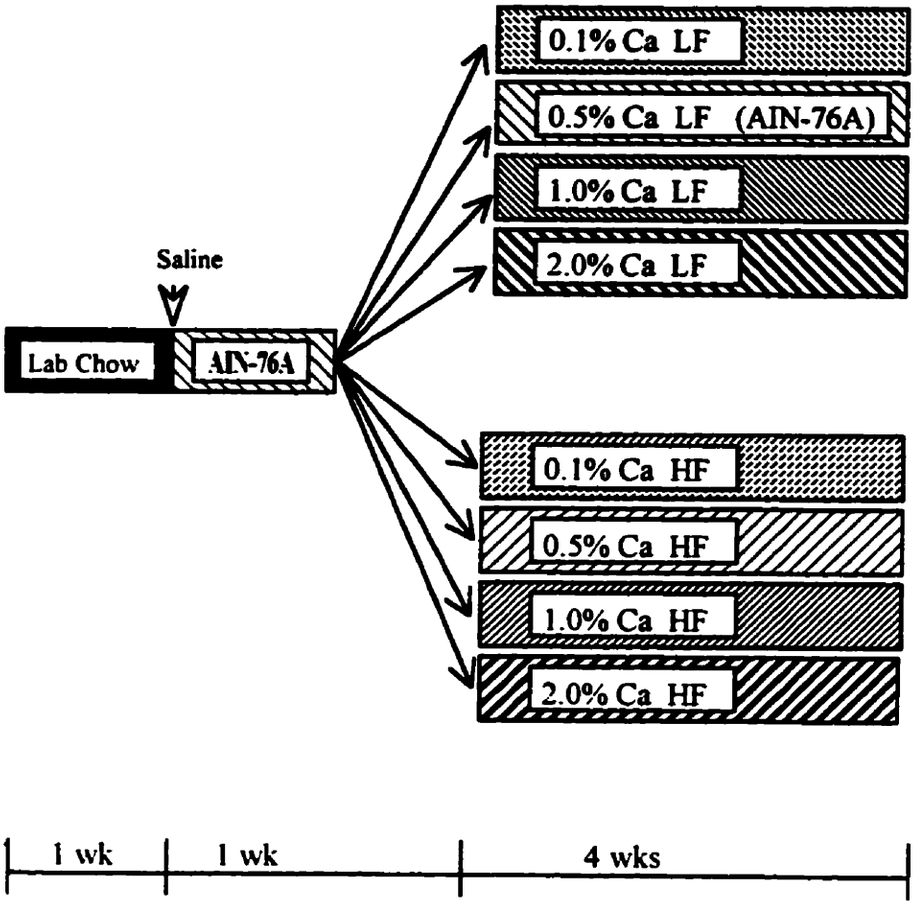
Animals. Female Sprague Dawley rats approximately five to six weeks old were acclimatized for one week prior to initiating experimental procedures.

Experimental Diets. The formulated diets were based on the AIN-76A diet (American Institute of Nutrition, 1977; 1980) with the exception of $CaHPO_4$. For the high-fat diet, an additional 15% fat by weight was added as beef tallow at the expense of carbohydrate. The nutrients per kilocalorie density of the diets were identical except for fat and carbohydrate. Calcium (Ca) levels were altered by adding $CaCO_3$ to the base $CaHPO_4$ in the mineral mix to maintain the phosphate level in the diet at control levels. To achieve the low calcium diet (0.1% Ca), 0.4% $NaHPO_4$ was added to 0.1% $CaHPO_4$ to maintain the phosphate level in the diet mix. All diets are itemized in Appendix I.

Study Design. Animals were injected with a single dose of saline and fed the AIN-76A control diet for one week. Animals were then randomized into eight dietary groups (5 rats/group) and fed the experimental diets for four weeks (Figure 15) and terminated by carbon dioxide asphyxiation. Two 0.5 cm segments were removed 2 and 5 cm from the rectal end of the colon and assessed for proliferative indices. The remaining tissue was scraped and PKC activity assessed.

Extraction, separation and assay of PKC from rat colonic tissue. Rat colonic PKC activity was measured according to the PKC assay previously described (Craven & DeRubertis, 1992b) with some modifications (Magnuson, 1993). Although this method does not exclude the Ca^{2+} -specific activity, a substantial portion of methods in published PKC studies are similar to this procedure (Ashendel et al., 1985; Craven & DeRubertis, 1987; Hashimoto et al., 1989; Kopp et al., 1991; Wali et al., 1991; Craven & DeRubertis,

Figure 15: **Schematic representation of the study design used in the study of varying levels of calcium on cellular signaling and proliferative status in the colonic epithelium.**


0.1% Ca LF
0.5% Ca LF (AIN-76A)
1.0% Ca LF
2.0% Ca LF
Saline
Lab Chow
AIN-76A
0.1% Ca HF
0.5% Ca HF
1.0% Ca HF
2.0% Ca HF
1 wk
1 wk
4 wks

1992b; Pence et al., 1995). As this was the first time that the effect of dietary calcium on PKC activity was to be investigated it was prudent to use a well established method. Furthermore, all tissues were analyzed with the same method and thus the results are valid relative to the control group. Briefly, colons were removed, flushed with cold Krebs Ringer solution, slit longitudinally and placed on a 0°C cooled surface to collect colonic mucosal scrapings. The tissue was homogenized in 25 mM Tris buffer (pH 7.5) containing 0.25 mM phenylmethylsulfonyl fluoride (PMSF), 15 mM ethyleneglycol-*bis* (ß-aminoethyl ether)-N,N,N',N'-tetraacetic acid, 0.25 M sucrose and 10 µg/mL trypsin inhibitor. The homogenized mixture was centrifuged at 100,000 x g for 60 min. Soluble PKC was partially purified from the obtained supernatant by separation on a pre-equilibrated DEAE-sephacel column. The column was washed with two bed volumes of Buffer A (lacking Triton-X 100) and eluted with a 25 mM Tris Buffer B containing 0.25 mM PMSF, 15 mM mercaptoethanol and 0.15 mM NaCl. The pellet was resuspended and homogenized in Buffer A with 0.5% Triton-X 100, centrifuged at 100,000 x g for 60 min to solubilize the particulate associated PKC, and then partially purified as described for the soluble fraction. Both soluble and particulate PKC were eluted with 0.15 M NaCl. All column procedures were carried out at 4°C unless otherwise noted.

Activity was determined by comparing the transfer of $^{32}PO_4^{3-}$ from γ-[^{32}P] adenosine triphosphate (ATP) to a histone protein in both the presence and absence of phosphatidylserine (PS) and diacylgycerol (DAG). All reaction mixtures contained 25 mM Tris buffer with 10 mM $MgCl_2$, 740 µg/mL histone, 2 mM $CaCl_2$, with (PS-DAG calcium-dependent) or without (non-PS-DAG-dependent) 80 µg/mL PS and 8 µg/mL DAG. A 75-µL aliquot of the reaction mixture was added to 25 µL of column eluate; the reaction was then initiated by addition of 10 µL of γ-[^{32}P] ATP (0.45 µCi/10 µL) and allowed to incubate at 37°C for 5 min. Reactions were then quenched in an ice bath, and then 50µL of the mixture was spotted onto small squares of P81 phosphocellulose paper.

The paper was immersed in 75 mM phosphoric acid, washed with dH_2O and the dried squares were counted for radioactivity on a Beckmann LS6000TA Liquid Scintillation Counter. Protein content was assayed using the Pierce Coomassie protein assay reagent and BSA was used as standard. All assays were duplicated.

Proliferation measurements. The number of proliferating cell nuclear antigen (PCNA) labeled cells as well as corresponding crypt height were determined as described previously.

Statistical Analysis. Two-way analysis of variance (ANOVA) was used to analyze the effect of dietary calcium and fat, which looks at the main effects of these nutrient in addition to the interactions between them on experimental variables, as carried out by the SAS statistical software for microcomputers. Duncan's Multiple Range test was used to separate treatment means when the ANOVA indicated significant differences. Differences were regarded as significant if $p<0.05$. Correlational analysis between the variables was performed using the Univariate Procedure as carried out by the SAS statistical software for microcomputers. Data which conformed to the normal distribution was analyzed using Pearson's Correlation Coefficient, Spearman's Correlation Coefficient was used to analyze data that did not conform to the normal distribution. Correlational plots were visualized to exclude the possibility of non linear relationships. A correlation was regarded as significant if $p<0.05$.

3. Results

Body weights. Animals body weights (Table 22) were similar initially and for the following two weeks. Analysis of the main effects revealed that by week three and four a significant fat and calcium interaction was modulating weight gain. Weight gains for the different levels of calcium were similar except for the 2.0%Ca fed animals where in the presence of high fat animals gained the most weight. Similarly, other researchers have

Table 22: The Effect of Varying Dietary Calcium in the Presence or Absence of High Fat on Weight Gain of Sprague Dawley Rat.[a]

	Wk 0	Wk 1	Wk 2	Wk 3	Wk 4
5% Low Fat					
0.1% Ca	142.8 ± 3.7	165.0 ± 3.9 [b]	189.6 ± 4.7	202.0 ± 4.9	221.3 ± 9.3
0.5% Ca	134.1 ± 2.6	147.8 ± 4.2 [c]	179.6 ± 4.4	195.8 ± 5.0 [c]	215.2 ± 5.4 [c]
1.0% Ca	146.8 ± 3.0	151.2 ± 6.5	178.2 ± 8.1	211.8 ± 11.9	235.3 ± 16.7
2.0% Ca	133.8 ± 5.4	153.3 ± 5.0	180.2 ± 6.0	199.0 ± 6.9 [c]	217.0 ± 12.3 [c]
20% High Fat					
0.1% Ca	131.5 ± 6.3	153.5 ± 6.8	178.0 ± 8.9	190.8 ± 9.1 [c]	209.0 ± 6.0 [c]
0.5% Ca	132.8 ± 4.2	154.1 ± 5.9	183.2 ± 6.3	202.3 ± 7.4	216.7 ± 6.4 [c]
1.0% Ca	141.0 ± 3.6	164.5 ± 4.8	192.8 ± 4.9	211.3 ± 4.0	234.7 ± 8.4
2.0% Ca	141.0 ± 5.4	167.3 ± 7.2	200.3 ± 10.4	224.5 ± 10.4 [b]	254.0 ± 19.2 [b]
Main Effects [d]					
Fat	p = 0.2488	p = 0.7725	p = 0.7450	p = 0.8992	p = 0.3411
Ca	p = 0.0645	p = 0.0497	p = 0.0632	p = 0.0123	p = 0.1129
Fat • Ca	p = 0.2001	p = 0.0602	p = 0.0625	p = 0.0408	p = 0.0443

[a] Values represent means (grams) ± S.E.

[b-c] : Means within a column with different superscripts differ at $p \leq 0.05$.

[d] Main Effects: Two Way Anova p values.

found that after four weeks of feeding 1.5%Ca in the presence of a high fat diet, body weights were significantly greater than control (0.5%) calcium fed animals (Awad et al., 1990).

Protein kinase C activity. It was observed that the relative effectiveness of dietary calcium on PKC activity was dependent on the level of fat in the diet (Table 23). A significant interaction was apparent between dietary calcium and fat with respect to the modulation of membranous PKC activity (Figure 16). Activity was greater in 0.1% and 0.5% calcium groups fed the high-fat diets relative to the low-fat groups, however, this difference was significantly larger when calcium reached 2.0% dietary intake. This pattern was reversed at 1.0% dietary calcium. Animals fed the 0.1% and 2.0% Ca diets in combination with high fat as well as 1.0% Ca low fat fed animals exhibited significantly greater PKC activity relative to the control group (0.5% Ca low fat). A significant interaction between the dietary nutrients and activity was noted for cytosolic PKC activity. In contrast to membrane PKC activity, cytosolic activity was greater in the colonic mucosa of animals fed the 0.1%, 0.5% and 1.0% Ca low fat diets relative to high fat counterparts. Animals fed the 2.0% Ca diet demonstrated a marked response reversal as activity jumped in the high fat group. Animals fed the 0.5% and 1.0% Ca diets in combination with high fat as well as 2.0% Ca low fat fed animals exhibited significantly lower PKC activity relative to the control group (0.5% Ca low fat). Total PKC activity was not significantly altered by dietary changes.

Cell proliferation measurements. Proliferation was measured by identifying cells expressing the PCNA antigen and results are shown in Table 24. The main modulating factor in the mid region LI was dietary calcium. The reduction of mid region LI in a dose dependent manner demonstrated the most consistent pattern of modulation by dietary calcium observed in the proliferation indices measured. The dose dependent decrease in LI was independent of the level of dietary fat. The rectal end LI was significantly

Table 23 : The Effect of Varying Dietary Calcium in the Presence or Absence of High Fat on Both the Particulate and Soluble Colonic Protein Kinase C Fractions . [a]

	Particulate Fraction	Soluble Fraction
5% Low Fat		
0.1% Ca	1.77 ± 0.19 [de]	6.96 ● 1.55 [b]
0.5% Ca	0.68 ± 0.11 [e]	3.97 ● 1.42 [b]
1.0% Ca	3.14 ± 0.46 [c]	5.70 ● 1.01 [b]
2.0% Ca	1.11 ± 0.19 [de]	0.02 ● 0.02 [c]
20% High Fat		
0.1% Ca	2.35 ± 0.32 [cd]	5.40 ± 1.40 [b]
0.5% Ca	1.68 ± 0.35 [de]	0.07 ± 0.05 [c]
1.0% Ca	1.05 ± 0.32 [de]	0.04 ± 0.04 [c]
2.0% Ca	5.28 ± 0.88 [b]	6.94 ± 2.12 [b]
Main Effects [f]		
Fat	p = 0.0041	p = 0.2327
Ca	p = 0.0005	p = 0.0110
Fat • Ca	p = 0.0001	p = 0.0001

[a] Values represent means ± S.E.

[b-e] : Means within a column with different superscripts differ at $p \leq 0.05$.

[f] Main Effects: Two Way Anova p values.

Figure 16: Modulation of Sprague Dawley rat colonic membranous PKC activity by varying levels of dietary calcium in the presence or absence of high fat. Values represent the mean, different superscripts differ $p<0.05$.

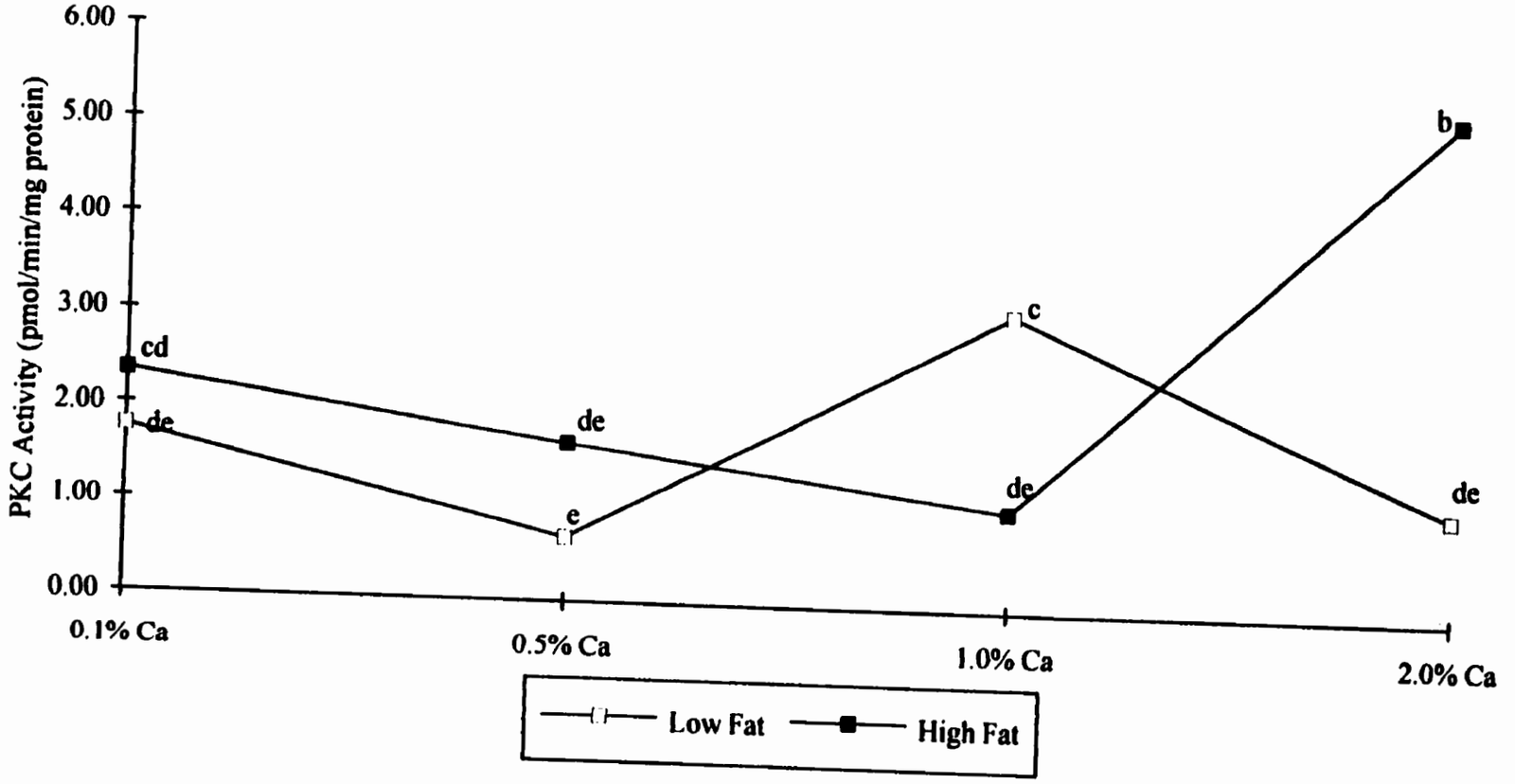

6.00
5.00
4.00
3.00
2.00
1.00
0.00
PKC Activity (pmol/min/mg protein)
cd
de
de
e
c
de
b
de
0.1% Ca
0.5% Ca
1.0% Ca
2.0% Ca
Low Fat
High Fat

Table 24 : The Effect of Varying Dietary Calcium in the Presence or Absence of High Fat on PCNA Labeling. [a]

	Rectal End		Mid Region	
	LI [b]	PZ [c]	LI [b]	PZ [c]
5% Low Fat				
0.1% Ca	14.9 ± 1.6 [f]	16.8 ± 1.1 [de]	21.3 ± 1.0 [de]	18.2 ± 0.2 [de]
0.5% Ca	23.4 ± 1.1 [e]	14.6 ± 0.9 [ef]	20.4 ± 1.7 [def]	16.5 ± 0.8 [ef]
1.0% Ca	18.2 ± 1.8 [f]	18.9 ± 1.4 [d]	17.8 ± 1.2 [ef]	20.3 ± 1.6 [d]
2.0% Ca	17.0 ± 1.8 [f]	16.5 ± 1.4 [de]	12.1 ± 1.4 [g]	19.2 ± 1.3 [de]
20% High Fat				
0.1% Ca	29.4 ± 3.0 [d]	16.0 ± 1.1 [de]	22.6 ± 1.9 [d]	19.6 ± 0.3 [de]
0.5% Ca	18.8 ± 1.4 [ef]	15.7 ± 1.3 [de]	19.3 ± 0.8 [def]	19.0 ± 0.8 [de]
1.0% Ca	23.6 ± 1.1 [e]	18.1 ± 1.0 [de]	16.6 ± 1.2 [f]	17.8 ± 1.6 [de]
2.0% Ca	5.3 ± 1.0 [g]	11.2 ± 1.2 [f]	5.8 ± 0.3 [h]	13.7 ± 1.1 [f]
Main Effects [i]				
Fat	p = 0.8489	p = 0.2097	p = 0.0819	p = 0.5932
Ca	p = 0.0001	p = 0.0101	p = 0.0001	p = 0.0686
Fat * Ca	p = 0.0001	p = 0.0767	p = 0.0977	p = 0.0013

[a] Values represent means ± S.E. See text for detailed description of segmental colonic divisions.

[b] LI: PCNA labeling index - the number of labelled cells per crypt divided by twice the number of cells per mid axial crypt then multiplied by 100.

[c] PZ: Proliferative zone - the highest position of the labeled cells per crypt per group.

[d-h] : Means within a column with different superscripts differ at $p \leq 0.05$.

[i] Main Effects: Two Way Anova p values.

modulated by interaction between calcium and fat, however no consistent pattern was evident. The low calcium (0.1% Ca) diet exhibited the clearest interaction where the LI was significantly lower in animals fed low fat diet but significantly greater in animals fed the high fat diet relative to the control (0.5% Ca) group. The LI was lowest in animals fed the high fat diet receiving the highest level of calcium (2.0% Ca) in both the rectal and mid colonic epithelium. A significant interaction was observed between dietary calcium and fat with respect to the modulation of the mid region PZ. High fat, compared to low fat, fed animals consuming the high calcium (1.0% & 2.0%) diets exhibited a lower PZ, however, in the presence of low and control (0.1% & 0.5%) calcium diets the PZ was slightly greater. The main modulating factor in the rectal end PZ was dietary calcium, however no consistent pattern was evident. The PZ was also lowest in animals fed the high fat diet receiving the highest level of calcium (2.0% Ca) in both the rectal and mid colonic epithelium.

Comparative analysis of biological endpoints. PCNA LI in the rectal region was negatively correlated with membranous ($R= -0.5181$ $p= 0.0006$) PKC activity indicating that these biological endpoints shared 27% of their variation in common. There was a lack of significant correlation between the PCNA LI in the rectal region and cytosolic ($R= -0.2973$ $p= 0.0625$) associated PKC activity. The PCNA LI in the mid region was not related to either the membranous ($R= -0.2569$ $p= 0.0830$) or cytosolic ($R= 0.0281$ $p= 0.8640$) associated PKC activity.

4. Discussion

The purpose of the present study was to determine whether alterations in dietary calcium and/or fat could modulate colonic protein kinase C (PKC) activity and whether these changes were linked to changes in mucosal cell proliferation. It was observed that a significant nutrient interaction exists in the modulation of PKC activity. The addition of

2.0% calcium in the presence of a high-fat diet resulted in significantly elevated membranous PKC activity, however, in the presence of a low-fat diet membranous PKC activity was significantly elevated in 1.0%Ca and not 2.0%Ca fed animals. Proliferating cell nuclear antigen (PCNA) labeling index (LI) and proliferative zone (PZ) were consistently lowest in animals fed the 2.0%Ca high-fat diet in both the rectal and mid regions of the colon. Although a dose dependent decrease in PCNA LI was observed in the mid region of the colon, the rectal end LI exhibited a significant nutrient interaction response. As a result only rectal PCNA LI correlated significantly with membranous PKC activity. The primary findings of this study suggest that a dose dependent increase in dietary calcium produces complex effects on PKC activity and these effects do not always produce similar effects in cellular proliferation measurements.

A substantial body of evidence supports a role for PKC in colon carcinogenesis (Ashendel, 1985; Baum et al., 1990; Craven & DeRubertis, 1988; Craven et al., 1987; Fitzer et al., 1987; Nishizuka, 1986). It has been suggested that PKC serves as a convenient intermediate end point for evaluating intervention agents (Pence, 1993; Weinstein, 1991). The enzyme is known to exist in various isoforms which exhibit differences in co-factor dependence and activator specificity (Kikkawa et al., 1989; Murray et al., 1993). The ability of PKC to remain associated with the membrane as the active form may depend on the lipid composition of that membrane. In the current investigation membrane PKC activity was higher in the colonic mucosa of high fat fed animals. The lower amount of cytosolic PKC activity suggests enhanced translocation of the enzyme to the membrane subfraction. This observation is compatible with the findings of others who have reported that tumor promoters appear to increase the translocation of cytosolic PKC to the membrane fraction (Castagna et al., 1982). Consistent with our findings, activation of PKC has been noted in mucosa exposed to free fatty acids (Craven and DeRubertis, 1988) and bile acids (Craven et al., 1987; Fitzer et al.,

1987), both of which are thought to be elevated in the colons of high fat fed animals. Furthermore, in a study investigating the role of dietary fat and PKC in skin carcinogenesis it was reported that the activity in the membrane fraction increased twofold, and the soluble PKC activity decreased dramatically in animals consuming the high fat diet (Donnelly et al., 1987). Baum et al. (1990) noted a significant increase in membranous PKC activity in DMH treated rat colonic mucosa at 10 weeks followed by a loss of activity by 15 weeks until eventual appearance of tumors, suggesting perhaps that an early increase in PKC activity predicts eventual loss of enzyme activity and ultimate tumor promotion. However, studies have yielded somewhat conflicting results as down regulation of PKC activity has been observed in chemically induced rat colonic cancer (Craven & DeRubertis, 1992a; Wali et al., 1991) and human colonic carcinomas (Guillem et al., 1987; Kopp et al., 1991), while others have found no differences (Hashimoto et al., 1989; McGarrity & Peiffer, 1994).

Much of the evidence that supports a role for PKC in colon carcinogenesis involves the actions of bile acids (O'Brian and Ward, 1989). A great deal of research indicates that calcium has an ameliorating effect on the immediate bile acid induced toxicity in the colonic epithelium (Baer & Wargovich, 1989; Bird et al., 1986; Wargovich et al., 1983). In the current investigation, PKC activity was affected by a complex interaction between dietary calcium and fat which was most pronounced at the high (1.0% & 2.0%) calcium levels. In the presence of a high fat diet calcium levels of 0.1% to 1.0% produced similar results on membranous PKC activity, which is currently thought to represent the active form of the enzyme. However, the addition of 2.0%Ca significantly elevated activity in the normal rat colonic epithelium. Consistent with these results, Fitzer et al. (1987) reported that the effects of bile acids on PKC activity were influenced by the concentrations of calcium and lipid in the medium. In the presence of bile acids PKC activity was reduced by 10% when the medium contained 1 mM Ca^{2+}, however, a

twofold increase was observed when the calcium concentration was doubled. In contrast, Pence et al. (1995) observed that rats fed a 2.0%Ca cholic acid diet exhibited significantly lower membranous PKC activity at 15 weeks post carcinogen treatment compared to those receiving a 0.5%Ca cholic acid diet. The differences between these and the current findings might be attributed to the high level of cholic acid (0.5%) used by Pence et al. (1995) which may elicit different biological effects than bile acids produced as a consequence of a high fat diet. In addition, Pence et al. (1995) exposed animals to a colon specific carcinogen whereas the current findings were observed in saline treated rat colonic mucosa. The functional significance of altered PKC activity is still speculative. Whitfield (1992) suggested that surges in membranous PKC activity signal stimulation of secretions, such as autocrine mitogens, which could in turn drive the proliferation of premalignant cells providing a selective growth advantage. Additionally, this surge could result in a swell of oxygen radicals, as a result of increased plasma membrane Nad(P)H oxidase activity stimulated by PKC, causing chromosomal damage associated with the transition from a benign to malignant state (Whitfield, 1992).

Dietary calcium has been purported to be a cellular differentiation agent (Hennings et al., 1980; McGrath and Soule, 1984; Whitfield, 1992). Several researchers have reported that increased dietary calcium suppresses hyperproliferation in colonic epithelial cells (Arlow et al., 1989; Bird et al., 1986; Reshef et al., 1990; Wargovich et al., 1983). These findings are consistent with the current observation that mid region cellular proliferation decreased in a dose dependent manner with increased dietary calcium and that the high (2.0%) calcium diet demonstrated the lowest LI in both the mid and rectal colonic regions. PKC has been recognized as having an important role in the regulation of cell growth and differentiation (Nishizuka, 1992; Olson et al., 1993; Whitfield, 1992). Craven et al. (1987) observed that the hyperproliferation induced by bile acids and tumor promoting phorbol esters involved the activation of PKC. Craven and DeRubertis (1987)

reported that a PKC inhibitor suppressed bile acid and phorbol ester enhanced proliferation suggesting that the enzyme was a positive intracellular signal for colonic epithelial cell growth. Many factors may affect PKC activity either directly or indirectly and although it has been suggested that colonic epithelial proliferation may be mediated by PKC the results of the current investigation indicate that PKC activity is independent of proliferative indices. The impact of altered dietary calcium and fat on the mid colon was a consistent decrease whereas the effect of PKC activity was variable. Correlational analysis revealed that only membrane PKC activity and rectal end LI displayed a negative but significant relationship. Furthermore, this analysis revealed that the similarity between the two variables was limited to 27%. Consistent with these findings are those of Craven and DeRubertis (1992b) which indicated that increased proliferation of the colonic epithelium was not mediated by PKC. The hyperproliferation induced by refeeding fasted rats was not reduced by PKC inhibitors. In view of the fact that an increase in proliferative indices and PKC activity have been purported to be risk markers of colon carcinogenesis (Pence, 1993; Weinstein, 1991) the lack of correlation between the variables is disconcerting. The validation of these purported risk markers must be considered paramount considering their wide inclusion in research investigations.

In the present investigation, PKC activity in the cytosolic and membrane fractions was measured using a method similar to those employed by others (Craven and DeRubertis, 1987; Craven and DeRubertis, 1992a; Craven and DeRubertis, 1992b; Craven et al., 1987; Donnelly et al., 1987; Fitzer et al., 1987; McGrath and Soule, 1984). Considerable advances have been made in the biochemical and molecular approaches to study various PKC isoforms and their role in cellular processes. The current observation that PKC activity was modulated in the normal mucosa by dietary fat and calcium in addition to the findings that the level of various PKC isoforms may differ in normal versus neoplastic colonic tissue (Craven and DeRubertis, 1992a; DeRubertis & Craven,

1987) lends support to the notion that PKC plays an important role in colon carcinogenesis. The complex nature of the nutrient interaction between dietary calcium and fat on PKC activity and its isoforms in normal and neoplastic tissue should be explored in future research.

The results of this study demonstrate that the nutrient interaction between calcium and fat is complex with regards to the modulation of PKC activity. A distinct reduction in mid colonic proliferation was demonstrated but poor correlation of the various end points suggests that further validation of these potential intermediate end points is required. To author's knowledge this is the first time that a gradient increase in dietary calcium has been shown to modulate rat colonic mucosal PKC activity and that the level of dietary fat is critical to its effect. The exact mechanisms by which varying levels of calcium and fat modulate the activity of PKC are unknown and requires further investigation.

Chapter 8.

RESPONSE OF SPRAGUE DAWLEY RAT COLONIC MEMBRANE LIPID COMPOSITION TO ALTERED DIETARY CALCIUM IN THE PRESENCE OR ABSENCE OF HIGH FAT

1. Introduction

Several studies have demonstrated that rodent diets containing 20% or more fat by weight from sources such as corn oil, safflower oil, beef fat, lard, or any combination thereof, promote chemically induced colon carcinogenesis (Carroll, 1991; Rao et al., 1993; Reddy, 1986; Reddy & Maeura, 1984). However, this effect is not seen when dietary fat is derived from fish oil or coconut oil (Rao et al., 1993; Reddy & Maruyama, 1986). Although the effect of fat has been well documented, the exact mechanism by which the high fat (HF) diet exerts this promotional effect on colon carcinogenesis remains poorly understood. The level and type of dietary fat has been shown to modify membrane fatty acid composition (Rao et al., 1993; Reddy and Maruyama, 1986). Changes in various cellular functions have been attributed to altered membrane fatty acid and phospholipid composition (Croft et al., 1985; Donnelly et al., 1987; Lee et al., 1988). It was previously (Chapter 7) demonstrated that varying levels of calcium in the diet had a profound modulating effect on the PKC activity. This observation suggested that dietary caclium was affecting events at the membrane level. Calcium has been implicated in phospholipid metabolism. The main objective of this study was to determine if different levels of dietary calcium altered colonic lipid composition. The hypothesis that calcium modifies membrane lipid composition was tested by evaluating colonic phospholipid fatty acid profiles in rats fed increasing dietary calcium.

2. Material and Methods

Animals. Female Sprague Dawley rats approximately five to six weeks old were acclimatized for one week prior to initiating experimental procedures.

Experimental Diets. The formulated diets were based on the AIN-76A diet (American Institute of Nutrition, 1977; 1980) with the exception of $CaHPO_4$. For the high-fat diet, an additional 15% fat by weight was added as beef tallow at the expense of carbohydrate. The nutrients per kilocalorie density of the diets were identical except for fat and carbohydrate. Calcium (Ca) levels were altered by adding $CaCO_3$ to the base $CaHPO_4$ in the mineral mix to maintain the phosphate level in the diet at control levels. To achieve the low calcium diet (0.1% Ca), 0.4% $NaHPO_4$ was added to 0.1% $CaHPO_4$ to maintain the phosphate level in the diet mix. All diets are itemized in Appendix I.

Study Design. All animals underwent 1 week of acclimatization and fed the AIN-76A control diet. After one week animals were randomized into eight dietary groups (5 rats/group) and fed the experimental diets for four weeks (Figure 16) and terminated by carbon dioxide asphyxiation. Colons were removed immediately and flushed with phosphate buffered saline (PBS), slit open from cecum to anus, mucosa scraped and frozen immediately at -80^{o}C until lipids were extracted.

Analysis of colonic phospholipid fatty acids. Lipids were immediately extracted as described by Folch et al. (1957) using chloroform/methanol (2:1, vol/vol). Phospholipids were separated by thin-layer chromatography using Silica Gel Merck 60 precoated plates and fatty acids separated by gas-liquid chromatography as described previously.

Statistical Analysis. Two-way analysis of variance (ANOVA) was used to analyze the effect of dietary calcium and fat, which looks at the main effects of these nutrient in addition to the interactions between them on experimental variables, as carried out by the SAS statistical software for microcomputers. Duncan's Multiple Range test

was used to separate treatment means when the ANOVA indicated significant differences. Differences were regarded as significant if $p<0.05$.

3. Results

Body weights. Animals body weights (Table 22) were similar initially and for the following two weeks. Analysis of the main effects revealed that by week three and four a significant fat and calcium interaction was modulating weight gain. Weight gains for the different levels of calcium were similar except for the 2.0%Ca fed animals where in the presence of high fat animals gained the most weight.

Membrane phospholipid composition. The relative effect of dietary intervention on the phosphatidylcholine (PC) content of the colonic mucosa was significantly affected by the nutrient interaction between calcium and fat (Table 25). The low fat (LF) fed animals PC content increased with increasing dietary calcium. The animals fed high fat (HF) with low (0.1%) calcium had the highest PC content compared to the remaining calcium groups. In the high (1.0% and 2.0%) calcium groups those fed the LF diet had significantly more PC than HF counterparts. The content of phosphatidylethanolamine (PE) was significantly affected by the level of calcium in the diet. Among the LF group, PE was elevated in low (0.1%) and high (1.0%) calcium groups compared to the control (0.5%CaLF) group, however, this effect was lost with the addition of the highest (2.0%) level of dietary calcium. No differences were observed in the HF animals although the 0.1%Ca, 1.0%Ca, and 2.0%Ca groups were greater than the control (0.5%CaLF) group. The phosphatidylserine (PS) content was not significantly affected by dietary modifications, however, PS was lowest in 0.5%Ca groups compared to all other levels of calcium in the LF and HF fed animals. The phosphatidylinositol (PI) content was fairly consistent particularly in LF fed animals. The PC:PE ratio was affected ($p\leq0.05$) by the nutrient interaction between fat and calcium. The 0.1%CaLF animals exhibited a

Table 25: The Effect of Varying Dietary Calcium in the Presence or Absence of High Fat on the Phospholipid Composition of Rat Colonic Mucosa. [a]

	PC	PE	PS	PI	PC:PE
5% Low Fat					
0.1% Ca	240.0 ± 34.1 [d]	589.0 ± 71.0 [cb]	83.5 ± 10.1	65.9 ± 15.6	0.4 ± 0.1 [c]
0.5% Ca	307.3 ± 50.1 [cd]	192.3 ± 22.8 [d]	41.2 ± 12.0	64.0 ± 11.0	1.6 ± 0.3 [b]
1.0% Ca	541.0 ± 76.4 [b]	524.1 ± 95.8 [cb]	74.3 ± 9.6	61.0 ± 8.5	1.0 ± 0.5
2.0% Ca	637.3 ± 99.9 [b]	375.7 ± 71.2 [bd]	70.4 ± 15.3	79.4 ± 17.4 b	1.7 ± 0.4 [b]
20% High Fat					
0.1% Ca	478.1 ± 68.2 [cb]	808.0 ± 157.3 [b]	75.5 ± 10.1	68.3 ± 11.2	0.6 ± 0.1 [c]
0.5% Ca	295.4 ± 50.1 [d]	309.2 ± 114.6 [bd]	43.1 ± 15.7	27.5 ± 11.4 [c]	1.0 ± 0.6
1.0% Ca	156.3 ± 13.5 [d]	508.6 ± 92.0 [cb]	58.5 ± 9.4	71.9 ± 13.4	0.3 ± 0.1 [c]
2.0% Ca	254.8 ± 38.6 [d]	581.6 ± 99.9 [cb]	73.1 ± 22.3	49.9 ± 21.3	0.4 ± 0.1 [c]
Main Effects [g]					
Fat	p = 0.0034	p = 0.0655	p = 0.5227	p = 0.1483	p = 0.0223
Ca	p = 0.1343	p = 0.0009	p = 0.0645	p = 0.4028	p = 0.0109
Fat * Ca	p = 0.0001	p = 0.6114	p = 0.8879	p = 0.3112	p = 0.0395

[a] Values represent means (µg/mg wet weight) ± S.E.

[b-f] : Means within a column with different superscripts differ at $p \leq 0.05$.

[g] : Two Way Anova p values.

significantly low ratio. The ratio was lower in HF compared to the respective LF fed animals likely due to the increased PE content of the HF group.

Phosphatidylcholine fatty acid profile. A significant effect of the calcium and fat interaction was observed on the polyunsaturated fatty acid (PUFA) content in the PC subfraction (Table 26). In general, PUFA content was significantly lower in the HF compared to the LF groups. The degree of saturation was unchanged except in the 1.0%Ca group where levels were decreased and increased in LF and HF fed animals, respectively. The stearic (18:0) acid (SA) content of the PC subfraction was significantly altered by the nutrient interaction (Table 27). HF fed animals had increased SA, particularly in the high (1.0% and 2.0%) calcium groups. Oleic (18:1) acid (OA) and linoleic (18:2) acid (LA) were relatively unchanged by dietary intervention with the exception of 1.0%Ca feeding where in a LF diet these fatty acids were elevated but in a HF diet their levels were depressed. AA levels were decreased 2-fold in a LF diet containing 0.1%Ca whereas in a HF diet a similar decrease was observed in the 1.0%Ca group (Table 28).

Phosphatidylethanolamine fatty acid profile. A significant nutrient interaction was exerted on the PUFA content of PE (Table 29). A dose dependent increase in PUFA from 0.5%Ca to 2.0%Ca was observed in the LF diet. No effect of calcium was observed in the presence of a HF diet, however, all dietary alterations significantly increased PUFA content of PE compared to the control (0.5%CaLF) diet. Changes in PUFA content were at the expense of saturated fatty acid (SATFA) content. The SA content was modulated by the nutrient interaction and a dose dependent decrease was observed in 0.5%Ca to 2.0%Ca in the presence of a LF diet (Table 30). The control (0.5%CaLF) group had the highest SA compared to all other dietary protocols. OA was significantly modulated by fat and calcium independently. In general, OA was greater in the HF groups irrespective of calcium, whereas animals consuming 0.5%Ca and 1.0%Ca diets exhibited the highest

Table 26: The Effect of Varying Dietary Calcium in the Presence or Absence of High Fat on the Percent Fatty Acid Composition of Phosphatidylcholine Categorized by the Degree of Saturation. [a]

	Saturated	Monounsaturated	Polyunsaturated
5% Low Fat			
0.1% Ca	65.7 ± 2.3 [c]	19.0 ± 2.2 [c]	15.3 ± 0.7 [cd]
0.5% Ca	60.5 ± 1.7 [c]	15.5 ± 1.0 [cd]	24.1 ± 1.3 [b]
1.0% Ca	46.8 ± 6.7 [d]	28.4 ± 6.0 [b]	24.8 ± 0.9 [b]
2.0% Ca	60.3 ± 2.0 [c]	15.1 ± 0.6 [cd]	24.6 ± 1.8 [b]
20% High Fat			
0.1% Ca	62.3 ± 2.6 [c]	17.6 ± 1.2 [c]	20.1 ± 2.6 [bc]
0.5% Ca	64.8 ± 1.7 [c]	16.6 ± 0.4 [cd]	18.6 ± 1.8 [c]
1.0% Ca	81.1 ± 1.8 [b]	8.7 ± 1.2 [d]	10.3 ± 0.8 [d]
2.0% Ca	69.1 ± 3.3 [c]	14.0 ± 0.4 [cd]	16.9 ± 2.9 [c]
Main Effects [e]			
Fat	p = 0.0001	p = 0.0096	p = 0.0001
Ca	p = 0.9100	p = 0.2153	p = 0.0947
Fat * Ca	p = 0.0001	p = 0.0012	p = 0.0001

[a] Values represent means (% of total) ± S.E.

[b-d] : Means within a column with different superscripts differ at $p \leq 0.05$.

[e] : Two Way Anova p values.

Table 27: The Effect of Varying Dietary Calcium in the Presence or Absence of High Fat on the Colonic Phosphatidylcholine Percent Fatty Acid Composition. [a]

	C 14:0	C 16:0	C 16:1	C 18:0	C 18:1	C 18:2
5% Low Fat						
0.1% Ca	0.6 ± 0.1 [d]	41.9 ± 0.7	0.7 ± 0.1 [cd]	23.2 ± 1.8 [cd]	9.8 ± 0.4 [cd]	4.3 ± 0.3 [cde]
0.5% Ca	1.2 ± 0.1 [b]	42.5 ± 1.2	1.7 ± 0.2 [bc]	16.8 ± 0.6 [ef]	13.3 ± 0.8 [cd]	7.7 ± 1.0 [c]
1.0% Ca	0.7 ± 0.1 [cd]	31.1 ± 4.4	1.9 ± 0.9 [b]	15.0 ± 2.2 [f]	25.7 ± 5.2 [b]	11.2 ± 2.6 [b]
2.0% Ca	1.1 ± 0.1 [b]	43.4 ± 1.8	1.6 ± 0.2	15.8 ± 0.6 [ef]	13.0 ± 0.5 [cd]	5.9 ± 0.3 [cd]
20% High Fat						
0.1% Ca	1.2 ± 0.0 [b]	40.9 ± 1.6	1.5 ± 0.1	20.2 ± 1.1 [de]	15.6 ± 1.1 [c]	6.1 ± 0.8 [cd]
0.5% Ca	1.3 ± 0.1 [b]	42.8 ± 1.2	1.2 ± 0.2	20.7 ± 0.7 [cde]	14.7 ± 0.4 [c]	5.4 ± 0.3 [cde]
1.0% Ca	1.1 ± 0.1 [b]	46.4 ± 0.3	0.5 ± 0.1 [d]	33.6 ± 2.0 [b]	6.9 ± 1.1 [d]	2.2 ± 0.4 [e]
2.0% Ca	1.0 ± 0.1 [bc]	41.4 ± 2.2	0.9 ± 0.2	25.3 ± 2.4 [c]	10.0 ± 2.3 [cd]	3.8 ± 0.3 [de]
Main Effects [g]						
Fat	p = 0.0027	p = 0.0539	p = 0.1148	p = 0.0001	p = 0.0309	p = 0.0010
Ca	p = 0.0118	p = 0.1450	p = 0.7947	p = 0.0350	p = 0.0852	p = 0.1603
Fat * Ca	p = 0.0059	p = 0.0009	p = 0.0252	p = 0.0001	p = 0.0001	p = 0.0003

[a] Values represent means (% of total) ± S.E.

[b-f] : Means within a column with different superscripts differ at $p \leq 0.05$.

[g] : Two Way Anova p values.

Table 28: The Effect of Varying Dietary Calcium in the Presence or Absence of High Fat on the Colonic Phosphatidylcholine Percent Fatty Acid Composition. [a]

	C 18:3	C 20:1	C 20:3	C 20:4	C 20:5
5% Low Fat					
0.1% Ca	1.8 ± 0.2 [b]	8.5 ± 1.8 [b]	2.7 ± 0.5	5.5 ± 0.1 [c]	1.0 ± 0.1 [b]
0.5% Ca	0.3 ± 0.1 [e]	0.5 ± 0.0 [c]	1.5 ± 0.1	14.3 ± 0.4 [b]	0.3 ± 0.1 [c]
1.0% Ca	0.6 ± 0.2 [de]	0.8 ± 0.1 [c]	1.0 ± 0.2 [c]	11.6 ± 2.1 [b]	0.4 ± 0.0 [c]
2.0% Ca	0.4 ± 0.0 [de]	0.5 ± 0.0 [c]	3.8 ± 2.2 [b]	14.2 ± 0.8 [b]	0.2 ± 0.1 [c]
20% High Fat					
0.1% Ca	0.3 ± 0.0 [e]	0.5 ± 0.0 [c]	1.6 ± 0.1	11.8 ± 2.7 [b]	0.3 ± 0.1 [c]
0.5% Ca	0.5 ± 0.1 [de]	0.6 ± 0.1 [c]	0.9 ± 0.1 [c]	11.6 ± 1.1 [b]	0.4 ± 0.1 [c]
1.0% Ca	1.6 ± 0.3 [bc]	1.3 ± 0.4 [c]	0.1 ± 0.0 [c]	5.4 ± 1.2 [c]	0.9 ± 0.2 [b]
2.0% Ca	1.1 ± 0.5 [cd]	1.3 ± 0.4 [c]	0.9 ± 0.2 [c]	11.2 ± 2.4 [b]	1.1 ± 0.3 [b]
Main Effects [f]					
Fat	p = 0.6679	p = 0.0015	p = 0.0309	p = 0.3206	p = 0.0836
Ca	p = 0.2610	p = 0.0001	p = 0.1394	p = 0.0134	p = 0.0991
Fat * Ca	p = 0.0001	p = 0.0001	p = 0.5033	p = 0.0048	p = 0.0001

[a] Values represent means (% of total) ± S.E.

[b-e] : Means within a column with different superscripts differ at $p \leq 0.05$.

[f] : Two Way Anova p values.

Table 29: The Effect of Varying Dietary Calcium in the Presence or Absence of High Fat on the Percent Fatty Acid Composition of Phosphatidylethanolamine Categorized by the Degree of Saturation. [a]

	Saturated	Monounsaturated	Polyunsaturated
5% Low Fat			
0.1% Ca	37.2 ± 2.5 [d]	22.7 ± 3.5 [c]	40.1 ± 1.7 [c]
0.5% Ca	58.9 ± 0.6 [b]	21.0 ± 0.9 [c]	20.1 ± 0.5 [d]
1.0% Ca	36.1 ± 1.8 [de]	28.5 ± 1.8	35.4 ± 2.7 [c]
2.0% Ca	22.3 ± 1.1 [f]	26.0 ± 2.4	51.7 ± 2.1 [b]
20% High Fat			
0.1% Ca	36.1 ± 3.5 [de]	25.2 ● 1.3	38.7 ± 3.7 [c]
0.5% Ca	29.5 ± 1.5 [e]	34.5 ± 2.3 [b]	36.0 ± 2.7 [c]
1.0% Ca	34.9 ± 2.1 [de]	34.0 ± 5.6 [b]	31.0 ± 4.7 [c]
2.0% Ca	45.8 ● 3.8 [c]	22.2 ± 5.0 [c]	32.0 ± 3.2 [c]
Main Effects [g]			
Fat	p = 0.2262	p = 0.0638	p = 0.2518
Ca	p = 0.0006	p = 0.0979	p = 0.0002
Fat * Ca	p = 0.0001	p = 0.8240	p = 0.0001

[a] Values represent means (% of total) ± S.E.

[b-f] : Means within a column with different superscripts differ at $p \leq 0.05$.

[g] : Two Way Anova p values.

Table 30: The Effect of Varying Dietary Calcium in the Presence or Absence of High Fat on the Colonic Phosphatidylethanolamine Percent Fatty Acid Composition. [a]

	C 14:0	C 16:0	C 16:1	C 18:0	C 18:1	C 18:2
5% Low Fat						
0.1% Ca	2.8 ± 0.1 [bcd]	18.1 ± 2.3 [bc]	3.7 ± 0.5 [cde]	16.4 ± 1.0 [d]	18.4 ± 3.9 [c]	11.5 ± 1.2 [c]
0.5% Ca	0.9 ± 0.2 [de]	11.7 ± 0.8 [e]	1.2 ± 0.3 [f]	46.4 ± 0.5 [b]	16.8 ± 0.9 [c]	3.4 ± 0.1 [d]
1.0% Ca	1.1 ± 0.2 [de]	17.2 ± 0.4 [bcd]	5.7 ± 0.7 [c]	17.9 ± 1.6 [cd]	22.1 ± 2.5	9.3 ± 1.4 [cd]
2.0% Ca	0.5 ± 0.1 [e]	11.8 ± 2.4 [e]	10.5 ± 1.3 [b]	10.0 ± 1.6 [e]	15.0 ± 1.2 [c]	19.2 ± 4.2 [b]
20% High Fat						
0.1% Ca	2.4 ± 0.6 [cde]	13.3 ± 1.1 [cde]	5.3 ± 0.6 [cd]	18.5 ± 0.7 [cd]	19.1 ± 1.8 [c]	6.6 ± 0.5 [cd]
0.5% Ca	1.7 ± 0.4 [cde]	12.5 ± 1.9 [de]	4.4 ± 0.8 [cde]	15.3 ± 0.9 [d]	29.3 ± 3.0 [b]	12.0 ± 1.4 [c]
1.0% Ca	3.4 ± 1.6 [bc]	14.6 ± 1.3	3.4 ± 0.3 [de]	16.9 ± 1.9 [d]	29.8 ± 5.4 [b]	11.4 ± 1.9 [c]
2.0% Ca	4.6 ± 0.4 [b]	19.1 ± 0.8 [b]	2.9 ± 0.5 [ef]	22.2 ± 3.2 [c]	18.4 ± 4.6 [c]	5.4 ± 1.5 [d]
Main Effects [g]						
Fat	p = 0.0007	p = 0.8697	p = 0.0149	p = 0.0005	p = 0.0140	p = 0.1477
Ca	p = 0.1823	p = 0.0618	p = 0.0001	p = 0.0001	p = 0.0367	p = 0.1183
Fat * Ca	p = 0.0090	p = 0.0027	p = 0.0001	p = 0.0001	p = 0.3118	p = 0.0001

[a] Values represent means (% of total) ± S.E.

[b-f] : Means within a column with different superscripts differ at $p \leq 0.05$.

[g] : Two Way Anova p values.

Table 31: The Effect of Varying Dietary Calcium in the Presence or Absence of High Fat on the Colonic Phosphatidylethanolamine Percent Fatty Acid Composition. [a]

	C 18:3	C 20:1	C 20:3	C 20:4	C 20:5
5% Low Fat					
0.1% Ca	0.3 ± 0.1 [c]	0.6 ± 0.0 [cd]	2.1 ± 0.2 [b]	25.8 ± 2.0 [bc]	0.5 ± 0.1 [c]
0.5% Ca	0.7 ± 0.1 [c]	3.0 ± 0.2 [b]	1.9 ± 0.1 [bc]	11.3 ± 0.5 [d]	2.9 ± 0.1 [b]
1.0% Ca	0.3 ± 0.1 [c]	0.7 ± 0.0 [cd]	1.4 ± 0.2	23.9 ± 3.7 [bc]	0.4 ± 0.1 [c]
2.0% Ca	6.8 ± 1.7 [b]	0.5 ± 0.1 [d]	1.3 ± 0.2 [cd]	23.6 ± 3.7 [bc]	0.6 ± 0.1 [c]
20% High Fat					
0.1% Ca	0.5 ± 0.2 [c]	0.7 ± 0.1 [cd]	2.0 ± 0.2 [bc]	31.0 ± 1.9 [b]	0.7 ± 0.1 [c]
0.5% Ca	0.4 ± 0.1 [c]	0.8 ± 0.1 [cd]	1.4 ± 0.2 [cd]	21.7 ± 3.6	0.4 ± 0.1 [c]
1.0% Ca	0.4 ± 0.1 [c]	0.8 ± 0.0 [c]	1.3 ± 0.4 [cd]	17.5 ± 6.0 [cd]	0.4 ± 0.1 [c]
2.0% Ca	0.6 ± 0.1 [c]	0.9 ± 0.2 [c]	1.1 ± 0.2 [d]	24.4 ± 4.0 [bc]	0.6 ± 0.2 [c]
Main Effects [e]					
Fat	p = 0.0010	p = 0.0001	p = 0.1196	p = 0.3222	p = 0.0001
Ca	p = 0.0001	p = 0.0001	p = 0.0041	p = 0.0147	p = 0.0001
Fat * Ca	p = 0.0001	p = 0.0001	p = 0.8202	p = 0.1268	p = 0.0001

[a] Values represent means (% of total) ± S.E.

[b-d] : Means within a column with different superscripts differ at $p \leq 0.05$.

[e] : Two Way Anova p values.

levels. LA and linolenic acid were significantly affected by the fat and calcium interaction with 2.0%CaLF having the largest proportion of each (Table 30 and Table 31). The percent composition of AA was significantly increased by altering dietary calcium in the presence of a LF diet (Table 31). In the presence of a HF diet the extreme ends of calcium modification (0.1%Ca and 2.0%Ca) resulted in elevated AA levels.

Phosphatidylserine fatty acid profile. A significant nutrient interaction effect was exerted on the PUFA content of PS (Table 32). A dose dependent decrease in LF PUFA was observed from 0.1%Ca to 1.0%Ca, but this was lost with the addition of 2.0%Ca to the diet. The 1.0%CaHF and 0.1%CaLF exhibited the greatest PUFA content, however, dietary modifications did not produce any significant changes compared to the control (0.5%CaLF) group. SATFAs were unchanged by dietary protocol. SA was not appreciably affected by dietary protocol (Table 33). A 3-fold increase in PS LA was observed in LF, but not HF, diets containing 0.1%Ca. PS derived AA was significantly altered by the fat and calcium interaction (Table 34). AA levels were lowest in 0.1%Ca fed animals. AA was greatest in animals fed 1.0%Ca in the presence of a HF diet.

Phosphatidylinositol fatty acid profile. The nutrient interaction significantly affected the PUFA content of PI (Table 35). All dietary modifications reduced PUFA content relative to the control (0.5%CaLF) group. This effect was at the expense of SATFA content as all dietary modifications increased levels relative to the control (0.5%CaLF) group. SA, significantly affected by the nutrient interaction, constituted a large proportion of the PI fatty acid profile (Table 36). In the LF group, increasing dietary calcium from 0.5% resulted in increasing SA content while in the presence of HF, calcium had no effect on SA content. OA was significantly modulated by the nutrient interaction where in a LF diet OA was elevated in 0.1%Ca and 1.0%Ca fed animals but low in 0.5%Ca and 2.0%Ca diets. In a HF diet OA decreased with increasing dietary calcium. LA content decreased with increasing calcium, reaching significance in the

Table 32: The Effect of Varying Dietary Calcium in the Presence or Absence of High Fat on the Percent Fatty Acid Composition of Phosphatidylserine Categorized by the Degree of Saturation. [a]

	Saturated	Monounsaturated	Polyunsaturated
5% Low Fat			
0.1% Ca	56.6 ● 1.7	17.0 ± 0.7 [e]	26.4 ± 1.3 [b]
0.5% Ca	62.3 ● 8.5	22.5 ● 1.5 [d]	15.2 ± 9.1 [bc]
1.0% Ca	62.7 ● 2.5	27.6 ● 2.1 [cd]	9.7 ± 1.0 [c]
2.0% Ca	55.6 ● 2.5	26.1 ● 1.1 [cd]	17.7 ± 2.5 [bc]
20% High Fat			
0.1% Ca	54.1 ● 3.7	34.9 ± 2.7 [b]	11.1 ● 1.0 [c]
0.5% Ca	58.2 ● 1.7	31.1 ± 1.0 [bc]	10.7 ± 0.7 [c]
1.0% Ca	58.9 ± 2.0	14.9 ± 0.2 [e]	26.3 ● 2.0 [b]
2.0% Ca	64.6 ± 3.1	24.1 ± 3.8 [d]	11.3 ± 5.1 [c]
Main Effects [f]			
Fat	p = 0.9352	p = 0.0404	p = 0.3363
Ca	p = 0.4288	p = 0.1177	p = 0.3314
Fat * Ca	p = 0.2330	p = 0.0001	p = 0.0012

[a] Values represent means (% of total) ± S.E.

[b-e] : Means within a column with different superscripts differ at $p \leq 0.05$.

[f] : Two Way Anova p values.

Table 33: The Effect of Varying Dietary Calcium in the Presence or Absence of High Fat on the Colonic Phosphatidylserine Percent Fatty Acid Composition. [a]

	C 14:0	C 16:0	C 16:1	C 18:0	C 18:1	C 18:2
5% Low Fat						
0.1% Ca	1.1 ± 0.1 [c]	39.8 ± 1.9 [b]	1.2 ± 0.1	15.8 ± 0.8 [c]	14.9 ± 0.6	8.0 ± 0.6 [b]
0.5% Ca	1.5 ± 0.5	21.4 ± 1.2 [cd]	1.9 ± 0.5 [bc]	26.8 ± 2.7 [b]	13.4 ± 0.9	2.5 ± 0.8 [de]
1.0% Ca	1.2 ± 0.3	25.0 ± 2.1 [cd]	1.1 ± 0.3	25.1 ± 0.3 [b]	15.0 ± 3.2	3.2 ± 1.1 [cde]
2.0% Ca	1.0 ± 0.1 [c]	20.9 ± 2.6 [cd]	1.2 ± 0.1	27.1 ± 1.4 [b]	16.8 ± 1.5	4.6 ± 0.9 [cd]
20% High Fat						
0.1% Ca	1.0 ± 0.1 [c]	24.0 ± 1.6 [cd]	1.6 ± 0.2 [bc]	25.5 ± 2.3 [b]	17.9 ± 0.9	3.1 ± 0.5 [cde]
0.5% Ca	1.9 ± 0.3 [b]	17.5 ± 2.8 [d]	1.1 ± 0.3 [cd]	30.6 ± 2.3 [b]	15.8 ± 0.5	4.0 ± 0.5 [cde]
1.0% Ca	1.1 ± 0.1 [c]	22.3 ± 0.5 [cd]	0.6 ± 0.1 [cd]	18.3 ± 1.2 [c]	13.4 ± 0.2	5.2 ± 0.2 [c]
2.0% Ca	1.3 ± 0.1	27.7 ± 4.1 [c]	2.0 ± 0.3 [b]	27.2 ± 3.5 [b]	14.6 ± 1.5	2.2 ± 0.6 [de]
Main Effects [f]						
Fat	p = 0.4584	p = 0.0235	p = 0.7658	p = 0.2510	p = 0.6932	p = 0.0783
Ca	p = 0.0505	p = 0.0001	p = 0.0583	p = 0.0007	p = 0.4252	p = 0.0100
Fat * Ca	p = 0.6591	p = 0.0004	p = 0.0198	p = 0.0036	p = 0.1821	p = 0.001

[a] Values represent means (% of total) ± S.E.

[b-e] : Means within a column with different superscripts differ at $p \leq 0.05$.

[f] : Two Way Anova p values.

Table 34: The Effect of Varying Dietary Calcium in the Presence or Absence of High Fat on the Colonic Phosphatidylserine Percent Fatty Acid Composition. [a]

	C 18:3	C 20:1	C 20:3	C 20:4	C 20:5
5% Low Fat					
0.1% Ca	0.2 ± 0.0 [c]	0.8 ± 0.1 [d]	2.0 ± 0.2 [b]	15.6 ± 1.1 [e]	0.6 ± 0.0 [de]
0.5% Ca	0.5 ± 0.0 [c]	1.6 ± 0.2 [c]	0.6 ± 0.4 [d]	28.5 ± 3.8 [c]	1.5 ± 0.2 [b]
1.0% Ca	0.9 ± 0.5	2.1 ± 0.2 [b]	1.1 ± 0.1 [cd]	23.8 ± 1.5 [cd]	1.7 ± 0.3 [b]
2.0% Ca	1.6 ± 0.5 [b]	2.1 ± 0.2 [b]	1.2 ± 0.2 [cd]	22.7 ± 1.4 [cd]	0.8 ± 0.2 [cd]
20% High Fat					
0.1% Ca	0.5 ± 0.0 [c]	2.4 ± 0.1 [b]	1.6 ± 0.2 [bc]	21.0 ± 1.2 [de]	1.4 ± 0.1 [bc]
0.5% Ca	0.9 ± 0.2	1.5 ± 0.2 [c]	0.9 ± 0.2 [cd]	23.8 ± 1.8 [cd]	1.2 ± 0.2 [bcd]
1.0% Ca	0.4 ± 0.0 [c]	0.9 ± 0.0 [d]	2.0 ± 0.2 [b]	34.8 ± 1.2 [b]	0.9 ± 0.1 [cd]
2.0% Ca	0.6 ± 0.1 [c]	0.6 ± 0.1 [d]	1.0 ± 0.2 [cd]	22.6 ± 1.8 [cd]	0.3 ± 0.1 [e]
Main Effects [f]					
Fat	p = 0.2512	p = 0.0144	p = 0.3661	p = 0.0377	p = 0.0964
Ca	p = 0.0591	p = 0.3574	p = 0.0002	p = 0.0001	p = 0.0007
Fat * Ca	p = 0.0231	p = 0.0001	p = 0.0414	p = 0.0016	p = 0.0009

[a] Values represent means (% of total) ± S.E.

[b-e] : Means within a column with different superscripts differ at $p \leq 0.05$.

[f] : Two Way Anova p values.

Table 35: The Effect of Varying Dietary Calcium in the Presence or Absence of High Fat on the Percent Fatty Acid Composition of Phosphatidylinositol Categorized by the Degree of Saturation. [a]

	Saturated	Monounsaturated	Polyunsaturated
5% Low Fat			
0.1% Ca	52.4 ● 0.9 [b]	22.1 ● 0.5 [bc]	25.5 ● 0.8 [c]
0.5% Ca	31.5 ± 0.9 [d]	19.3 ± 0.7 [cd]	49.1 ± 1.0 [b]
1.0% Ca	42.8 ± 1.3 [c]	26.1 ● 2.9 [b]	31.2 ± 3.0 [c]
2.0% Ca	53.7 ± 2.1 [b]	16.1 ± 1.2 [d]	30.1 ± 1.7 [c]
20% High Fat			
0.1% Ca	46.2 ± 3.9 [bc]	25.6 ± 1.9 [b]	28.1 ± 2.1 [c]
0.5% Ca	42.9 ± 0.8 [c]	25.2 ± 0.6 [b]	31.9 ± 1.1 [c]
1.0% Ca	48.0 ± 5.8 [bc]	24.8 ● 0.5 [b]	27.1 ± 6.3 [c]
2.0% Ca	46.7 ± 2.0 [bc]	21.2 ± 1.7 [bc]	32.1 ± 1.8 [c]
Main Effects [e]			
Fat	p = 0.6854	p = 0.0047	p = 0.1139
Ca	p = 0.0001	p = 0.0006	p = 0.0001
Fat * Ca	p = 0.0016	p = 0.1239	p = 0.0006

[a] Values represent means (% of total) ± S.E.

[b-d] : Means within a column with different superscripts differ at $p \leq 0.05$.

[e] : Two Way Anova p values.

Table 36: The Effect of Varying Dietary Calcium in the Presence or Absence of High Fat on the Colonic Phosphatidylinositol Percent Fatty Acid Composition. [a]

	C 14:0	C 16:0	C 16:1	C 18:0	C 18:1
5% Low Fat					
0.1% Ca	1.3 ± 0.2 [b]	10.2 ± 0.7 [c]	0.7 ± 0.1 [c]	40.9 ± 1.7 [b]	19.7 ± 0.7 [bcd]
0.5% Ca	1.4 ± 0.1 [b]	9.9 ± 0.5 [c]	7.1 ± 0.5 [b]	20.2 ± 0.4 [d]	11.4 ± 0.4 [f]
1.0% Ca	0.6 ± 0.1 [c]	10.4 ± 0.3 [c]	1.2 ± 0.3 [c]	31.8 ± 1.2 [c]	23.4 ± 2.8 [b]
2.0% Ca	0.6 ± 0.0 [c]	17.6 ± 2.7 [b]	0.6 ± 0.0 [c]	35.5 ± 2.2 [bc]	13.7 ± 1.2 [ef]
20% High Fat					
0.1% Ca	0.4 ± 0.1 [c]	13.2 ± 1.7	1.5 ± 0.4 [c]	32.5 ± 5.1 [c]	21.6 ± 1.7 [bc]
0.5% Ca	1.1 ± 0.2 [b]	11.5 ± 1.3 [c]	1.3 ± 0.2 [c]	30.3 ± 1.9 [c]	21.9 ± 0.8 [bc]
1.0% Ca	0.6 ± 0.0 [c]	10.4 ± 1.7 [c]	1.0 ± 0.1 [c]	29.5 ± 0.4 [c]	18.5 ± 0.5 [cd]
2.0% Ca	0.7 ± 0.2 [c]	11.0 ± 2.5 [c]	1.2 ± 0.2 [c]	35.1 ± 1.1 [bc]	15.9 ± 1.7 [de]
Main Effects [g]					
Fat	p = 0.0082	p = 0.7211	p = 0.0001	p = 0.9235	p = 0.0234
Ca	p = 0.0001	p = 0.0945	p = 0.0001	p = 0.0001	p = 0.0002
Fat * Ca	p = 0.0119	p = 0.0286	p = 0.0001	p = 0.0031	p = 0.0002

[a] Values represent means (% of total) ± S.E.

[b-f] : Means within a column with different superscripts differ at $p \leq 0.05$.

[g] : Two Way Anova p values.

Table 37: The Effect of Varying Dietary Calcium in the Presence or Absence of High Fat on the Colonic Phosphatidylinositol Percent Fatty Acid Composition. [a]

	C 18:2	C 20:1	C 20:3	C 20:4	C 20:5
5% Low Fat					
0.1% Ca	4.6 ± 0.4 [cde]	1.7 ± 0.6 [cd]	0.7 ± 0.3 [d]	19.4 ± 0.9 [ef]	0.8 ± 0.2 [d]
0.5% Ca	5.2 ± 0.2 [bcd]	0.8 ± 0.0 [e]	2.7 ± 0.2 [b]	40.3 ± 1.0 [b]	1.0 ± 0.0 [cd]
1.0% Ca	4.8 ± 0.7 [cde]	1.5 ± 0.2 [de]	1.6 ± 0.1 [c]	23.6 ± 2.7 [de]	1.2 ± 0.2 [cd]
2.0% Ca	3.0 ± 0.3 [de]	1.8 ± 0.3 [cd]	2.1 ± 0.3 [bc]	23.5 ± 1.8 [de]	1.5 ± 0.1 [bc]
20% High Fat					
0.1% Ca	6.9 ± 1.2 [bc]	2.6 ± 0.3 [c]	2.0 ± 0.3 [bc]	18.1 ± 1.1 [f]	1.1 ± 0.2 [cd]
0.5% Ca	7.4 ± 1.5 [b]	2.0 ± 0.2 [cd]	1.6 ± 0.2 [c]	20.9 ± 0.6 [def]	2.0 ± 0.3 [b]
1.0% Ca	2.4 ± 0.1 [e]	3.7 ± 0.1 [b]	2.2 ± 0.2 [bc]	30.6 ± 0.6 [c]	1.0 ± 0.3 [cd]
2.0% Ca	2.6 ± 0.3 [e]	4.1 ± 0.3 [b]	2.5 ± 0.4 [b]	25.5 ± 2.2 [d]	1.5 ± 0.1 [bc]
Main Effects [g]					
Fat	p = 0.3323	p = 0.0001	p = 0.0988	p = 0.0061	p = 0.0259
Ca	p = 0.0002	p = 0.0001	p = 0.0066	p = 0.0001	p = 0.0243
Fat * Ca	p = 0.0152	p = 0.0409	p = 0.0019	p = 0.0001	p = 0.0130

[a] Values represent means (% of total) ± S.E.

[b-f] : Means within a column with different superscripts differ at $p \leq 0.05$.

[g] : Two Way Anova p values.

presence of HF (Table 37). AA was modulated by the complex nutrient fat and calcium interaction with the control (0.5%CaLF) group exhibiting the highest AA content.

4. Discussion

The purpose of the present investigation was to determine whether altering the level of dietary calcium modulated colonic membrane lipid composition and whether the effect was evident in both a low (LF) and high (HF) fat diet. Although many significant differences were found the salient findings include that the effect of calcium was most prominent in the LF diet and obscured by the HF diet. This is evident in colonic mucosal phosphatidylcholine (PC) content where increasing calcium resulted in a dose dependent PC increase in the presence of the LF but not the HF diet. In addition, increased dietary calcium in a LF diet led to increased arachidonic acid (AA) in phospholipid fractions except in phosphotidylinositol (PI). Animals fed the HF diet yielded similar AA values to those fed the high calcium LF diets. The primary findings of this study indicate that dietary calcium alters membrane lipid composition, however, the effect on fatty acid composition of the various phospholipids was variable.

Calcium has been implicated as a chemopreventive agent in colon carcinogenesis and the primary hypothesis contends that it ameliorates the toxic effects of free fatty acids and bile acids associated with a high fat diet (Newmark et al., 1984). Recently, evidence of non luminal protective effects of calcium have been reported (Lans et al., 1991; Lapre et al., 1993; Llor et al., 1991) suggesting the effects of this nutrient may be more complex than originally hypothesized. It has been demonstrated that HF diets that promote colon tumorigenesis produce different membrane fatty acid profiles than those that do not promote the disease (Rao et al., 1993). Moreover, linoleic acid (LA) and AA levels were significantly decreased in the colonic membrane of animals fed a non promoting HF diet. This suggests that changes in membrane composition may play a role

in enhancement or inhibition of tumorigenesis. The current findings that calcium modulates fatty acid composition of the various phospholipid subfractions suggests an additional mechanism by which calcium ameliorates the disease process. The modulation of tissue lipid composition by dietary calcium has been addressed by only a few studies. In an early investigation low calcium increased cholesterol, phospholipid, sphingomylein, and phosphatidylethanolamine (PE) in liver tissue (Dougherty & Iacono, 1979). Another investigation demonstrated elevated total plasma membrane phospholipids in response to increased dietary calcium with no effect on cholesterol or triglycerides (Foley et al., 1990). Only one study has investigated the effect of altered calcium on colonic membrane lipid composition and the authors reported that high (1.5%) calcium in a HF diet had no effect on fatty acid composition (Awad et al., 1990). In both the latter and present study the effects of calcium were investigated on saline treated rats fed experimental diets for 4 weeks. However, in the present investigation the effect of calcium was determined on the various subclasses of phospholipids whereas Awad et al. (1990) examined the fatty acid composition of total colonic lipid. The effect of calcium may be limited to alteration in phospholipid composition (Foley et al., 1990) suggesting that examining fatty acid composition in total colonic lipid may have masked a modulating effect of calcium. Overall, the present investigation demonstrates that calcium clearly alters membrane phospholipid composition, however, the biological significance of this finding in terms of disease development remains to be established.

It has been suggested that multistage carcinogenesis involves a progressive disorder in signal transduction pathways (Weinstein, 1988). A great deal of interest has been focused on the protein kinase C (PKC) pathway since the finding that it was directly activated by 12-*O*-tetradecanoylphorbol-13-acetate (TPA), a known tumor promoter (Ashendel, 1985; Nishizuka, 1986). Furthermore, this enzyme has been implicated in colon cancer because of its connection to cell growth and division (Berridge,

1987). Although HF diets have been positively correlated with colon carcinogenesis (Willet et al., 1990) little to no information has been confirmed regarding the mechanism of action. One possibility is that membrane phospholipid composition, which has been shown to be modulated by dietary fat level and type (Donnelly et al., 1987; Rao et al., 1993), may regulate the activity of PKC and thus affect the disease process. Fatty acids such as AA, LA, and oleic acid (OA) have been shown to activate PKC (McPhail et al., 1984; Oishi et al., 1990; Belury et al., 1993). It has been suggested that the alteration of colonic membrane fatty acid composition could influence tumor development by altering the phospholipase C cell signaling system (Awad et al., 1993). The activation of this system results in the hydrolysis of phospholipids, production of inositol triphosphate and diacylglycerol, and activation of PKC. In the present investigation calcium was able to modulate both phospholipid composition and the fatty acid composition of each subclass. In addition, the fatty acids AA, LA, and OA, which have been reported to activate PKC *in vitro*, were affected *in vivo* by altered dietary calcium. Thus it is plausible that calcium alters PKC activity through modulation of membrane phospholipid composition. In support of this contention Donnelly et al. (1987) demonstrated that alterations in dietary fat which influenced the activity of PKC were accompanied with changes in epidermal cell membrane phospholipid subclass fatty acid composition, predominantly increased LA.

An additional mechanism by which a HF diet may modulate colon carcinogenesis is through the alteration of membrane phospholipids and eicosanoid synthesis (Rao & Reddy, 1993). The fatty acids LA and AA form eicosanoids, such that metabolism of LA and AA yields lipoxygenase products and metabolism of AA yields cyclooxygenase products (Mayes, 1988). Eicosanoids have been implicated in colon carcinogenesis and several prostaglandins (PG) as well as leukotriene B_4 have been shown to stimulate the proliferation of human colon carcinoma cells *in vitro* (Qiao et al., 1995). Furthermore,

AA metabolism blockers such as aspirin, piroxicam, and indomethacin have been shown to suppress colon tumorigenesis (Polard & Luckert, 1983; Nigro et al., 1986; Reddy et al., 1993). Rao et al. (1996) reported that animals fed a HF fish oil diet compared to a corn oil HF diet, which have been previously shown to produce marked differences in colonic phospholipid fatty acid composition, exhibited lower PG and thromboxane levels. Moreover, there was a reduction in phospholipase A_2 and phosphatidylinositol-specific phospholipase C, both dominant pathways for AA release and formation of eicosanoids, in rats fed HF fish oil diets. These results suggest that perhaps changes in phospholipid fatty acid composition may mediate colon carcinogenesis through enhancement or inhibition of eicosanoid synthesis. The present finding that calcium altered AA and LA levels in colonic phospholipids may provide a non luminal mechanism by which dietary calcium mediates the disease process.

To the author's knowledge this is the first time that modifying the level of dietary fat and calcium has been shown to alter membrane phospholipid composition in non carcinogen treated rat colon. Inspite of the myriad of changes observed it was demonstrated that: in general there was no dose dependent effect of calcium; an effect of calcium was observed in the LF; the effect of calcium was lost in the presence of a HF diet; and that the similar results between the high calcium LF and the HF groups suggests that these components may affect a common pathway to elicite similar changes. It is plausible that membrane composition may affect colon carcinogenesis through changes in PKC activity or eicosanoid synthesis. Further research is required to elucidate the connection between membrane composition and biochemical changes in the colonic mucosa.

Section VI

THE EFFECT OF CALCIUM IN AN ESTABLISHED DISEASE STATE

Chapter 9.

THE EFFECT OF DIETARY CALCIUM ON THE GROWTH OF ESTABLISHED ABERRANT CRYPT FOCI AND COLONIC TUMORS

1. Introduction

In view of the fact that carcinogenesis is a multistep process it is reasonable to propose that the response of preneoplastic or neoplastic lesions in their early developmental stages would differ to growth regulatory stimuli than when in a more advanced stage. In other words a preneoplastic lesion progressing from one growth state to another may differ from its preceding state with regard to its growth requirements. Animal models provide the unique opportunity to explore this concept. Previous studies have demonstrated that ACF with varying growth features represent pre-adenomatous stages (Pretlow et al., 1992; Zhang et al., 1992; Magnuson et al., 1993). As a result, the study of alterations in the growth features of ACF, adenoma, or adenocarcinomas and their incidence enables one to investigate the effect of growth modulators on the multistep process of colon carcinogenesis. Animals exposed to azoxymethane harbor a number of ACF, preneoplastic lesions (Bird, 1987; McLellan & Bird, 1988; Bird, 1995). Several weeks after exposure to single or multiple injections of AOM rodent colons may possess ACF exhibiting different growth features as well as adenomatous exophytic lesions. Therefore, intervention efficacy can be assessed on presumably all stages of colon carcinogenesis by introducing a specific growth modulator of the disease process several weeks after the initiation (AOM injection). Calcium has been advocated to play an important role in cell growth as well as differentiation (Hennings et al., 1980; McGrath and Soule, 1984; Whitfield, 1992). The hypothesis that calcium intervention could augment the development of established preneoplastic and neoplastic lesions was tested

by examining the modulating effects of this nutrient on the sequential growth of ACF and eventual tumor outcome on lesions that were initiated in the rat colon 12 weeks earlier and growing in a high fat environment.

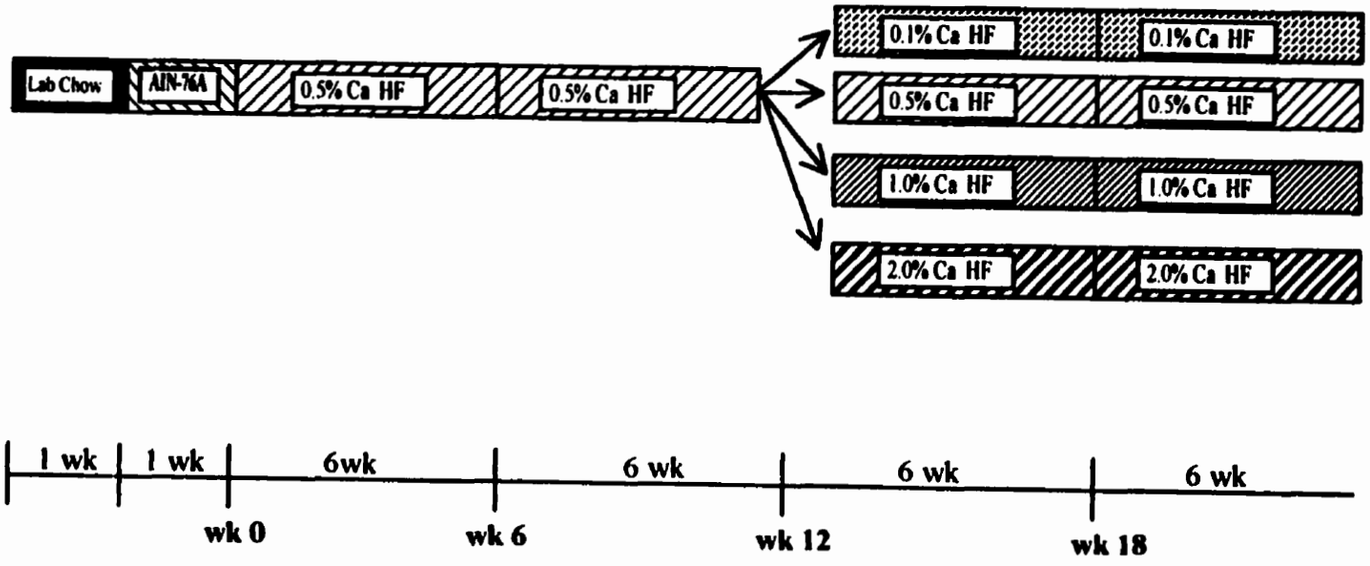
2. Materials and Methods

Animals. Male Sprague Dawley rats approximately five to six weeks old were acclimatized for one week prior to initiating experimental procedures.

Experimental Diets. The formulated diets were based on the AIN-76A diet (American Institute of Nutrition, 1977; 1980) with the exception of $CaHPO_4$. To achieve the high-fat diet, an additional 15% fat by weight was added as beef tallow at the expense of carbohydrate. The nutrients per kilocalorie density of the diets were identical except for fat and carbohydrate. Calcium (Ca) levels were altered by adding $CaCO_3$ to the base $CaHPO_4$ in the mineral mix to maintain the phosphate level in the diet at control levels. To achieve the low calcium diet (0.1% Ca), 0.4% $NaHPO_4$ was added to 0.1% $CaHPO_4$ to maintain the phosphate level in the diet mix. All diets are itemized in Appendix I.

Study Design. Animals were injected with a two doses of azoxymethane (15 mg/kg) 1 weeks apart and fed the AIN-76A control diet till one week after the last carcinogen treatment. Animals were then fed the high fat diet for twelve weeks. At twelve weeks animals were fed 0.1%, 0.5%, 1.0% and 2.0% calcium AIN modified diets for the remainder of the study (Figure 17). Animals (8 animals/treatment group) were terminated at 6, 12, and 18 week time points for ACF evaluation. At 24 weeks the remainder of the animals were terminated and evaluated for ACF and tumor pathology. All animals were terminated by carbon dioxide asphyxiation. Colons were removed immediately after termination and flushed with phosphate buffered saline (PBS), slit open from cecum to anus, and fixed in 70% ethanol and assessed for aberrant crypt foci (ACF).

Figure 17: Schematic representation of the study design used in the study of varying levels of dietary calcium on the growth of established aberrant crypt foci and colonic tumor development.


Lab Chow
AIN-76A
0.5% Ca HF
0.5% Ca HF
0.1% Ca HF
0.1% Ca HF
0.5% Ca HF
0.5% Ca HF
1.0% Ca HF
1.0% Ca HF
2.0% Ca HF
2.0% Ca HF
1 wk
1 wk
6wk
6 wk
6 wk
6 wk
wk 0
wk 6
wk 12
wk 18

Quantification of aberrant crypt foci. The number, distribution, multiplicity, and size of ACF were determined for the entire length of the colon as described previously.

Tumor Pathology. Animals developing tumors were killed after 24 weeks as several began showing fecal blood. Animals were coded to conceal the identity of the treatment group until histopathological evaluation was completed. The location, appearance and dimensions of all suspicious lesions were recorded. Abnormal lesions included visible tumors and enlarged lymphoid aggregates. Each lesion was dissected out with 0.5 cm surrounding mucosa and fixed in 10% neutral buffered formalin. Lesions were embedded in paraffin, sectioned at 5 microns and stained with hematoxylin and eosin. The existence of neoplasms was identified microscopically and categorized as adenoma or adenocarcinoma. Adenocarcinomas showed marked nuclear pleomorphism and/or invasion of the submucosa. As described by Clinton et al. (1988), histological evaluation of suspected lesions was necessary as the occurrence of exophytic lesions felt by palpation including areas of abnormal hyperplasia and enlarged lymphoid follicles were macroscopically indistinguishable from adenomas and would have falsely increased tumor incidence.

Statistical Analysis. χ^2 analysis was used to determine the effect of diet on tumor incidence, adenoma incidence, adenocarcinoma incidence, tumor incidence along the colon length, and the number of tumors along the colon length. Analysis of variance (ANOVA) was used to determine the effect of diet on all remaining parameters. SAS statistical software for microcomputers was used for all statistical analysis. Duncan's Multiple Range test was used to separate treatment means when the ANOVA indicated significant differences. Differences were regarded as significant if $p<0.05$.

3. Results.

Body weights. At the time of the first AOM injection, the average body weight was 149 g (Figure 18). At 18 wks, 6 wks after introducing the different levels of calcium, animals consuming the 2.0%Ca diet had a significantly ($p<0.05$) lower body weight gain compared to those consuming the 1.0%Ca diet. While 2.0%Ca fed animals remained the lowest to the end of the experiment this difference was no longer significant. Animals consuming the 2.0%Ca diet did appear to lag in weight gain and this phenomenon has been reported by others (Karkare et al., 1991; McSherry et al., 1989; Pence et al., 1995). However, it has been reported that high levels of supplemental dietary calcium did not significantly alter mean daily food consumption and thus as body weight did not correlate with tumor outcome (McSherry et al., 1989; Pence et al., 1995).

Characteristics of ACF. Intervention with varying levels of calcium was initiated in a group of rats injected with AOM (a colon carcinogen) 12 weeks earlier. Appearance of ACF in AOM induced rat colon peaked at week 12 (467) and declined in all groups throughout the remainder of the experiment (Table 38). At week 18 (6 weeks on the various calcium feeding regimes) the total number of ACF among the groups ranged from 284 to 371 in the order of 1.0%Ca > 0.5%Ca > 0.1%Ca > 2.0%Ca (Table 38 - 18 wks). The number of AC/foci at week 12 was 2.9 and increased by week 18 in the order of 2.0%Ca > 0.1%Ca > 0.5%Ca > 1.0%Ca groups. The number of ACF with a crypt multiplicity of one or two (AC1-2) ranged from 112 to 187. The number of ACF with a crypt multiplicity of 6 or more (AC≥6) remained consistent throughout. The 1.0%Ca regime resulted in an increased number and size of AC≥6 (Figure 19). At week 24 further decline was noted in the total number of ACF as well as the number of AC1-2 and AC3-5 when compared to the values at week 12 and 18 with the 2.0%Ca exhibiting the lowest values. The category of average size indicates the area occupied by each ACF in the various growth stages. The average size of ACF increased with increasing crypt

Figure 18: The effect of varying levels of calcium, introduced 12 weeks post carcinogen administration, on the weight gain of Sprague Dawley rats. Values represent mean group weight (grams) ± S.E.

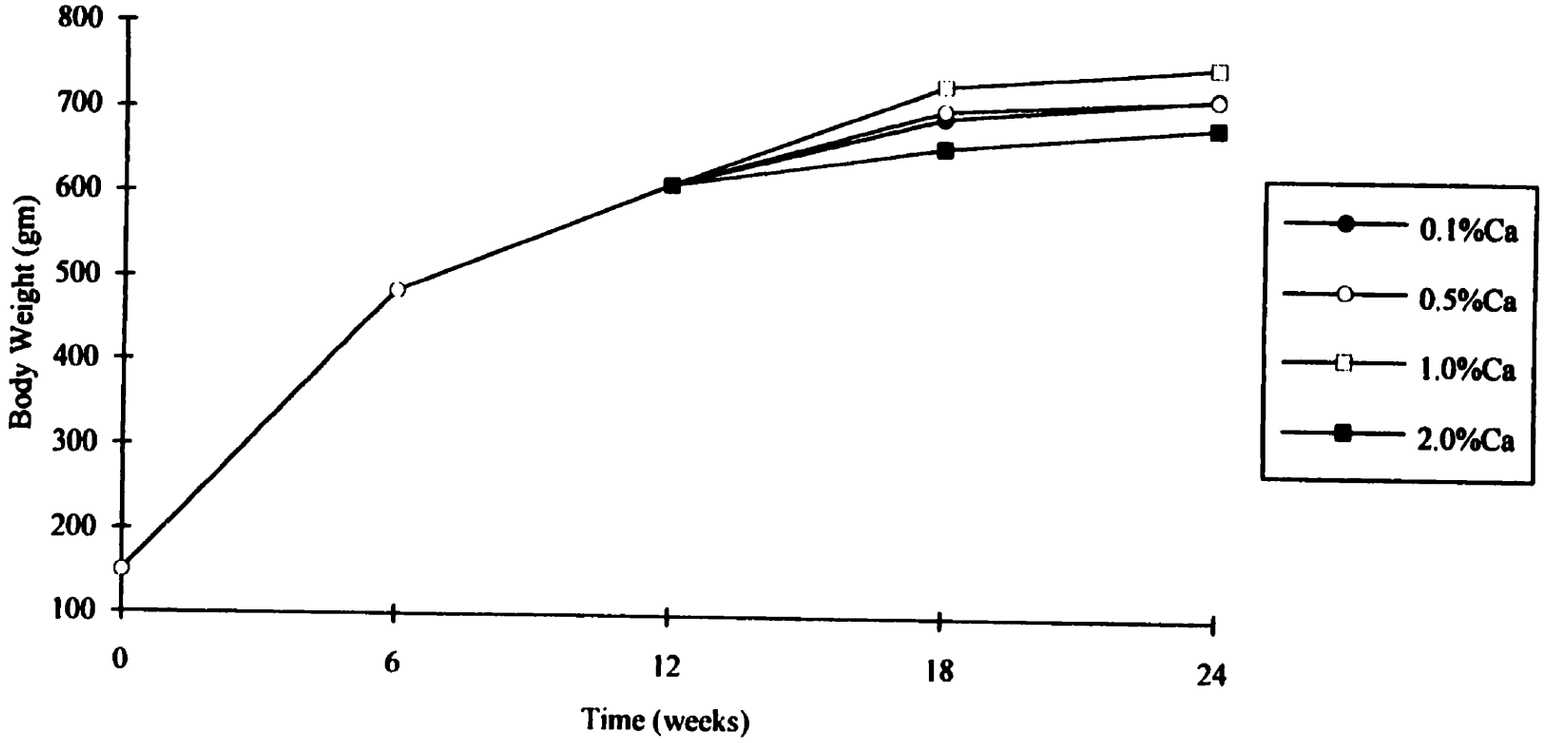

800
700
600
500
400
300
200
100
Body Weight (gm)
0
6
12
18
24
Time (weeks)
0.1%Ca
0.5%Ca
1.0%Ca
2.0%Ca

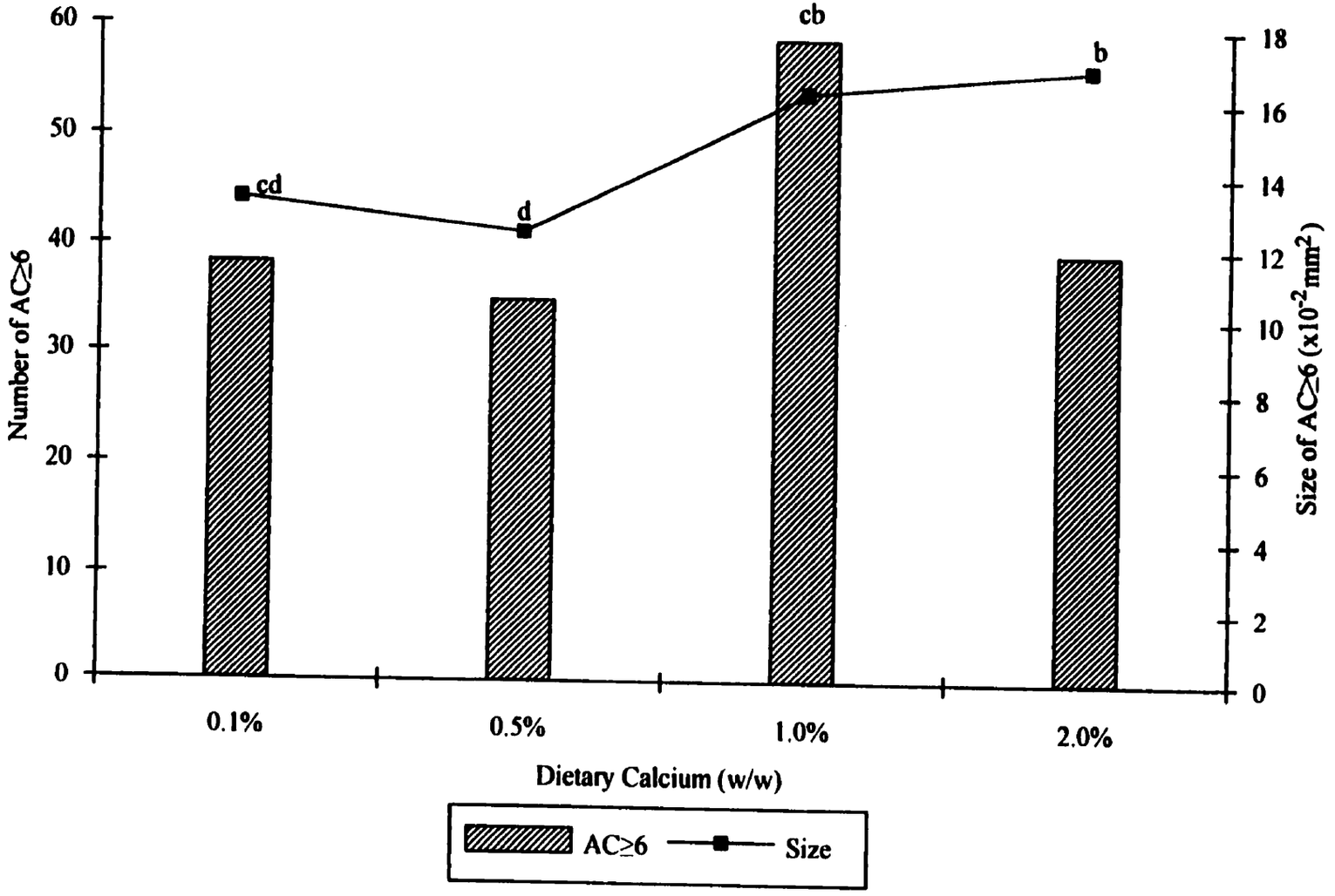
Table 38: The Effect of Varying Levels of Calcium Fed 12 Weeks Post Carcinogen on the Growth and Modulation of Aberrant Crypt Foci with Various Growth Features. [a]

			AC 1-2		AC 3-5		AC≥6	
	Total	AC/focus	Number	Size	Number	Size	Number	Size
6 wks								
0.5% Ca	385.7 ± 24.7	2.9 ± 0.2	199.2 ± 19.5	2.3 ± 0.1	150.7 ± 14.0	5.6 ± 0.1	35.8 ± 9.5	11.4 ± 0.7
12 wks								
0.5% Ca	467.4 ± 105.6	2.9 ± 0.3	262.0 ± 5.7	2.1 ± 0.1	149.4 ± 12.8	6.0 ± 0.3	56.0 ± 17.3	14.0 ± 0.8
18 wks								
0.1% Ca	284.4 ± 18.9 [c]	3.5 ± 0.5	130.2 ± 31.0	2.3 ± 0.3	109.2 ± 9.1	7.2 ± 0.5	45.0 ± 12.6	17.0 ± 2.0
0.5% Ca	314.7 ± 31.0	3.2 ± 0.1	154.5 ± 12.7	2.4 ± 0.2	121.0 ± 13.4	6.8 ± 0.3	39.2 ± 7.9	14.9 ± 1.7
1.0% Ca	371.6 ± 35.6 [b]	3.1 ± 0.3	187.6 ± 25.3 [b]	2.2 ± 0.2	136.6 ± 12.7	7.5 ± 0.7	47.4 ± 12.7	15.8 ± 1.1
2.0% Ca	279.0 ± 16.8 [c]	3.9 ± 0.5	112.0 ± 14.1 [c]	2.4 ± 0.1	116.4 ± 8.6	7.3 ± 0.5	50.6 ± 14.3	17.0 ± 2.2
24 wks								
0.1% Ca	230.4 ± 17.4	3.6 ± 0.3	90.2 ± 7.4 [b]	2.4 ± 0.1 [c]	101.8 ± 12.3	5.8 ± 0.3 [c]	38.4 ± 10.3	13.3 ± 0.8 [cd]
0.5% Ca	250.6 ± 29.9	3.3 ± 0.2	120.0 ± 17.2 [b]	2.2 ± 0.1 [c]	95.6 ± 10.2	5.6 ± 0.2 [c]	35.0 ± 8.2	12.4 ± 0.8 [d]
1.0% Ca	277.2 ± 25.3 [b]	3.9 ± 0.2	96.0 ± 7.8 [b]	2.6 ± 0.1 [c]	122.4 ± 13.7	6.4 ± 0.3	58.8 ± 11.9	16.2 ± 0.9 [cb]
2.0% Ca	195.8 ± 8.8 [c]	4.0 ± 0.2	56.7 ± 9.4 [c]	3.1 ± 0.2 [b]	99.7 ± 2.4	7.5 ± 0.6 [b]	39.5 ± 7.0	16.9 ± 1.4 [b]

[a] Values represent the means ± S.E. Size (x 10^2 mm^2)

[b-d]: Means within a column with different superscripts differ at $p \leq 0.05$.

Figure 19: The number and size of AC≥6 in Sprague Dawley rats treated with azoxymethane after 12 weeks of receiving 0.1%, 0.5%, 1.0%, or 2.0% calcium (24 week time point). Values represent groups means. Means with different superscripts differ from corresponding calcium groups ($p<0.05$).


60
50
40
30
20
10
0
Number of AC≥6
18
16
14
12
10
8
6
4
2
0
Size of AC≥6 (x10^{-2}mm^2)
cd
d
cb
b
0.1%
0.5%
1.0%
2.0%
Dietary Calcium (w/w)
AC≥6
Size

multiplicity (Figure 20). By week 24 the 2.0%Ca group had larger (dilated) crypts compared to the 0.1%Ca or 0.5%Ca groups for both AC3-5 and AC≥6 ($p<0.05$) as well as the 0.1%Ca, 0.5%Ca, or 1.0%Ca groups for AC1-2 ($p<0.05$). The effect of dietary intervention on ACF in various segments of the colon is presented in Table 39. In general, at 18 weeks the largest proportion of ACF were found in the 8.0-11.9 cm region of the colon and this was increased from the 12 week time point. In contrast, the number of ACF in the 0.0-3.9 cm and 4.0-7.9 cm regions dropped significantly at 18 weeks in all levels of calcium relative to the 12 week time point. At 24 weeks animals fed the 2.0%Ca diet harbored fewer ACF in the 0.0-3.9 cm and 4.0-7.9 cm regions, and exhibited ACF with significantly dilated crypts in all segment divisions of the colon. Table 40 illustrates the growth pattern of ACF with advanced growth features, AC≥6, along the length of the colon. At 18 weeks the majority of AC≥6 were found in the 4.0-7.9 cm and 8.0-11.9 cm colonic regions, although most predominantly in the latter section. At 24 weeks 1.0%Ca and 2.0%Ca fed animals exhibited large dilated crypts in each region of the colon. ACF categorized by size are presented in Table 41. At 6 and 12 weeks ACF were predominantly (62% and 66%, respectively) classified in the smallest size grouping while only 2% and 4% were found in the largest size category. At 18 weeks only 55% of the ACF remained in this size grouping and by 24 weeks animals fed the 2.0%Ca diet had significantly fewer (36.9%) small ACF. In contrast the number of ACF in the large size categories had grown in the 1.0%Ca and 2.0%Ca fed animals to almost double those of the 0.1%Ca and 0.5%Ca diets.

Tumor incidence and number. Induction of tumors in the present investigation was high but a recent study with a similar carcinogen protocol (2 x 12 mg/kg b.w. AOM) in the presence of a tumor promoting diet produced an 85% colonic tumor incidence (Pence et al., 1995) which is in keeping with the observed tumor incidence. The incidence of tumors was not statistically affected by delayed calcium intervention, however, growth

Figure 20: The modulating effect of various levels of calcium on the size of ACF at various growth stages after 12 weeks exposure (24 week time point). Values represent group means. Mean with different superscripts differ from corresponding calcium groups ($p<0.05$).

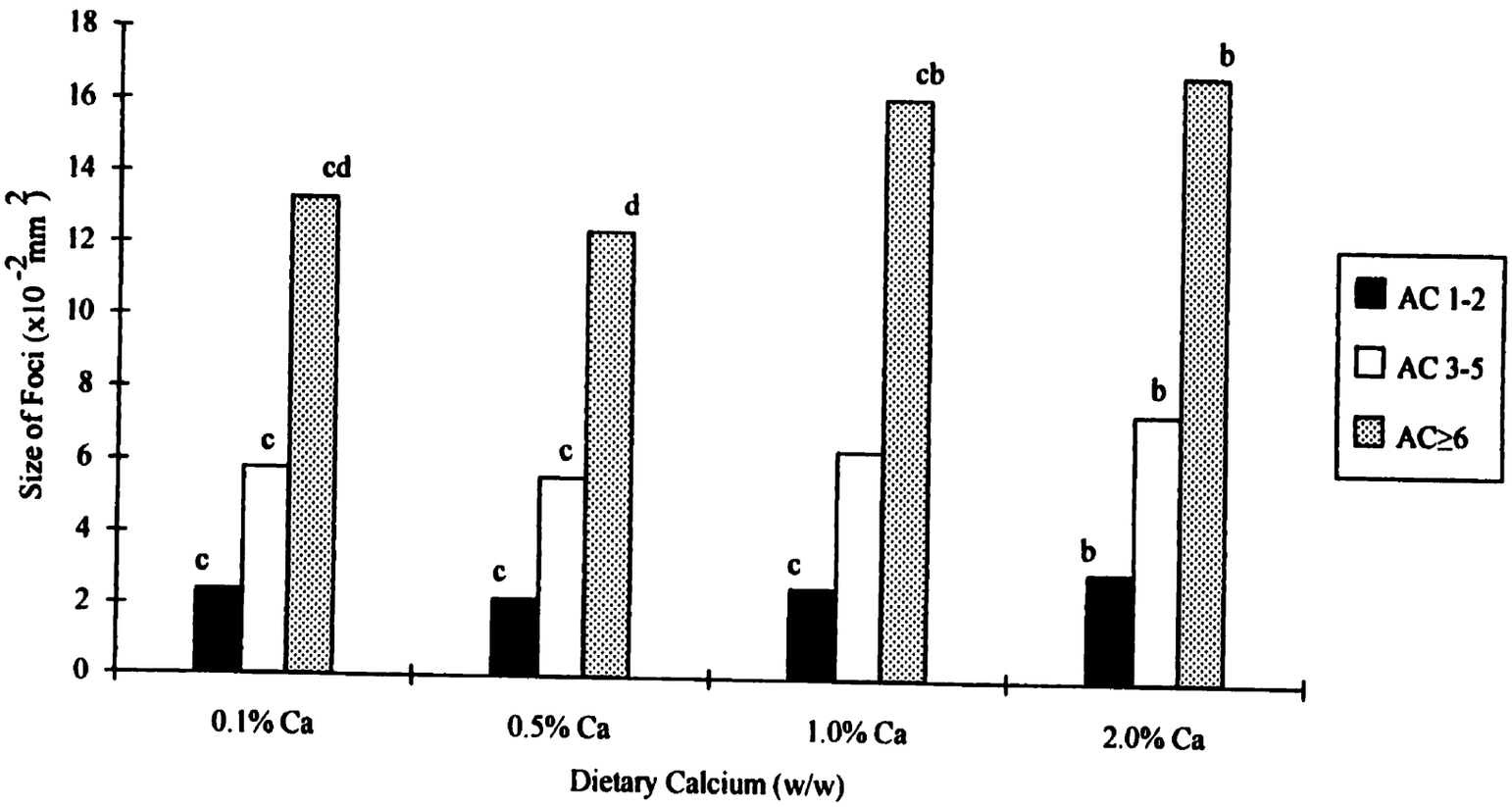

Size of Foci (x10^{-2}mm^2)
18
16
14
12
10
8
6
4
2
0
c
c
cd
c
c
d
c
cb
b
b
b
0.1% Ca
0.5% Ca
1.0% Ca
2.0% Ca
Dietary Calcium (w/w)
AC 1-2
AC 3-5
AC≥6

Table 39: The Effect of Varying Levels of Calcium Fed 12 Weeks Post Carcinogen on the Growth of Aberrant Crypt Foci Along the Rat Colon. [a]

	0.0 - 3.9 cm		4.0 - 7.9 cm		8.0 - 11.9 cm		12.0 + cm	
	Number	Size	Number	Size	Number	Size	Number	Size
6 wks								
0.5% Ca	89.8 ± 13.8	4.3 ± 0.5	133.3 ± 22.8	4.6 ± 0.4	111.7 ● 8.8	4.6 ± 0.4	50.8 ± 18.8	3.8 ± 1.0
12 wks								
0.5% Ca	162.4 ± 25.4	4.6 ± 0.3	188.6 ± 12.0	5.1 ± 0.3	93.8 ± 30.7	4.0 ± 0.6	22.6 ± 8.1	2.7 ± 0.2
18 wks								
0.1% Ca	69.6 ± 18.9	6.9 ± 1.1 [b]	86.6 ± 15.0	7.7 ± 1.2	97.2 ± 8.2	8.2 ± 1.5	31.0 ± 9.9	4.3 ± 0.8
0.5% Ca	70.5 ± 9.8	4.4 ± 0.4 [c]	85.2 ± 14.1	5.4 ± 0.4	113.8 ± 24.5	6.1 ± 1.0	45.2 ± 27.2	4.1 ± 1.2
1.0% Ca	74.0 ± 11.5	4.1 ± 0.6 [c]	82.8 ± 15.1	6.1 ± 0.5	133.4 ● 10.1	6.9 ± 0.9	81.4 ± 17.6	5.0 ± 0.8
2.0% Ca	52.6 ± 12.2	5.8 ± 0.7	93.6 ± 13.1	8.8 ± 2.2	120.8 ± 19.1	8.0 ± 1.1	33.6 ± 12.9	3.4 ± 0.6
24 wks								
0.1% Ca	81.0 ± 12.9	4.9 ± 0.6	87.6 ± 12.3	6.7 ± 0.8	50.2 ± 11.8 [c]	5.1 ± 0.6 [c]	11.6 ± 7.6	2.9 ± 0.3 [c]
0.5% Ca	66.0 ± 12.1	3.5 ± 0.2 [c]	78.8 ± 17.1	5.1 ± 0.4 [c]	92.2 ± 6.7 [b]	5.7 ± 0.8 [c]	13.6 ± 4.3	3.5 ± 0.6 [c]
1.0% Ca	75.8 ± 10.0	5.2 ± 0.6	99.2 ± 13.1 [b]	7.7 ± 1.0	80.8 ± 11.5 [b]	8.3 ± 0.3 [b]	21.4 ± 5.7	4.3 ± 0.3
2.0% Ca	55.5 ± 8.3	6.0 ± 0.8 [b]	51.5 ± 5.6 [c]	8.3 ± 0.9 [b]	73.5 ± 7.7	10.3 ± 1.2 [b]	15.3 ± 1.6	5.5 ± 0.5 [b]

[a] Values represent the means ± S.E. Size (x 10^2 mm^2)

[b-c] : Means within a column with different superscripts differ at $p \leq 0.05$.

Table 40: The Effect of Varying Levels of Calcium Fed 12 Weeks Post Carcinogen on the Growth of Aberrant Crypt Foci ≥6 Along the Rat Colon.[a]

	0.0 - 3.9 cm		4.0 - 7.9 cm		8.0 - 11.9 cm		12.0 + cm	
	Number	Size	Number	Size	Number	Size	Number	Size
6 wks								
0.5% Ca	5.5 ± 1.8	7.8 ± 1.6	12.3 ± 3.3	11.2 ± 1.0	12.3 ± 4.3	12.1 ± 0.6	5.7 ± 3.9	7.9 ± 2.7
12 wks								
0.5% Ca	16.7 ± 11.2	7.6 ● 3.8	20.3 ± 8.2	14.0 ± 2.2	11.0 ± 9.1	9.4 ± 4.8	0.0 ± 0.0	0.0 ± 0.0
18 wks								
0.1% Ca	4.4 ± 1.0	13.2 ± 1.7	18.6 ± 6.3	16.2 ± 2.2	19.6 ± 6.8	17.8 ± 2.1	2.4 ± 0.9	8.1 ± 2.8
0.5% Ca	4.3 ± 2.3	9.3 ± 3.5	8.7 ± 2.9	13.6 ± 2.1	18.7 ± 5.5	13.1 ± 3.1	7.5 ± 5.2	8.3 ± 3.9
1.0% Ca	4.0 ± 2.0	11.4 ± 2.2	9.0 ± 3.0	16.7 ± 2.0	25.2 ± 7.7	16.8 ± 1.1	9.2 ± 3.0	11.2 ± 3.1
2.0% Ca	5.6 ± 2.4	6.4 ± 2.7	22.0 ± 8.9	17.4 ± 2.6	25.0 ± 5.1	17.6 ± 2.5	2.6 ± 2.1	5.3 ± 3.4
24 wks								
0.1% Ca	10.4 ± 3.9	8.3 ± 2.2	20.0 ± 5.5	14.5 ± 1.5	7.6 ± 3.0 [c]	12.0 ± 3.1	0.4 ± 0.4	2.2 ± 2.2 [c]
0.5% Ca	3.2 ± 1.6	7.9 ± 2.2	12.2 ± 5.2	12.4 ± 1.1 [c]	18.4 ± 5.1	12.1 ± 1.3	1.2 ± 1.2	2.1 ± 2.1 [c]
1.0% Ca	7.8 ± 1.8	13.0 ± 2.2	24.0 ± 6.1	17.0 ± 1.3 [b]	24.4 ± 5.1 [b]	15.7 ± 0.5	2.6 ± 1.1	11.4 ± 4.0 [b]
2.0% Ca	5.2 ± 2.2	11.6 ± 1.5	9.2 ± 2.1	16.3 ± 1.2	23.5 ± 5.0	18.1 ± 1.8	1.7 ± 0.4	12.3 ± 2.8 [b]

[a] Values represent the means ± S.E. Size (x 10^2 mm^2)

[b-c]: Means within a column with different superscripts differ at $p \leq 0.05$.

Table 41: The Effect of Varying Levels of Calcium fed 12 Weeks Post Carcinogen on Aberrant Crypt Foci in Various Size (x 10^{-2} mm^2) Categories [a]

	1.0 - 4.9	5.0 - 9.9	10.0 - 14.9	15.0 +
6 wks				
0.5% Ca	240.7 ± 19.4	115.7 ± 11.4	21.3 ± 5.2	8.0 ± 4.1
12 wks				
0.5% Ca	293.8 ± 13.3	112.6 ± 1.6	18.4 ± 3.6	20.3 ± 4.7
18 wks				
0.1% Ca	145.8 ± 31.2	73.4 ± 5.0	32.2 ± 7.4	31.6 ± 9.0
0.5% Ca	172.5 ± 17.4	90.0 ± 11.6	32.3 ± 5.3	19.7 ± 4.7
1.0% Ca	210.2 ± 27.8	96.6 ± 12.3	39.4 ± 7.2	30.4 ± 7.6
2.0% Ca	144.0 ± 23.6	82.6 ± 9.5	39.8 ± 3.1	33.8 ± 10.3
24 wks				
0.1% Ca	122.8 ● 8.9 [b]	72.6 ± 9.2	20.4 ± 5.2 [c]	14.4 ● 6.3
0.5% Ca	150.8 ± 18.7 [b]	71.2 ± 10.0	18.6 ± 3.5 [c]	18.6 ± 3.1
1.0% Ca	126.0 ± 12.0 [b]	82.8 ± 10.1	39.4 ± 7.4 [b]	29.0 ● 4.7
2.0% Ca	72.2 ± 10.6 [c]	64.0 ● 3.4	32.3 ● 4.1	27.3 ± 6.7

[a] Values represent means ± S.E.

[b-c] :Means within a column with different superscripts differ at $p \leq 0.05$.

patterns exhibited a varied rather than dose-dependent response to dietary changes (Table 42). Total tumor incidence, the incidence of adenomas, and the size of adenocarcinomas was highest in the 0.5%Ca (AIN recommended level) fed animals and lowest in the latter two categories for animals fed the 2.0%Ca diets. Animals fed the 1.0%Ca diet had the highest number of tumors per tumor-bearing rat and incidence of adenocarcinomas. The incidence of adenomas exhibited a biphasic response to altered dietary calcium where animals consuming the 2.0%Ca diet harbored the fewest adenomas. Tumor incidence along the colon length is shown in Table 43. It was important to note that the 0.1%Ca group had considerably lower (5 versus 11-12) number of tumors in the most proximal region and higher in the most distal region (10 versus 6-8) compared to the other groups. The total number of tumors identified and their average size are shown in Table 44. An important observation was that average size of tumors was smallest in the 2.0%Ca group. Most importantly the variability among the size of the tumors was consistently lower in the 2.0%Ca group than the other groups. This trend was quite evident in the most proximal region when tumor number and sizes were considered. Among 0.5%Ca, 1.0%Ca, and 2.0%Ca groups the number of tumors were 12 or 14 the average size ranged from 27 - 29.5 and the S.E. ranged from 6.3 to 9.4. The lowest value was for the 2.0%Ca group. In general, tumors were found primarily in the 8.0-11.9 cm region of the colon. The frequency distribution of tumor incidence, number of tumors, AC≥6 at 18 and 24 weeks is shown in Figure 21 and indicates that tumor development along the length of the colon correlates well with aberrant crypts with advanced growth characteristics. Tumor size was generally smallest in animals fed the 2.0%Ca diet except in the 12.0+ cm of the colon.

Table 42: The Effect of Varying Levels of Calcium Fed 12 Weeks Post Carcinogen on Tumor Pathology. [a]

	Total Tumor Incidence [a]	# Tumors/Rat with Tumors	Tumor Burden [b]	Adenomas		Adenocarcinomas	
				Rats with Tumors [a]	Size [c]	Rats with Tumors [a]	Size [c]
0.1% Ca	25/33 (75.8%)	2.1 ± 0.3	48.8 ± 20.5	19/33 (57.6%)	5.6 ± 0.7	12/33 (36.4%)	43.1 ± 8.0
0.5% Ca	25/31 (80.6%)	2.0 ± 0.3	40.0 ± 12.1	20/31 (64.5%)	4.2 ± 0.6	12/31 (38.7%)	53.9 ± 13.3
1.0% Ca	22/31 (71.0%)	2.4 ± 0.3	51.2 ± 15.4	14/31 (45.2%)	4.1 ± 0.6	17/31 (54.8%)	45.2 ± 11.2
2.0% Ca	26/35 (74.3%)	2.0 ± 0.3	34.5 ● 4.7	14/35 (40.0%)	5.2 ± 0.6	13/31 (37.1%)	31.5 ± 3.2
	p = 0.757			p = 0.092		p = 0.304	

[a] Number of rats with tumors/number of rats (% of total).

[b] Total tumor area (mm^2) ± S.E. in rats with tumors.

[c] Mean size of tumors (mm^2) ± S.E.

Table 43: The Effect of Varying Levels of Calcium Fed 12 Weeks Post Carcinogen on Tumor Incidence Along the Length of the Rat Colon. [a]

	n =	0.0 - 3.9 cm		4.0 - 7.9 cm		8.0 - 11.9 cm		12.0 + cm	
0.1% Ca	33	10	(30.3%)	9	(27.3%)	17	(51.5%)	5	(15.2%)
0.5% Ca	31	8	(25.8%)	5	(16.1%)	17	(54.8%)	11	(35.5%)
1.0% Ca	31	7	(22.6%)	11	(35.5%)	12	(38.7%)	12	(38.7%)
2.0% Ca	35	6	(17.1%)	7	(20.0%)	18	(51.4%)	11	(31.4%)
		p = 0.286		p = 0.316		p = 0.416		p = 0.180	

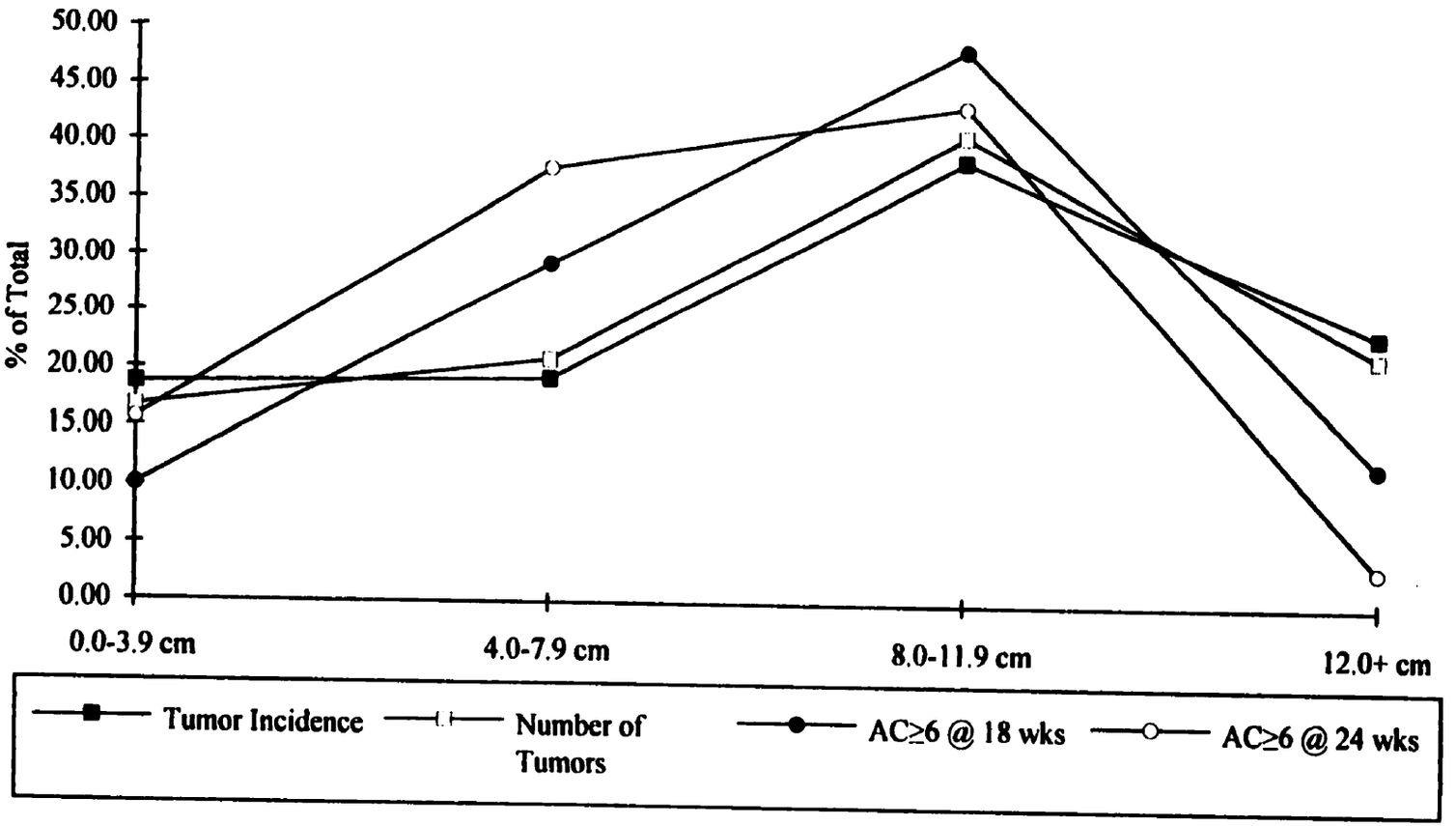
[a] Number of rats with tumors (% of total).

Table 44: The Effect of Varying Levels of Calcium Fed 12 Weeks Post Carcinogen on the Total Number of Tumors and Tumor Size Along the Length of the Rat Colon[a]

	Total Colon	0.0 - 3.9 cm	4.0 - 7.9 cm	8.0 - 11.9 cm	12.0+ cm
0.1% Ca	(53) 20.6 ± 4.1	(12) 25.7 ± 14.4	(14) 19.9 ± 7.1	(22) 19.0 ± 4.2	(5) 22.7 ± 13.0
0.5% Ca	(51) 19.2 ± 5.0	(9) 28.1 ± 24.6	(8) 17.1 ± 7.3	(21) 11.2 ± 3.7	(13) 28.8 ± 9.4
1.0% Ca	(54) 21.6 ± 5.5	(7) 19.9 ± 15.5	(14) 10.0 ± 2.6	(19) 26.7 ± 12.5	(14) 27.1 ± 10.1
2.0% Ca	(51) 17.6 ± 2.4	(7) 10.0 ± 4.7	(8) 13.4 ± 3.0	(23) 14.6 ± 3.0	(13) 29.5 ± 6.3
	p = 0.680	p = 0.511	p = 0.193	p = 0.630	p = 0.087

[a] (Number of tumors) and mean tumor size (mm^2) ± S.E.

Figure 21: Frequency distribution of tumor incidence, number of tumors, and number of AC≥6 at both 18 and 24 week time points along the length of the rat colon. Values represent group means.


50.00
45.00
40.00
35.00
30.00
25.00
20.00
15.00
10.00
5.00
0.00
% of Total
0.0-3.9 cm
4.0-7.9 cm
8.0-11.9 cm
12.0+ cm
Tumor Incidence
Number of Tumors
AC≥6 @ 18 wks
AC≥6 @ 24 wks

4. Discussion

The purpose of the present study was to systematically examine the effects of delayed calcium intervention on the development of colon carcinogenesis. It was observed that delayed introduction of dietary calcium modulates the disease process, as indicated by significant changes in ACF, and disease outcome, as indicated by changes in tumor characteristics albeit not statistically significant. The findings illustrate that the effects of supplemental calcium are complex and that cancer development does not respond in a predictable manner to a dose-dependent increase in dietary calcium. Evaluation of ACF in separate divisions of the colon revealed that a majority of ACF and the select population with advanced growth features were found in the same region where the number of tumors and tumor incidence was greatest. The addition of 2.0%Ca to the diet of animals consuming a tumor promoting high fat diet led to changes in ACF with a decrease in the total number primarily due to a significant decrease in the appearance of small ACF. Animals receiving the highest calcium dose had consistently large dilated crypts in: each ACF growth category; the various regions along the length of the colon for both the total number of ACF and ACF with advanced growth characteristics; and the number of ACF grouped into large size categories. The incidence of adenomas was lowest in 2.0%Ca fed animals, however, this effect was not seen in total tumor incidence or incidence of adenocarcinomas. Conversely, animals consuming a 1.0%Ca diet exhibited elevated tumor growth characteristics evidenced by an increased number of tumors per tumor bearing rat and incidence of adenocarcinomas. The size of adenocarcinomas, but not adenomas, was reduced in the highest (2.0%Ca) calcium group. In addition, the variability within experimental groups was dramatically lower in the 2.0%Ca group. Overall, although there was no statistically significant effect of dietary calcium on tumor incidence some similarities between dietary modulation patterns of ACF and tumor outcome were observed. The primary findings of this study suggest that delayed calcium

intervention in a high risk dietary environment may modulate the disease process. However, the effects of dietary manipulation are subtle and may not be able to alter the course of well established premalignant lesions but may modulate the growth of established ACF in their early stages (AC1 or AC2). Further research is required to define the role of calcium as a chemopreventive agent in the multistep process of colon carcinogenesis. The possibility that a high calcium diet may retard the development of select colonic tumors of specific phenotype must be considered. A very high calcium diet (2.0%) suppressed the growth of tumors in the rectal regions but not in the proximal regions. This is supported by the findings of Karkare et al. (1991) where 2.0%Ca diets fed from the initiation of the experiment reduced distal and not proximal colonic tumor outcome. High risk individuals with adenomatous lesions mainly in the rectal regions may benefit from calcium supplementation.

It is proposed that ACF represent preneoplastic lesions of colon cancer useful as biological end points in identifying modulators of colon carcinogenesis (Bird, 1987; Bird et al., 1989; McLellan & Bird, 1991; McLellan & Bird, 1988) and in understanding the stepwise development of colon cancer. Phenotypic and genotypic atypia demonstrated in ACF have supported the contention that ACF are preneoplastic lesions (McLellan et al., 1991a; Smith et al., 1994; Stopera et al., 1992a; Stopera et al., 1992b). Understanding the growth characteristics of ACF is critical in establishing the reliability of ACF as study end points (Bird & Lafave, 1995). ACF with increased crypts per focus are thought to represent lesions with advanced growth characteristics and this increased crypt multiplicity has been found to be an early and persistent predictor of eventual tumor outcome (Pretlow et al., 1992; Zhang et al., 1992; Magnuson et al., 1993). In the present investigation the distribution of ACF and tumors along the length of the colon was evaluated. At 18 weeks the majority of ACF and AC$\geq$6 were found in the mid colon (8.0-11.9 cm) which was also the region which had higher number of tumors by week 24.

Shivapurkar et al. (1992) also reported that the distribution of tumors along the length of the colon resembled the distribution pattern of ACF. Recently, Carter et al. (1994) reported that numerical density of lymphoid nodules predicted distribution of tumors along the length of the colon and that ACF had little malignant potential. Several factors may explain why these conclusions differ from those of the present study and that of Shivapurkar et al. (1992). Carter et al. (1994) employed a multiple injection protocol, as compared to the two injection protocol used in the present investigation. It has been demonstrated that variances in carcinogen administration produce altered biological responses (Bird and Lafave, 1995). Carter et al. (1994) utilized the murine colon carcinogenesis model whereas the rat model was employed in the present investigation. Response differences to carcinogen and dietary modifications have been reported between the murine and the rat model (Augellone, 1992). Carter et al. (1994) compared ACF and tumor distribution simultaneously at 24 weeks, thus the authors evaluated the coincident appearance, rather than the predictive value, of ACF. The ability of ACF with higher crypt multiplicity to predict eventual tumor outcome has been reported at 8, 12, and 14 weeks with eventual tumor outcome measured at 18, 36, and 34 weeks (Pretlow et al., 1992; Zhang et al., 1992; Magnuson et al., 1993). The results of the present study indicate that ACF with advanced growth characteristics appear to predict overt tumor formation along the length of the colon and thus supports AC≥6 as a valid indicator of overall tumor risk.

The results of the present investigation lend further support to the sequential growth characteristic of ACF. The total population of ACF increased up to 12 weeks and then dropped in the two subsequent time points. This is similar to the cyclic ACF growth pattern reported in rats treated with both a single (McLellan et al., 1991b) and double (Magnuson et al., 1993) carcinogen injection protocol. These findings together support the contention that some lesions are prone to regress or remodel and are thus

eliminated. New ACF continue to form long after carcinogen exposure. Magnuson et al. (1993) reported a dramatic increase in small ACF twelve weeks post-carcinogen administration and McLellan et al. (1991b) documented a similar increase 20-32 weeks post-carcinogen administration. Accordingly, in the present investigation an increase in small ACF was observed at 12 weeks post-carcinogen treatment. Six and 12 weeks post-dietary intervention 2.0%Ca fed animals harbored significantly fewer small ACF. This finding suggests that perhaps 2.0%Ca inhibits the development of new ACF. The finding that the total ACF population in animals fed the 1.0%Ca diet was accompanied by elevated values first in small ACF (18 weeks) progressing to those with higher crypt multiplicity (24 weeks) further illustrates the complex nature of calcium supplementation.

The currently held dogma of the multistage development of cancer involves the emergence of a few select cells from a larger population of altered cells to continue on the path toward cancer, the ultimate manifestation of the disease. From each population only a select group continue such that at each stage some cells have greater malignant potential than others. The ability to identify cells with greater or lesser malignant potential would greatly enhance our understanding of the early changes occurring in carcinogenesis. ACF are composed of a heterogeneous group of lesions some of which appear to be prone to regress or remodel whereas others seem to have a selective altered growth advantage. Magnuson et al. (1993) found that a 0.2% cholic acid diet promoted only those lesions with the highest potential (ACF with higher crypt multiplicity) to progress to malignancy. Bird and Lafave (1995) found that a high carcinogen dose, known to produce significant tumor incidence, resulted in the formation of ACF with constricted crypts as compared to a low carcinogen dose which produced very dilated (large in size) ACF. This suggested that dilated crypts could be less stable and represent a segment of the ACF population amenable to regression or remodeling. Shirtliff and Bird (1996) reported that the 0.2% cholic acid diet, which had been shown to promote only ACF with a high

malignant potential, eliminated ACF with dilated crypts leaving only those with constricted crypts to continue to the next stage. This supported the contention that constricted, and not dilated, ACF crypts possessed the high potential to progress to malignancy. In the present investigation animals fed the 2.0%Ca diet possessed ACF with dilated crypts in all growth categories at 24 weeks without an increase in the number of AC≥6. In addition the incidence of adenomas displayed a biphasic response where the 0.5%Ca group expressed the highest and the 2.0%Ca group the lowest incidence levels. This finding suggests that the delayed introduction of calcium alters the progress of certain lesions. One possibility is that the observation indicates that the high calcium diet stagnates the growth development of ACF. Another possibility is that the dilated crypts represent a stage of regression or remodeling being promoted by the high calcium diet. Whether these ACF with dilated crypts represent a differentiated phenotype needs to be assessed and would have to be considered in concert with other growth features of ACF.

The biological effect of calcium on colon carcinogenesis is complex and dependent on the level in the diet. It was observed that while feeding 2.0%Ca reduced the total number of ACF and those with 1 or 2 crypts per focus the exact opposite response was demonstrated in those animals consuming the 1.0%Ca diet. To our knowledge this is the first report that has investigated four levels of calcium on colon tumorigenesis. Tumor incidence was found to be reduced in animals fed a 1.0%Ca (Pence and Buddingh, 1988) and 1.5%Ca (Skrypec and Bursey, 1988) diet whereas no effect was observed in those fed a 1.6%Ca (McSherry et al., 1989) diet. Karkare et al. (1991) investigated two levels of calcium supplementation (1.0% & 2.0%) but found no effect on tumor incidence. The aforementioned studies all evaluated the effects of calcium intervention immediately after carcinogen administration. Recently, Pence et al. (1995) delayed calcium intervention by 38 weeks and found no protective effect by dietary calcium on tumor incidence. Moreover, animals receiving delayed supplemental (2.0%) calcium exhibited a

significantly greater number of tumors per rat. In the present investigation the 1.0%Ca, but not the 2.0%Ca, group exhibited an elevated number of tumors per rat, however, no significant effect of calcium on tumor parameters was demonstrated. While the two study protocols were similar with respect to carcinogen type and dose, the study by Pence et al. (1995) delayed calcium intervention by 38 weeks. At this point the authors suggested that many lesions were at the treatment stage and in fact 20% of animals had died by this time interval. It has been suggested that once cells have been transformed a high calcium environment selectively enhances the growth of these cells while suppressing the proliferation of normal cells (Whitfield, 1992). Consistent with our results, Karkare et al. (1991) found that although the effect of calcium did not achieve statistical significance, animals receiving 1.0%Ca (but not 2.0%Ca) had an increased incidence of adenocarcinoma and that the incidence of adenomas demonstrated a biphasic response to the dose increase in dietary calcium introduced 1 week after carcinogen administration. This continuity in findings suggests that while delayed calcium intervention may have potential for harm, a delay of 12 weeks does not seem to alter the modulating effect drastically from immediate intervention.

The results of this study demonstrate that delayed calcium introduction does affect growth characteristics of ACF, however, the effects may be too subtle to significantly alter established premalignant growth. The highest calcium diet appeared to exert a possible chemopreventive effect exhibited by the inhibition of small ACF formation and the build up of ACF with dilated crypts, which are reported to be easily eliminated or remodeled (Shirtliff & Bird, 1996). To our knowledge this is the first study to examine the sequential dietary calcium increase in a delayed intervention design protocol. It would be valuable to sequentially delay calcium introduction to investigate the optimal timing for maximizing the biological effect of calcium on disease outcome.

Chapter 10.

A ROLE FOR DIETARY CALCIUM AS A SYSTEMIC MODULATOR

1. Introduction

The proposed protective effect of dietary calcium was first thought to involve luminal binding of free fatty acids and bile acids by calcium (Newmark et al., 1984). Subsequently, many non-luminal mechanisms have been proposed (Pence, 1993). Considering that dietary calcium has been shown to decrease mammary gland cell proliferation (Jacobson et al., 1989; Zhang et al., 1987) a non-luminal mechanism of action was plausible. Studies on calcium metabolism indicate that only 30-50% of ingested calcium is normally absorbed and efficiency increases or decreases based on physiologic need leaving the unabsorbed calcium to be voided in feces (Arnaud & Sanchez, 1990). This would suggest that once dietary calcium requirements are achieved the excess calcium should spill over into the colon thus preventing further systemic outcomes. However, this proposal does not explain marked changes in the mammary gland with respect to proliferative status or carcinogenesis in response to low or a high calcium diets. A variety of eicosanoids have been implicated in colon cancer (Rigas et al., 1993). A potential role of leukotriene B_4 (LTB_4) in carcinogenesis is supported by such findings as the stimulation of colon carcinoma cells *in vitro* by LTB_4 (Qiao et al., 1995) and the stimulation of LTB_4 production by known colon tumor promoters lithocholic acid and deoxycholic acid (Dias et al., 1994). Although several cell types are capable of producing LTB_4 from arachidonic acid, neutrophils produce LTB_4 as their major arachidonic acid metabolite (Feinstein & Sha'afi, 1983). The activation of neutrophils and subsequent production of LTB_4 is brought about by the mobilization of calcium (Feinstein & Sha'afi, 1983). In spite of an association made for calcium in the development of colon cancer,

very little is actually known about the mechanism by which it may modulate the disease process. The main objective of this study was to investigate a distinctly non-luminal effect of dietary calcium. The hypothesis that the dietary calcium exerts a systemic effect was tested by examining varied levels of dietary calcium on LTB_4 production and associated membrane fatty acid profiles. Blood neutrophils were selected as the target tissue and stimulated with calcium ionophores A23187 and thapsagargin. Both of these compounds mobilize calcium from intracellular stores and stimulate LTB_4 production (Mead et al., 1986; Hakii et al., 1986).

2. Materials and Methods

Animals. Male Sprague Dawley rats approximately five to six weeks old were acclimatized for one week prior to initiating experimental procedures.

Experimental Diets. The formulated diets were based on the AIN-76A diet (American Institute of Nutrition, 1977; 1980) with the exception of $CaHPO_4$. The basal low fat diet was made according to the specifications of the AIN-76A diet. To achieve the high-fat diet, an additional 15% fat by weight was added as beef tallow at the expense of carbohydrate. The nutrients per kilocalorie density of the diets were identical except for fat and carbohydrate. Calcium (Ca) levels were altered by adding $CaCO_3$ to the base $CaHPO_4$ in the mineral mix to maintain the phosphate level in the diet at control levels. To achieve the low calcium diet (0.1% Ca), 0.4% $NaHPO_4$ was added to 0.1% $CaHPO_4$ to maintain the phosphate level in the diet mix. All diets are itemized in Appendix I.

Study Design. Animals were injected with a two doses of azoxymethane (15 mg/kg) 1 weeks apart and fed the AIN-76A basal diet till one week after the last carcinogen treatment. Animals were then randomized into 2 dietary groups and fed the basal diet (10 animals/group) or the high fat diet (30 animals/group). At twelve weeks animals fed the high fat diet were further randomized into 3 groups and fed 0.1%, 0.5%,

and 1.0% calcium AIN modified diets (10 animals/group) for the remainder of the study (Figure 22). Animals on the basal low fat diet remained on this diet for the full 24 weeks. Animals were terminated at 24 weeks by carbon dioxide asphyxiation. Colons were removed immediately after termination and flushed with phosphate buffered saline (PBS), slit open from cecum to anus, scraped and mucosa from 2 animals combined for neutrophil isolation.

Leukotriene Analysis. Neutrophil isolation. Neutrophils were prepared as previously described with some slight modifications (Mahavdepa et al., 19??). Blood was centrifuged at 300 x g for 20 min at 19°C. The top layer platelet rich plasma was removed up to 5 mm from the top of the red blood cell surface. Five mL of 6% dextran T-500 in a 0.9% NaCl solution was added, the volume made up to 50 mL with 0.9% NaCl solution, mixed well, and red blood cells allowed to settle out at room temperature for 45 min. The supernatant was immediately removed and centrifuged at 300 x g for 12 min at 19°C. The top layer was aspirated off to approximately 5 mm from the top of the pellet and the pellet then resuspended in 8 mL 0.9% NaCl solution. The suspension was then layered on 3 mL of Ficoll-Paque and then centrifuged at 750 x g for 25 min at 19°C. All remaining steps required ice cold solutions and centrifugation at 4°C. The upper layer was removed up to approximately 4 mm above the pellet, 5 mL of ice cold ddH_2O was added to the pellet solution, and centrifuged at 300 x g for 6 min at 4°C. Contaminating red cells were removed by repeating the above lysis steps. The supernatant was removed, the pellet resuspended in 10 mL PBS, and centrifuged at 300 x g for 6 min at 4°C. The supernatant was removed, the pellet resuspended in a known small volume of PBS, and neutrophils counted. The volume was then brought up to give a cell count of 1.2 x 109 cells/mL. The neutrophils were activated with either 3 μM A23187 or 1μM thapsagargin at 37°C in a shaking water bath for 5 min. The reaction was stopped with 400 μL of ice cold methanol and stored in a -80°C freezer until analyzed.

Figure 22: Schematic representation of the study design used in the study of varying levels of calcium fed 12 weeks post carcinogen on *ex vivo* stimulated neutrophil LTB_4 production and membrane fatty acid composition.

Leukotrienes were analyzed by precolumn extraction HPLC. A Millipore-Waters Model 600E gradient controller was used to deliver the mobile phases. Products were detected at 235 nm by use of a Millipore-Waters Model 490E programmable multi-wavelength detector. The stationary phase was a Millipore-Waters Novapak C_{18} column (4 μM particle size; 3.9 x 150 nm). The mobile phase consisted of a linear gradient between water:acetonitrile:acetic acid (80:20:0.02) and water: acetonitrile:methanol:acetic acid (28:33:39:0.02) over 60 min. The flow rate was 2 mL/min. The sample was loaded onto a Millipore-Waters Novapak C_{18} Guard-PAK precolumn by the use of a Beckman 110B solvent delivery module in line with a Millipore-Waters 700 satellite WISP automatic injector. 19-HydroxyPGB_2 was used as an internal standard to correct for recovery.

Analysis of neutrophil fatty acids. Lipids were immediately extracted as described by Folch et al. (1957) using chloroform/methanol (2:1, vol/vol). Phospholipids were separated by thin-layer chromatography using Silica Gel Merck 60 precoated plates and fatty acids separated by gas-liquid chromatography as described previously.

Statistical Analysis. Statistical analysis of the data was performed using the analysis of variance (ANOVA) as carried out by the SAS statistical software for microcomputers. Duncan's Multiple Range test was used to separate treatment means when the ANOVA indicated significant differences. Differences were regarded as significant if $p<0.05$.

3. Results

Body weights. Animals gained weight in a uniform manner and dietary calcium did not alter weight gain at the end time point (data not shown).

Leukotriene products. The effects of altered dietary calcium levels on LTB_4 and other derivatives is shown in Table 45. In general a dose dependent decrease in A23187-

Table 45: The Effect of Varying Levels of Calcium Fed 12 Weeks Post Carcinogen on 5-Lipoxygenase Metabolites (ng's per 1.2 x 10^9 cells/mL) formed in Response to Stimulation with A23187 or Thapsigargin [a]

	20-COOH-LTB$_4$	20-OH-LTB$_4$	6-trans-LTB$_4$	6-trans,12 epi-LTB$_4$	LTB$_4$	Total LTB$_4$
A23187						
0.5% CaLF	180.5 ± 38.8	58.5 ± 8.2 [cb]	92.0 ± 18.4	136.5 ± 30.7 [c]	986.5 ± 252.9 [dc]	1454.0 ± 331.2 [dc]
0.1% CaHF	259.8 ± 35.8	73.0 ± 11.3 [cb]	162.0 ± 13.5	257.3 ± 23.6 [b]	1573.3 ± 91.4 [b]	2325.3 ± 139.2 [b]
0.5% CaHF	245.3 ± 21.1	93.2 ± 16.5 [b]	136.0 ± 19.7	196.5 ± 21.0 [cb]	1451.3 ± 114.6 [cb]	2122.3 ± 141.0 [cb]
1.0% CaHF	174.5 ± 26.8	47.5 ± 7.0 [c]	111.5 ± 43.7	117.0 ± 27.0 [c]	717.8 ± 162.5 [d]	1168.3 ± 232.7 [c]
Thapsigargin						
0.5% CaLF	133.0 ± 28.2	41.8 ± 6.2	57.5 ± 13.1	60.3 ± 16.2	586.3 ± 151.2	878.8 ± 193.6
0.1% CaHF	193.5 ± 25.2	61.5 ± 11.0	74.5 ± 21.6	124.0 ± 47.4	771.0 ± 185.0	1224.5 ± 284.5
0.5% CaHF	199.8 ± 37.9	57.8 ± 17.8	127.8 ± 34.5	120.3 ± 19.5	1030.8 ± 258.5	1536.3 ± 334.7
1.0% CaHF	131.0 ± 17.6	38.5 ± 4.6	78.2 ± 51.0	61.5 ± 31.2	560.0 ± 134.6	869.3 ± 220.7

[a] Values represent means ± S.E.

[b-d] :Means within a column with different superscripts differ at $p \leq 0.05$.

stimulated LTB_4 was observed in response increased dietary calcium (Figure 23). Furthermore, high (1.0%) calcium reduced LTB_4 levels on par with animals receiving the low fat (basal) diet throughout the experiment. The other LTB_4 products exhibited similar response patterns to the altered dietary calcium and as such significant changes in total LTB_4 were the same as described for LTB_4. *Ex vivo* stimulation of neutrophils with thapsagargin did not produce any significant differences in LTB_4 levels. However, LTB_4 levels were consistently lowest in both the basal animals and those fed the 1.0% calcium diets.

Membrane lipids. The phospholipid profile of the neutrophil membrane is shown in Table 46. Phosphatidylcholine (PC) and sphingomylein (Sphing) were unaffected by diet. Low (0.1%) or high (1.0%) calcium increased the membrane phosphatidylethanolamine (PE) content similar to the basal diet group relative to the 0.5%CaHF group. Compensating for these low levels was the significantly elevated level of phosphatidylinositol (PI) in the 0.5%CaHF group. Low (0.1%) or high (1.0%) calcium decreased the membrane PC:PE ratio similar to the basal diet group relative to the 0.5%CaHF group.

Modulation of calcium on the fatty acid profile of PC is shown in Table 47. Modifying dietary calcium resulted in an increased membrane PUFA content. These changes were at the expense of saturated fatty acids (SATFA). Stearic (18:0) acid (SA) was lower ($p \leq 0.05$) in the basal group compared to all other groups regardless of dietary calcium levels. Similarly, linoleic (18:2) acid (LA) was lowest in the basal fed animals. Arachidonic (20:4) acid (AA) was lowest in the 0.5%CaHF diet relative to all other diets, however, this represented only a 3% change in overall fatty acid composition (Figure 24).

The fatty acid profile of PE as modulated by diet is shown in Table 48. The PUFA content was significantly reduced in the 1.0%Ca group with a concomitant

Figure 23: The effect of varying levels of calcium fed 12 weeks post carcinogen on leukotriene B_4 activity stimulated by either 123187 or thapsigargin. Values represent group means. Mean with different superscripts differ from corresponding calcium groups ($p<0.05$).

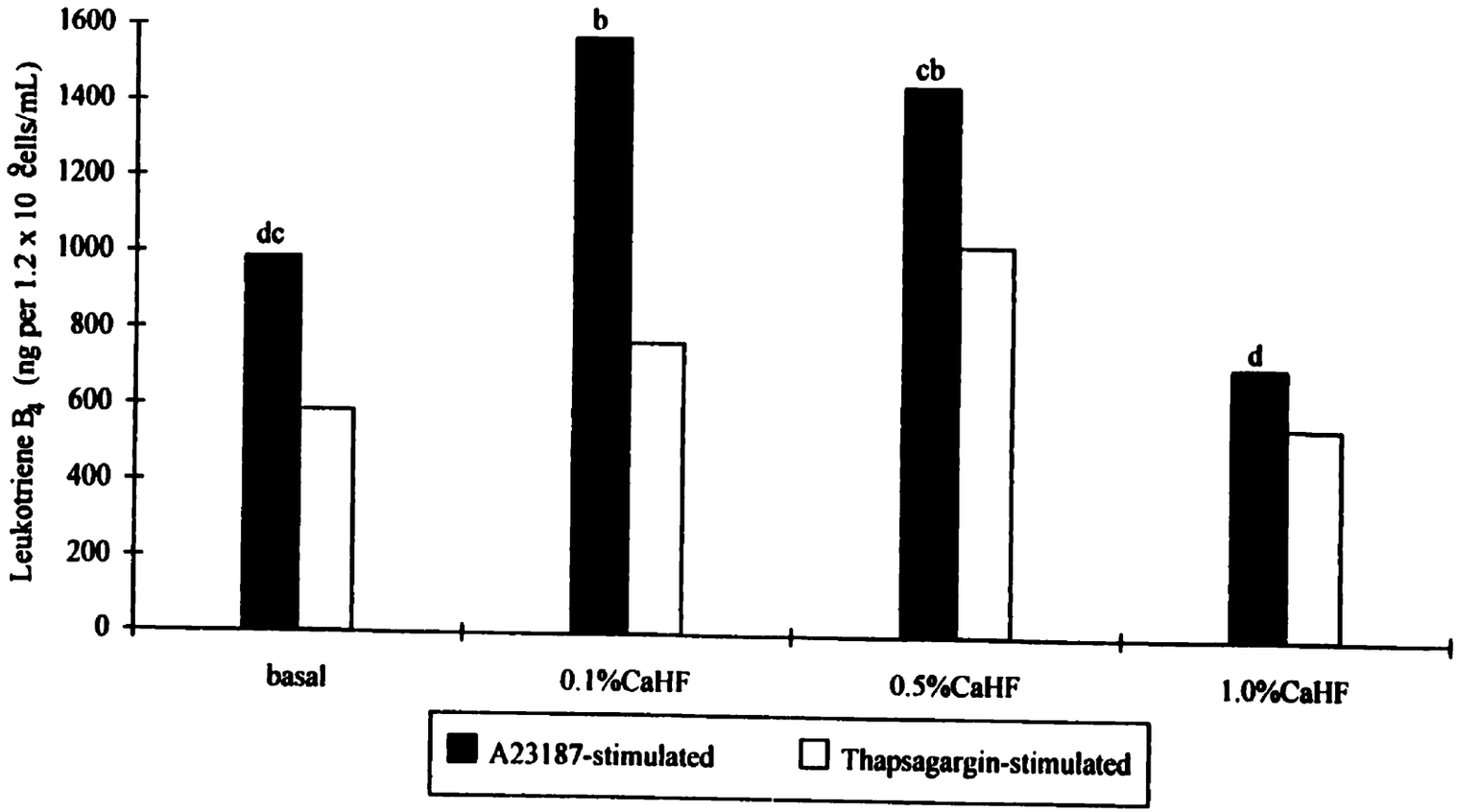

Leukotriene B_4 (ng per 1.2 x 10 cells/mL)
1600
1400
1200
1000
800
600
400
200
0
dc
b
cb
d
basal
0.1%CaHF
0.5%CaHF
1.0%CaHF
A23187-stimulated
Thapsagargin-stimulated

Table 46: The Effect of Varying Levels of Calcium fed 12 Weeks Post Carcinogen on the Phospholipid Composition of Neutrophil Membranes in the Sprague Dawley Rat. [a]

	PC	PE	PS	PI	Sphing	PC:PE
0.5% CaLF	30.9 ± 0.4	21.6 ± 0.5 [b]	8.7 ± 0.7	8.7 ± 0.8 [c]	10.9 ± 0.9	1.4 ± 0.0 [c]
0.1% CaHF	31.4 ± 2.7	16.7 ± 3.0 [b]	11.8 ± 1.3 [b]	13.7 ± 2.3	12.6 ± 1.7	2.1 ± 0.5 [c]
0.5% CaHF	33.8 ± 5.1	8.8 ± 0.9 [c]	7.4 ± 0.1 [c]	19.6 ± 4.3 [b]	9.3 ± 2.0	3.8 ± 0.5 [b]
1.0% CaHF	34.4 ± 2.5	21.6 ± 0.8 [b]	11.4 ± 1.3 [b]	8.1 ± 0.3 [c]	11.9 ± 0.8	1.6 ± 0.1 [c]

[a] Values represent means (μg/mg wet weight) ± S.E.

[b-c] : Means within a column with different superscripts differ at $p \leq 0.05$.

Table 47: The Effect of Varying Levels of Calcium Fed 12 Weeks Post Carcinogen on Neutrophil Membrane Phosphatidylcholine Percent Fatty Acid Composition. [a]

	C 14:0	C 16:0	C 16:1	C 18:0	C 18:1	C 18:2	C 18:3
0.5% CaLF	2.2 ± 0.7 [b]	31.2 ± 0.8 [b]	1.9 ± 0.1 [b]	8.5 ± 0.3 [c]	18.3 ± 0.7	9.1 ± 0.2 [c]	2.5 ± 0.2
0.1% CaHF	0.6 ± 0.0 [c]	26.9 ± 0.6 [c]	1.0 ± 0.1 [c]	10.1 ± 0.2 [b]	18.7 ± 0.1	11.5 ± 0.2 [b]	2.3 ± 0.0
0.5% CaHF	0.9 ± 0.2 [c]	30.4 ± 0.6 [b]	1.1 ± 0.0 [c]	10.4 ± 0.4 [b]	19.3 ± 0.7	11.5 ± 0.1 [b]	2.3 ± 0.1
1.0% CaHF	0.7 ± 0.0 [c]	28.0 ± 0.2 [c]	1.0 ± 0.0 [c]	10.1 ± 0.3 [b]	17.8 ± 0.4	11.8 ± 0.2 [b]	2.3 ± 0.1

	C 20:1	C 20:3	C 20:4	C 20:5	C 22:4	C 22:5	C 22:6
0.5% CaLF	2.6 ± 0.2 [b]	2.8 ± 0.1 [c]	18.1 ± 0.4 [b]	1.6 ± 0.1 [b]	1.5 ± 0.0	1.4 ± 0.1 [b]	1.1 ± 0.0 [b]
0.1% CaHF	0.9 ± 0.0 [c]	6.2 ± 0.8 [b]	18.6 ± 0.7 [b]	0.8 ± 0.3 [c]	1.8 ± 0.3 [b]	0.8 ± 0.1 [c]	0.8 ± 0.1
0.5% CaHF	1.0 ± 0.3 [c]	5.3 ± 0.7 [b]	15.2 ± 1.0 [c]	1.2 ± 0.1	1.1 ± 0.2 [c]	0.8 ± 0.1 [c]	0.7 ± 0.1 [c]
1.0% CaHF	1.0 ± 0.3 [c]	5.3 ± 1.0 [c]	18.3 ± 0.5 [b]	1.3 ± 0.1 [b]	1.5 ± 0.0	1.0 ± 0.1 [c]	0.9 ± 0.1

	Saturated	Monounsaturated	Polyunsaturated
0.5% CaLF	40.9 ± 1.3 [bc]	22.0 ± 0.9 [b]	37.1 ± 0.5 [c]
0.1% CaHF	37.3 ± 0.7 [d]	20.4 ± 0.1	42.3 ± 0.6 [b]
0.5% CaHF	41.1 ± 0.8 [b]	21.2 ± 0.5	37.7 ± 0.4 [c]
1.0% CaHF	38.4 ± 0.4 [cd]	19.7 ± 0.6 [c]	41.9 ± 0.5 [b]

[a] Values represent means (% of total) ± S.E.

[b-c] : Means within a column with different superscripts differ at $p \leq 0.05$.

Figure 24: The effect of varying levels of calcium fed 12 weeks post carcinogen on neutrophil membrane arachidonic acid levels in various phospholipid fractions. Values represent group means. Mean with different superscripts differ from corresponding calcium groups ($p<0.05$).

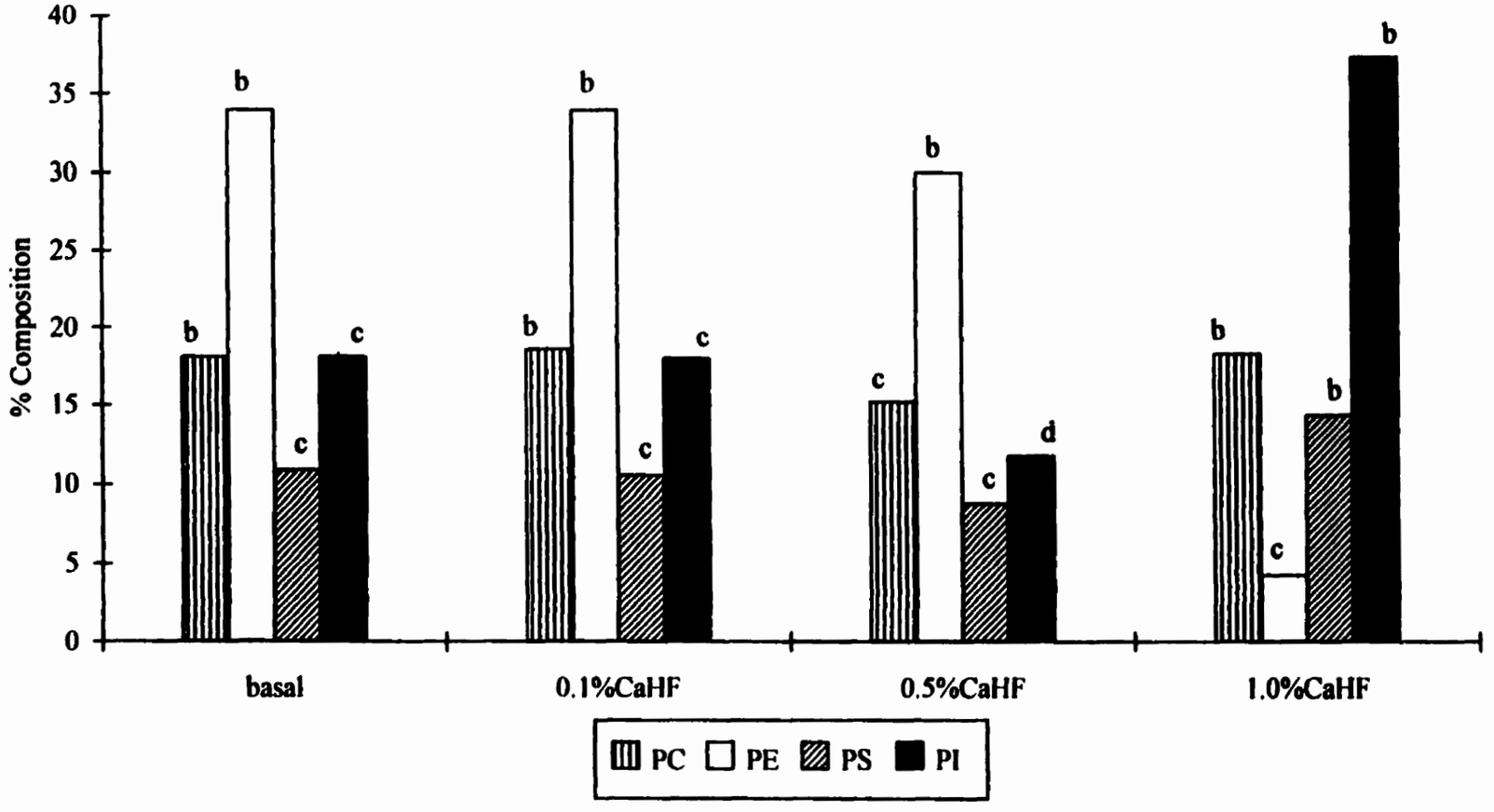

40
35
30
25
20
15
10
5
0
% Composition
b
b
c
c
b
b
c
c
c
b
d
c
b
c
b
b
basal
0.1%CaHF
0.5%CaHF
1.0%CaHF
PC
PE
PS
PI

Table 48: The Effect of Varying Levels of Calcium Fed 12 Weeks Post Carcinogen on Neutrophil Membrane Phosphatidylethanolamine Percent Fatty Acid Composition. [a]

	C 14:0	C 16:0	C 16:1	C 18:0	C 18:1	C 18:2	C 18:3
0.5% CaLF	2.1 ± 0.2 [b]	9.1 ± 0.8 [c]	1.6 ± 0.5 [c]	13.2 ± 0.3	17.2 ± 0.3 [cd]	5.8 ± 0.2 [d]	2.1 ± 0.1 [c]
0.1% CaHF	2.0 ± 0.0 [b]	9.9 ± 0.4 [c]	3.7 ± 0.2 [b]	12.4 ± 0.3 [c]	16.2 ± 0.0 [d]	6.5 ± 0.0 [cd]	1.8 ± 0.1 [c]
0.5% CaHF	1.9 ± 0.1 [b]	9.7 ± 0.3 [c]	2.4 ± 0.4 [c]	13.3 ± 0.4	19.0 ± 1.4 [c]	7.4 ± 0.6 [c]	2.3 ± 0.2 [c]
1.0% CaHF	1.4 ± 0.2 [c]	18.1 ± 0.3 [b]	1.7 ± 0.2 [c]	13.9 ± 0.4 [b]	24.3 ± 0.3 [b]	16.7 ± 0.2 [b]	5.8 ± 0.2 [b]

	C 20:1	C 20:3	C 20:4	C 20:5	C 22:4	C 22:5	C 22:6
0.5% CaLF	2.0 ± 0.3	3.7 ± 1.3	34.0 ± 1.7 [b]	1.4 ± 0.1 [c]	7.5 ± 0.5 [b]	1.4 ± 0.1 [c]	1.1 ± 0.1 [b]
0.1% CaHF	1.4 ± 0.3 [c]	3.3 ± 0.5	34.0 ± 0.2 [b]	1.0 ± 0.1 [c]	7.4 ± 0.4 [b]	1.1 ± 0.1 [c]	0.8 ± 0.1 [c]
0.5% CaHF	2.1 ± 0.5	4.5 ± 0.4	30.0 ± 2.0 [b]	1.3 ± 0.0 [c]	6.0 ± 0.7 [b]	1.2 ± 0.1 [c]	0.8 ± 0.0 [c]
1.0% CaHF	3.5 ± 1.0 [b]	5.6 ± 1.6	4.3 ± 2.1 [c]	2.1 ± 0.4 [b]	3.0 ± 0.6 [c]	2.2 ± 0.1 [b]	1.1 ± 0.2 [b]

	Saturated	Monounsaturated	Polyunsaturated
0.5% CaLF	23.9 ± 0.6 [c]	20.3 ± 0.7 [c]	55.8 ± 0.8 [b]
0.1% CaHF	23.9 ± 0.3 [c]	21.2 ± 0.4 [c]	54.9 ± 0.7 [b]
0.5% CaHF	24.4 ± 0.5 [c]	23.0 ± 1.9 [c]	52.6 ± 2.4 [b]
1.0% CaHF	32.2 ± 0.8 [b]	28.4 ± 0.7 [b]	39.4 ± 0.2 [c]

[a] Values represent means (% of total) ± S.E.

[b-d] : Means within a column with different superscripts differ at $p \leq 0.05$.

elevation in SATFA content. A dose dependent increase in oleic (18:1) acid (OA) was observed in response to increased dietary calcium. The addition of supplemental calcium (1.0%) resulted in a 2-fold increase in LA and appeared to be at the expense of AA (Figure 24).

Table 49 depicts the modulation of the PS fatty acid profile by dietary calcium. The addition of calcium (1.0%Ca) resulted in a significant PUFA increase. SATFA content was not significantly modified by dietary protocol. Palmitic acid (16:0) and stearic acid were decreased and increased ($p \leq 0.05$), respectively, in normal to high (0.5% & 1.0%) calcium groups compared to low (0.1%Ca) calcium and basal groups. While LA levels were unchanged, AA was marginally elevated ($p \leq 0.05$) high (1.0%) calcium groups (Figure 24).

Table 50 illustrates the modulation of PI fatty acids by altered dietary calcium. PUFA content was increased ($p \leq 0.05$) with either low or high (0.1% or 1.0%) calcium similar to the basal group. High (1.0%Ca) calcium nutriture resulted in a 3-fold AA increase and a 2-fold increase relative to the basal group (Figure 24). This increase appeared to be at the expense of palmitic, stearic, and oleic acid.

The fatty acid profile of Sphing is presented in Table 51. The PUFA content was greatest in the 1.0%Ca group. The fatty acid profile of neutrophil Sphing was relatively unchanged by diet.

Table 52 shows the correlation between LTB_4 and various phospholipid AA content levels. Leukotrienes are categorized as AA metabolites and as such were correlated with AA levels in membrane phospholipids. A23187-stimulated LTB_4 was negatively ($R = -0.5175$) correlated ($p = 0.04$) with AA levels in PS and this almost reached significance when all LTB_4 products ($R = -0.4743$) were included. When thapsagargin was used to stimulate neutrophil leukotriene activity a negative ($R = -0.6035$) association ($p = 0.01$) was found with the content of AA in the PC subfraction.

Table 49: The Effect of Varying Levels of Calcium Fed 12 Weeks Post Carcinogen on Neutrophil Membrane Phosphatidylserine Percent Fatty Acid Composition. [a]

	C 14:0	C 16:0	C 16:1	C 18:0	C 18:1	C 18:2	C 18:3
0.5% CaLF	0.8 ± 0.0	19.3 ± 1.1 [b]	0.7 ± 0.0	31.3 ± 0.9 [cd]	25.7 ± 0.6	4.4 ± 0.2	1.3 ± 0.2
0.1% CaHF	0.7 ± 0.1	21.1 ± 0.2 [b]	0.7 ± 0.0	29.4 ± 1.4 [d]	25.1 ± 0.3 [c]	4.3 ± 0.6	1.6 ± 0.2
0.5% CaHF	0.8 ± 0.1	12.1 ± 1.5 [c]	0.8 ± 0.1	37.9 ± 1.9 [b]	28.0 ± 1.0 [b]	5.1 ± 0.3	1.6 ± 0.2
1.0% CaHF	0.7 ± 0.1	10.7 ± 0.5 [c]	0.7 ± 0.0	35.3 ± 0.7 [bc]	26.1 ± 0.9	3.9 ± 0.8	1.4 ± 0.0

	C 20:1	C 20:3	C 20:4	C 20:5	C 22:4	C 22:5	C 22:6
0.5% CaLF	1.1 ± 0.2	1.6 ± 0.1 [c]	10.9 ± 1.3 [c]	1.0 ± 0.1	1.1 ± 0.1	0.8 ± 0.1 [c]	1.1 ± 0.2 [b]
0.1% CaHF	0.8 ± 0.1	2.3 ± 0.2	10.6 ± 0.6 [c]	1.0 ± 0.1	1.5 ± 0.2 [b]	0.5 ± 0.1 [cd]	1.3 ± 0.2 [b]
0.5% CaHF	1.1 ± 0.3	2.8 ± 0.4 [b]	8.8 ± 1.0 [c]	1.0 ± 0.1	0.6 ± 0.1 [c]	0.2 ± 0.0 [d]	0.5 ± 0.1 [c]
1.0% CaHF	1.0 ± 0.2	1.7 ± 0.1 [c]	14.4 ± 1.0 [b]	1.0 ± 0.0	1.5 ± 0.2 [b]	2.1 ± 0.3 [b]	0.6 ± 0.1 [c]

	Saturated	Monounsaturated	Polyunsaturated
0.5% CaLF	55.3 ± 4.4	24.7 ± 2.3	20.0 ± 2.4 [c]
0.1% CaHF	47.9 ± 2.9	27.7 ± 1.4	24.4 ± 1.6
0.5% CaHF	50.2 ± 0.5	29.5 ± 1.0	20.3 ± 1.0 [c]
1.0% CaHF	46.3 ± 1.0	27.4 ± 1.1	26.3 ± 2.0 [b]

[a] Values represent means (% of total) ± S.E.

[b-d] : Means within a column with different superscripts differ at $p \leq 0.05$.

Table 50: The Effect of Varying Levels of Calcium Fed 12 Weeks Post Carcinogen on Neutrophil Membrane Phosphatidylinositol Percent Fatty Acid Composition. [a]

	C 14:0	C 16:0	C 16:1	C 18:0	C 18:1	C 18:2
0.5% CaLF	1.1 ± 0.2 [c]	19.8 ± 1.6 [c]	2.2 ± 0.5 [b]	28.1 ± 1.9 [b]	15.8 ± 0.6 [b]	5.6 ± 0.2
0.1% CaHF	0.9 ± 0.1 [c]	24.4 ± 1.7 [b]	1.0 ± 0.2 [c]	27.6 ± 1.4 [b]	16.2 ± 0.8 [b]	5.8 ± 0.5
0.5% CaHF	1.1 ± 0.0 [c]	26.7 ± 0.7 [b]	1.0 ± 0.1 [c]	30.5 ± 0.5 [b]	17.0 ± 0.7 [b]	5.9 ± 0.2
1.0% CaHF	1.9 ± 0.3 [b]	10.1 ± 1.3 [d]	2.9 ± 0.1 [b]	18.4 ± 0.7 [c]	13.4 ± 0.2 [c]	5.2 ± 0.1

	C 18:3	C 20:1	C 20:3	C 20:4	C 20:5	C 22:4
0.5% CaLF	1.9 ± 0.2 [b]	1.6 ± 0.4 [b]	4.0 ± 0.6 [b]	18.1 ± 2.0 [c]	1.4 ± 0.2	2.0 ± 0.2 [c]
0.1% CaHF	1.7 ± 0.1 [b]	1.1 ± 0.2	1.6 ± 0.3 [c]	18.0 ± 1.4 [c]	0.8 ± 0.1	2.0 ± 0.4 [c]
0.5% CaHF	1.8 ± 0.1 [b]	1.3 ± 0.1 [b]	1.5 ± 0.3 [c]	11.8 ± 0.4 [d]	1.5 ± 0.0	1.2 ± 0.0 [c]
1.0% CaHF	0.7 ± 0.0 [c]	0.5 ± 0.0 [c]	1.2 ± 0.0 [c]	37.4 ± 0.4 [b]	1.6 ± 0.9	7.4 ± 0.5 [b]

	Saturated	Monounsaturated	Polyunsaturated
0.5% CaLF	48.2 ± 0.5 [d]	19.2 ± 1.3	32.6 ± 1.2 [c]
0.1% CaHF	52.4 ± 0.3 [c]	18.1 ± 1.0	29.5 ± 1.2 [c]
0.5% CaHF	57.5 ± 0.7 [b]	19.1 ± 0.9	23.4 ± 0.4 [d]
1.0% CaHF	30.3 ± 1.6 [e]	16.7 ± 0.2	53.0 ± 1.7 [b]

[a] Values represent means (% of total) ± S.E.

[b-e]: Means within a column with different superscripts differ at $p \leq 0.05$.

Table 51: The Effect of Varying Levels of Calcium Fed 12 Weeks Post Carcinogen on Neutrophil Membrane Sphingomyelin Percent Fatty Acid Composition. [a]

	C 14:0	C 16:0	C 16:1	C 18:0	C 18:1	C 18:2
0.5% CaLF	1.8 ± 0.4	38.8 ± 6.1	1.8 ± 0.4	17.4 ± 2.5	18.8 ± 1.9	6.7 ± 0.8
0.1% CaHF	1.3 ± 0.2	37.2 ± 5.0	0.8 ± 0.0	19.1 ± 1.2	18.3 ± 1.9	6.7 ± 0.9
0.5% CaHF	1.4 ± 0.2	40.8 ± 1.1	0.7 ± 0.0	21.1 ± 1.9	16.3 ± 1.2	5.9 ± 0.6
1.0% CaHF	1.0 ± 0.0	44.2 ± 1.0	2.3 ± 0.1	13.2 ± 0.7	17.4 ± 0.8	6.7 ± 0.3

	C 18:3	C 20:1	C 20:3	C 20:5
0.5% CaLF	2.5 ± 0.3	2.6 ± 0.4	3.6 ± 1.4	8.6 ± 0.8
0.1% CaHF	2.8 ± 0.3	2.8 ± 0.3	4.3 ± 0.8	9.5 ± 0.7
0.5% CaHF	2.8 ± 0.2	2.6 ± 0.3	2.8 ± 0.2	8.3 ± 1.4
1.0% CaHF	3.0 ± 0.2	3.0 ± 0.1	2.2 ± 0.6	10.0 ± 0.5

	Saturated	Monounsaturated	Polyunsaturated
0.5% CaLF	39.6 ± 3.2	15.7 ± 1.2	44.7 ± 2.3
0.1% CaHF	41.6 ± 3.1	15.7 ± 1.3	42.7 ± 1.9
0.5% CaHF	47.3 ± 2.8	14.6 ± 1.2	38.1 ± 3.5 [c]
1.0% CaHF	37.7 ± 1.3	14.3 ± 1.1	48.1 ± 2.2 [b]

[a] Values represent means (% of total) ± S.E.

[b-c] : Means within a column with different superscripts differ at $p \leq 0.05$.

Table 52: Comparison of Leukotriene B4 and Arachidonate from Various Phospholipid Fractions.

	R Value	P Value
A23187 Stimulated		
LTB_4 • PC 20:4 n-6	-0.3434	0.1928
LTB_4 • PE 20:4 n-6	0.2090	0.4373
LTB_4 • PS 20:4 n-6	-0.5175	0.0401
LTB_4 • PI 20:4 n-6	0.1232	0.6495
Thapsagargin Stimulated		
LTB_4 • PC 20:4 n-6	-0.6035	0.0133
LTB_4 • PE 20:4 n-6	-0.0588	0.8287
LTB_4 • PS 20:4 n-6	-0.4940	0.0518
LTB_4 • PI 20:4 n-6	0.1884	0.4847
A23187 Stimulated		
Total LTB_4 • PC 20:4 n-6	-0.3221	0.2232
Total LTB_4 • PE 20:4 n-6	0.2411	0.3682
Total LTB_4 • PS 20:4 n-6	-0.4743	0.0635
Total LTB_4 • PI 20:4 n-6	0.0978	0.7186
Thapsagargin Stimulated		
Total LTB_4 • PC 20:4 n-6	-0.5622	0.0234
Total LTB_4 • PE 20:4 n-6	-0.0309	0.9095
Total LTB_4 • PS 20:4 n-6	-0.4641	0.0701
Total LTB_4 • PI 20:4 n-6	0.1365	0.6141

4. Discussion

The purpose of the present study was to examine the possibility that varying levels of dietary calcium exert a systemic effect. It was observed that dietary calcium clearly inhibited neutrophil production of LTB_4 and its metabolites in a dose dependent manner. Furthermore, lipid metabolism was modified by the level of calcium in the diet. High (1.0%Ca) calcium nutriture altered ($p \leq 0.05$) the arachidonic acid (AA) composition of various phospholipid fractions. AA levels derived from the phosphatidylserine (PS) and phosphatidylcholine (PC) fractions negatively ($p \leq 0.05$) correlated with A23187 and thapsagargin stimulated neutrophil LTB_4 production, respectively. The primary findings of this study indicate that dietary calcium exerts a distinctly inhibitory effect on the production of LTB_4 in neutrophils and alters neutrophil membrane lipid metabolism. In concert, these findings demonstrate a systemic role for dietary calcium.

Solid tumors, like colonic tumors, frequently contain immunological cells unrelated to necrosis (Winawer et al., 1992), implicating the involvement of immune products in neoplastic events. Polymorphonuclear cells compose the majority of circulating blood leukocytes and play an important role in stimulating an inflammatory response (Feinstein & Sha'afi, 1983). Neutrophils represent the major cell type of polymorphonuclear cells and the leukotriene LTB_4 its primary arachidonic acid metabolite (Feinstein & Sha'afi, 1983). The interrelationship between calcium and arachidonic acid-derived metabolites, such as leukotrienes, involves the role of calcium in the release of arachidonic acid from membrane phospholipids as well as altered cellular calcium flux in response to these metabolites (Feinstein & Sha'afi, 1983). Therefore, the study of neutrophils and their product LTB_4 provided a unique opportunity to evaluate a distinct systemic effect of dietary calcium.

A role for eicosanoids has been implicated in the development of colon cancer. Lipoxygenase metabolites, hydroxyeicosatetraenoic acids (HETEs), have been shown to

stimulate tumor cell spreading, tumor cell adhesion, and enhanced tumor cell metastatic potential (Honn et al., 1989; Timer et al., 1992). Some lipoxygenase products have been implicated in the stimulation of the human leukemia cell line HL-60 (Miller et al., 1989). The human gastric cancer cell line AGS has been shown to direct AA metabolism through the 5-lipoxygenase pathway leading to the production of growth-stimulatory autocoids (Shimakura & Boland, 1992). In addition, transformed hepatoma cells have been shown to preferentially take up and metabolize LTB_4 (Leier et al., 1992). Rao et al. (1993a) demonstrated that HETE levels were significantly greater in AOM treated rat colon than saline counterparts. The addition of a chemopreventive agent, curcumin, significantly reduced HETE levels and ACF, purported preneoplastic lesion of colon cancer, compared to controls. It has been suggested that eicosanoids contribute to colon carcinogenesis by stimulating the proliferation rate. Lipoxygenase products were found to be elevated in the proliferative compartment of colonic epithelial cells (Craven & DeRubertis, 1986). Similarly, LTB_4 was shown to stimulate proliferation of human colon carcinoma cells *in vitro* (Qiao et al., 1995). Dias et al. (1994) demonstrated that bile salts, which are known to increase colonic cell proliferation, stimulate the synthesis of LTB_4 in an intestinal epithelial cell line. Specific inhibitors of the 5-lipoxygenase enzyme have been shown to reduce the proliferation rate and induce differentiation in chronic myelogenous leukemia blast cells (Anderson et al., 1995). Calcium has been purported to be a differentiating agent and has been shown to suppress both animal and human colonic epithelial proliferation (Arlow et al., 1989; Bird et al., 1986; Rozen et al., 1989; Wargovich et al., 1984). In particular calcium's ameliorating action is thought to protect against the damage of a high fat diet where bile acids and free fatty acids irritate the epithelium causing hyperproliferation. Since tissue damage of the colonic epithelium elicits an inflammatory response it is plausible to suggest that neutrophils, drawn to this region by chemotaxis, might release a suppressed concentration of LTB_4 as a result of high calcium nutriture.

Growing evidence indicates that membrane phospholipids, once relegated to the role of passive barrier, actively influence various cellular functions (Berridge, 1982). The results of the present study are of considerable interest in view of the fact that dietary calcium was demonstrated to significantly alter fatty acid composition of neutrophil membrane phospholipids. In general, AA levels were similar in the 0.1%Ca and 0.5%Ca high fat fed animals, however, at a dietary intake of 1.0%Ca AA levels were significantly greater in the PS and PI subfractions. In addition, a dramatic increase in linoleic acid (LA) was observed in the PE subfraction of animals fed the high (1.0%) calcium diet. Perturbations in calcium concentrations altered the polyunsaturated fatty acid (PUFA) content of the various phospholipid subfractions. PUFA levels were increased in the PC, PS, PI, and sphingomylein (Sphing) fractions if dietary calcium was either lowered or raised in the diet. The ability of the high fat compared to the basal diet to increase the PC/PE ratio is similar to findings where high fat diets of either corn oil or beef tallow increased the ratio in the rodent colonic mucosa (Robblee et al., 1988; Lafave et al., 1994). Interestingly in the present study this effect was reversed in response to increasing or decreasing dietary calcium. Fatty acid analysis on the colonic mucosa of tumor bearing rats also demonstrated a modulating effect of calcium, however, the trends lacked identifiable patterns (Appendix II). A paucity of information exists on the role of dietary calcium in the modulation of membrane fatty acid composition. Only a handful of studies have investigated the effect of calcium on tissue lipid levels and these predominantly support a role for modulation by dietary calcium. Microsporum gypseum cells were found to increase phospholipid synthesis in the presence of calcium (Giri et al., 1995). Young pigs fed three times the dietary requirement of calcium exhibited increased plasma phospholipid concentrations but did not yield changes in plasma cholesterol or triglycerides (Foley et al., 1990). It was demonstrated that liver tissue contained elevated cholesterol, total phospholipid, Sphing, and PE in rabbits fed a low (0.02%) calcium high

(20%) beef tallow diet (Dougherty & Iacono, 1979). In contrast, a study comparing high (1.5%) versus recommended (0.5%) calcium in the presence of a high (16%) beef tallow diet reported that high calcium had no effect on cholesterol, total phospholipid, or fatty acid distribution of colonic apical membranes (Awad et al., 1990). These differences might be attributed to the variation in experimental protocols employed. A major difference is that Awad et al. (1990) reported no effect of calcium after feeding diets for 4 weeks whereas the current results were obtained after 12 weeks of dietary treatments in rats. In addition, changes in fatty acid composition were reported for neutrophil membranes whereas Awad et al. (1990) studied colonic membranes. Lastly, changes in fatty acid profiles were determined from various phospholipid fractions compared to Awad et al. (1990) which determined fatty acid composition in total colonic lipid. This would include triglycerides, which have been reported unchanged by dietary calcium (Foley et al., 1990), perhaps obscuring the effect of calcium. In general, this suggests a novel role for dietary calcium as a modulator of membrane lipid composition.

Membrane fatty acids, which are precursors of bioactive eicosanoids, when altered affect a wide variety of cell functions (Bruckner et al., 1984; Croft et al., 1985; Iritani & Narita, 1984; Lee et al., 1988). It is plausible that the high calcium diet exerts its effect by altering fatty acid composition thus mediating changes in eicosanoid synthesis. In the present investigation rats fed the 1.0%Ca diet exhibited the lowest LTB_4 values as well as the highest levels of its precursor, AA, in the PS and PI fractions. Dias et al. (1992) reported that altering the AA content of the culture medium resulted in altered LTB_4 synthesis. Bell et al. (1992) demonstrated modifying dietary composition resulted in increased leucocyte membrane phospholipid AA composition and increased whole blood LTB_4 levels. The contradiction in the current finding is that neutrophils from the high calcium (1.0%Ca) group produced the lowest levels of LTB_4, however, the neutrophil membranes exhibited elevated AA levels. One possible explanation is that high AA

levels result from a decreased phospholipid turnover thus inhibiting the metabolism of AA to LTB_4. Inhibition of phospholipase A_2 activity would reduce the release of AA from phospholipids thus resulting in a reduction of LTB_4 (Dannhardt and Lehr, 1992). Alternatively, a high calcium diet may initially stimulate LTB_4 and eventually exhaust its production. Tumor promoters have been shown to stimulate enzymes to depletion thus mediating their disregulation of normal growth control (Kischel et al., 1989; Hansen et al., 1990). Lastly, perhaps elevated AA do indeed represent enhanced tumor potential and low LTB_4 levels are indicative of a shift in AA metabolism away from the lipoxygenase pathway. Ali et al. (1994) reported that an eosinophilic substrain of HL-60 cells upon stimulation of PKC by phorbol 12-myristate 13-acetate, a known tumor promoter, down regulated synthesis of cysteinyl leukotrienes and stimulated prostaglandin synthesis. Dietary calcium exhibited a clear effect on leukotriene production and the implications of this finding require further investigation and clarification.

The results of this study clearly demonstrate that dietary calcium exerts a systemic effect in the animal model. This was evidenced by profound alterations in the neutrophil membrane phospholipid profile, namely eicosanoid precursors AA and LA, in response to modified levels of dietary calcium. Further, this was demonstrated by the distinct inhibitory effect of a high calcium diet on the production of LTB_4 in neutrophils of carcinogen treated rats. To our knowledge this is the first time dietary calcium has been shown to affect neutrophil membrane composition or production the eicosanoid LTB_4. These findings suggest that, in addition to the proposed luminal effect, a systemic mechanism of action is in play for dietary calcium. The role of dietary calcium as a systemic modulator of colon carcinogenesis is relatively new and is a rich area for further research.

Section VII

GENERAL DISCUSSION

Chapter 11. GENERAL DISCUSSION AND CONCLUSIONS

A. Calcium as Modulator of Colon Cancer

The main purpose of this dissertation was to study the development and biological markers of colon cancer in response to varied levels of dietary calcium. Additionally, mechanistic explanations of the biological effects of calcium were investigated. The primary concerns involved diet composition and age. To this end several questions were addressed.

Prior to commencement of calcium studies it was prudent to investigate potentially confounding variables. Two important issues to be resolved regarding dietary composition included (i) what level of phosphorus to use? (Chapter 4) and (ii) which calcium salt to use when making a high calcium diet? (Chapter 5). The next critical step involved determining whether altered levels of dietary calcium would modulate colon carcinogenesis and whether this effect would change depending on the level of fat in the diet. The dose increase in calcium provided the unique opportunity to evaluate the early stages of colon cancer development in response to various states of dietary calcium requirement (Chapter 6). In the search to explain the effect of varied calcium and fat on disease progression two potential mechanistic avenues investigated were (1) the effect of this diet on a signal transduction pathway implicated in the disease process (Chapter 7) and (2) the effect of this diet on lipid composition of the colonic membrane (Chapter 8). The final emphasis of this dissertation was to explore the effect of dietary calcium on older animals with an established disease state. Evaluating animals several weeks after carcinogen administration provided the novel occasion to investigate the response of well established preneoplastic and neoplastic lesions to changes in dietary calcium (Chapter 9). A potential role for dietary calcium as a systemic modulator was investigated by studying the response of neutrophils to a dose calcium increase (Chapter 10). The results of these

studies have been discussed in each chapter. The main findings of this dissertation as they relate to furthering the understanding of dietary calcium as a modulator of colon cancer will be related.

The most important finding of this dissertation was the inability of a gradient increase in dietary calcium to induce a dose dependent response in a variety of biological parameters. This finding was consistently observed whether calcium was introduced early or late in disease development. Early intervention with calcium produced complex effects on colon carcinogenesis where 0.1%Ca and 1.0%Ca versus 0.5%Ca and 2.0%Ca diets modulated aberrant crypt foci (ACF) induction and growth in a similar but opposite manner (Chapter 6). In addition, a dose response was not observed in colonic protein kinase C (PKC) activity, lipid composition, or cellular proliferation markers. Intervention in the later stages of disease development failed to produce a dose dependent response in ACF or tumor growth (Chapter 9). The dose dependent response observed in neutrophil leukotriene B_4 (LTB_4) production was perhaps due to the fact that this leukotriene is directly activated by the calcium ion (Chapter 10). In addition, the proliferating cell nuclear antigen labeling index (PCNA LI) in the mid colonic region decreased in a dose dependent manner (Chapter 7). However, this response was not consistent as it was not demonstrated in the rectal end or supported by other cellular proliferation markers (Chapter 6). Therefore, investigating only one level of calcium is insufficient to elucidate the effect of calcium on a particular disease marker or disease development. For example, it has been reported that calcium decreases PKC activity in rat colonic mucosa (Pence et al., 1995). However, our findings indicate that high calcium nutriture results in either a reduction (2.0%CaLF) or an increase (1.0%CaLF) in PKC activity depending on the level of supplementation. A great deal of conflicting literature has been produced on this topic and this may be due to the non-linear effect of calcium.

A significant finding of the research presented was that the phase response exhibited by ACF with advanced growth features, AC≥4, was mirrored (Appendix III; R = + 0.9892, p = 0.0001) by membrane PKC activity (Chapter 7) in response to altered dietary calcium. PKC has been implicated in the disease process by its ability to exert control over cellular differentiation and growth via the phosphorylation of target proteins. Moreover, PKC has been shown to play a role in growth control of colon cancer cells (Choi et al., 1990; Guillem et al., 1987; Kopp et al., 1991; Wali et al., 1991). A somewhat perplexing result was the increased AC≥4 and membrane PKC activity in the high calcium high fat (2.0%CaHF) group with concomitant suppressed colonic mucosal proliferation (Chapter 6 & 7). This finding is supportive of the proposal that high concentrations of the calcium ion enhance the clonal expansion of preneoplastic lesions (Whitfield, 1992). Normal cells continue to respond to the "proliferating-stopping differentiation-triggering calcium switch" (Whitfield, 1992) thus presenting a quiescent colonic mucosa. However, in response to high levels of luminal calcium initiated or transformed cells increase proliferation and pile up on the mucosal surface because they no longer respond to calcium induced apoptosis (Cameron et al., 1990; Deschner & Lipkin, 1975; Risio et al., 1988; Whitfield, 1992). In point of fact, deletion or mutation of the familial adenomatous polyposis (FAP) gene results in an unresponsive colonic mucosal cell population that, unlike normal cells, do not stop proliferating at high physiological levels of calcium (Friedman, 1990; Whitfield, 1992). Furthermore, increased membrane PKC activity represents an additional premalignant change (Whitfield, 1992). Continual activation of secretion factors by membrane PKC contributes to the selective expansion of transformed cells by disconnecting intercellular gap junctions thus inhibiting restraining cross talk (Friedman et al., 1984; Friedman, 1985; Whitfield, 1992). Further, tumor promoter stimulated membrane PKC activity results in a surge of oxygen radicals in the plasma membrane (Whitfield, 1990). Through a series of events these radicals cause mutations

associated with the transition to malignancy (Baker et al., 1989; Paraskeva et al., 1990; Whitfield, 1992). The finding that calcium modulates preneoplastic lesions and PKC activity in a similar manner is intriguing and warrants further investigation perhaps at various time intervals in disease development.

An additional important finding was the dependency of calcium's biological effect on the level of fat in the diet. The complex phase pattern exhibited by AC≥4 and PKC was inversely modulated by a high fat compared to a low fat diet (Chapter 6 & 7). For example, if feeding high calcium (2.0%) in a low fat mixture yielded a decreased number of preneoplastic lesions then in a high fat mixture this number was significantly elevated. In addition, the action of calcium on proliferation biomarkers was influenced by the level of dietary fat, most notably in the bromodeoxyuridine (BrdU) and PCNA labeled cells (Chapter 6). It has been speculated that dietary calcium's mechanism of action relates to the ability of calcium to bind lipid during colonic transition. However, this does not explain the action of calcium in a low fat milieu or the modulating effect of the calcium and fat interaction. These findings illustrate the complex nature of the biological response to altered calcium intake casting doubt on the simple approach of the "calcium soaps" hypothesis. The complex effect of calcium has baffled researchers and has been dismissed by suggesting that a "clear cut inhibitory" effect is demonstrated only in the presence of high fat (Karkare et al., 1991). The results presented here indicate that this sweeping generalization does not withstand scrutinization. Indeed, calcium does not function independently of dietary fat and thus future calcium studies must take fat level into consideration when interpreting results.

The composition of the lipid bilayer was clearly modulated by dietary calcium in both the colonic and neutrophil cell membranes. Changes in the lipid profile suggest that high calcium and high fat exert their effect along a common pathway. For example, arachidonic acid (AA) levels were greater in a low fat high calcium diets but generally

elevated in all high fat diets regardless of calcium intake (Chapter 8). This finding also indicates that the effects are independent and not synergistic as AA levels in the high calcium groups were similar regardless of the level of dietary fat. Neutrophil membrane composition was also modulated by dietary calcium intake. For example, AA levels decreased in phosphatidylethanolamine but increased in phosphatidylinositol fractions in response to a high calcium diet (Chapter 10). Research in this area is sparse and only one study to date has investigated the effect of calcium on colonic membrane lipid composition thus making conclusions hard to deduce. Complicating matters further the present investigations were carried out at one time point on small amounts of lipid which were further fractionated into the various phospholipid samples. The extensive changes to the membrane bilayer in response to altered dietary calcium indicate that this is a rich area for future research. Our understanding of the effect of dietary calcium on membrane structure would be enhanced by phospholipid and fatty acid turnover studies at various developmental time points.

It was demonstrated that cellular proliferation biomarkers as modulated by dietary calcium were not a good predictors of disease outcome. ACF with increased crypts per focus represent lesions with advanced growth characteristics and this increased crypt multiplicity has been found to be an early and persistent predictor of tumor outcome (Pretlow et al., 1992; Zhang et al., 1992; Magnuson et al., 1993). Proliferation measured at various cell cycle points by BrdU labeling, PCNA labeling, and metaphase arrest (Chapter 2, 3 & 4) did not predict the growth response of ACF with advanced growth features. While increased proliferation may indeed precede cancer development, tumor development occurs in only a few select colonic locations and thus there is no guarantee that biopsy segments examined for proliferative status contain areas of abnormal cell growth. Early markers of cancer, termed "intermediate end points", are enticing as they monitor early changes in the neoplastic process and thus provide an opportunity for

intervention (Weinstein, 1991). However, biomarkers such as cell division and signal transduction markers must be validated before being used routinely as end points in prevention studies (Weinstein, 1991).

A slightly more obscure finding of high calcium nutriture was its ability to tightly regulate the biological parameters as evidenced by a smaller standard error of the mean (S.E.). This was most notable in the modulation of established growth lesions where after 12 weeks of intervention (24 wk time point) the S.E. for total ACF, AC3-5, and AC≥6 was lowest for animals fed the 2.0%Ca diet (Chapter 9). Similarly, the S.E. was lowest for tumor burden and size of adenocarcinoma in the high calcium group. This pattern of tight regulation was also demonstrated in total ACF and BrdU labeling in both the rectal and mid region (Chapter 6). This finding suggests that perhaps an additional characteristic of high calcium intake is that fewer biological parameters escape the modulating effect of this diet. The significance of this finding is unknown and requires further investigation.

Lastly, a systemic effect of calcium was clearly demonstrated. Results from this dissertation have shown that calcium nutriture modulates colonic membrane proliferation, PKC activity, and the development of colon cancer. However, these findings do not distinguish whether the effects are a result of a reduction in luminal fat (calcium-lipid complexes), direct action of luminal calcium (unabsorbed free calcium), or a systemic mode of action. Changes in neutrophil membrane lipid composition and production of LTB_4 confirm the systemic potential of dietary calcium (Chapter 10). The debate on calcium's mechanism action is the subject of review (Pence, 1993). Although the associations between dietary factors and cancer have been drawn there is a lack of understanding at the mechanistic level (Weinstein, 1991). Dietary calcium as systemic modulator was demonstrated, now further investigation is required to understand this systemic relationship with regard to the disease process.

B. Implications of the Findings.

A dose dependent response was not elicited with a sequential increase in dietary calcium. This finding has implications for future calcium studies such that the response of biological parameters to various levels of calcium can not be predicted. Therefore, a response in a given direction is specific to the level of calcium employed. One dilemma is the ambiguity of the terms "low" or "high" without specific composition values. For example, although the American Institute of Nutrition (AIN) dictates that a normal rodent diet contains 0.5%Ca the term "high" calcium has been ascribed to anything from a 0.32%Ca diet (Weisburger et al., 1994) to a 2.0%Ca (Pence et al., 1995). To elucidate and enhance our understanding of the role played by calcium in colon carcinogenesis it is important to have consensus as to the "normal" level of dietary calcium in the animal model.

The level of fat in the diet clearly influenced the modulating effect of calcium on disease progression. The level of dietary fat must be considered when interpreting results. In animal model studies dietary parameters are routinely controlled. However, in human clinical trials details on participant dietary intake other than calcium is rarely, if ever, reported. The results of this dissertation indicate that this needs to be addressed as it poses a potential confounding factor in calcium investigations.

The demonstration that dietary calcium significantly modulated the growth of established preneoplastic lesions but not tumor development suggests that calcium's effect is subtle and dependent on the stage of neoplastic growth. This finding indicates that the time of calcium intervention is important to its modulating capabilities. The inference drawn from this finding suggests that perhaps this provides a clue to pinpointing calcium's mechanism of action. Future calcium investigations should examine

early carcinogen induced cellular changes in ACF with advanced growth features and variation with altered dietary calcium.

C. Conclusions

A great deal of variability in epidemiological, experimental, and clinical studies on calcium nutriture suggested that a systematic investigation of calcium as a modulator of colon cancer was needed. Firstly, variables affecting dietary calcium supplementation were examined. Although various calcium salts did not appreciably differ with respect to the early stages of colon cancer, altering dietary phosphorus had a significant impact on disease development. Secondly, it was demonstrated that a gradient calcium increase did not produce a dose dependent response in various biological parameters including ACF, preneoplastic lesions of colon cancer. Furthermore, any effect of calcium was shown to be mediated by the level of dietary fat. Lastly, it was demonstrated that the developmental stage of the disease affected calcium's modulating impact. In addition, a distinct systemic effect of dietary calcium was demonstrated. The findings of this dissertation have enhanced the understanding of dietary calcium's effect on the whole organism. Dietary calcium was shown to exert a multi-level effect. At the tissue level changes were observed in colonic mucosal proliferation and ACF growth of the colonic mucosa. At the cellular level changes were observed in cell membrane composition and intracellular enzyme function. The effect of calcium was shown to be complex such that a biological response at a given concentration was not predictive of the response to an increase/decrease in intake.

Section VIII

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Section IX

APPENDICES

Appendix I

- Table A: Weight composition of the American Institute of Nutrition, AIN-76 semipurified diet, rat or mouse.
- Table B: Weight composition of animal experimental diets in chapter 4.
- Table C: Weight composition of animal experimental diets in chapter 5.
- Table D: Weight composition of animal experimental diets in chapter 6, 7, & 8.
- Table E: Weight composition of animal experimental diets in chapter 9 & 10.

Table B: Weight (g/kg diet) Composition of Animal Experimental Diets in Chapter 4.

	0.1%Ca & 0.1%P	0.1%Ca & 0.5%P	0.5%Ca & 0.5%P
Dextrose	514	502	500
Casein	200	200	200
Dextrin	150	150	150
Corn Oil	50	50	50
Alphacel	50	50	50
AIN-76 Min Mix (calcium free)	17.5	17.5	17.5
Calcium Phosphate	3.5	3.5	17.5
Sodium Phosphate	0	12.34	0
AIN-76 Vitamin Mix	10	10	10
DL - Methionine	3	3	3
Choline Bitartrate	2	2	2

Table C: Weight (g/kg diet) Composition of Animal Experimental Diets in Chapter 5.

	CaPhos	CaLact	CaCarb	CaGluco	CaGluca
Dextrose	500	483	495	461	480
Casein	200	200	200	200	200
Dextrin	150	150	150	150	150
Corn Oil	50	50	50	50	50
Alphacel	50	50	50	50	50
AIN-76 Min Mix (calcium free)	17.5	17.5	17.5	17.5	17.5
Sodium Phosphate	0	12.3	12.3	12.3	12.3
Calcium Phosphate	17.5	0	0	0	0
Calcium Lactate	0	22.4	0	0	0
Calcium Carbonate	0	0	10.3	0	0
Calcium Gluconate	0	0	0	44.2	0
Calcium Glucarate	0	0	0	0	25.5
AIN-76 Vitamin Mix	10	10	10	10	10
DL - Methionine	3	3	3	3	3
Choline Bitartrate	2	2	2	2	2

Table D: Weight (g/kg diet) Composition of Animal Experimental Diets in Chapter 6, 7, & 8.

	Low Fat				High Fat			
	0.1%Ca	0.5%Ca	1.0%Ca	2.0%Ca	0.1%Ca	0.5%Ca	1.0%Ca	2.0%Ca
Dextrose	500	500	488	463	250	250	235	206
Casein	200	200	200	200	245	245	245	245
Dextrin	150	150	150	150	184	184	184	184
Corn Oil	50	50	50	50	50	50	50	50
Beef Tallow	0	0	0	0	150	150	150	150
Alphacel	50	50	50	50	61	61	61	61
AIN-76 Min Mix (calcium free)	17.5	17.5	17.5	17.5	21.2	21.2	21.2	21.2
Calcium Phosphate	3.5	17.5	17.5	17.5	4.2	21.2	21.2	21.2
Sodium Phosphate	12.5	0	0	0	15.2	0	0	0
Calcium Carbonate	0	0	12.5	37.4	0	0	14.7	44
AIN-76 Vitamin Mix	10	10	10	10	12	12	12	12
DL - Methionine	3	3	3	3	4	4	4	4
Choline Bitartrate	2	2	2	2	2	2	2	2

Table E: Weight (g/kg diet) Composition of Animal Experimental Diets in Chapter 9 & 10.

	0.1%Ca	0.5%Ca	1.0%Ca	2.0%Ca
Dextrose	250	250	235	206
Casein	245	245	245	245
Dextrin	184	184	184	184
Corn Oil	50	50	50	50
Beef Tallow	150	150	150	150
Alphacel	61	61	61	61
AIN-76 Min Mix (calcium free)	21.2	21.2	21.2	21.2
Calcium Phosphate	4.2	21.2	21.2	21.2
Sodium Phosphate	15.2	0	0	0
Calcium Carbonate	0	0	14.7	44
AIN-76 Vitamin Mix	12	12	12	12
DL - Methionine	4	4	4	4
Choline Bitartrate	2	2	2	2

Appendix II

- Table A: The effect of varying levels of dietary calcium on the phosphatidylcholine percent fatty acid composition in the colonic mucosa of tumor bearing rats.
- Table B: The effect of varying levels of dietary calcium on the phosphatidylethanolamine percent fatty acid composition in the colonic mucosa of tumor bearing rats.
- Table C: The effect of varying levels of dietary calcium on the phosphatidylserine percent fatty acid composition in the colonic mucosa of tumor bearing rats.
- Table D: The effect of varying levels of dietary calcium on the phosphatidylinositol percent fatty acid composition in the colonic mucosa of tumor bearing rats.

Table A: The Effect of Varying Levels of Dietary Calcium on the Phosphatidylcholine Percent Fatty Acid Composition in the Colonic Mucosa of Tumor Bearing Rats. [a]

	C 14:0	C 16:0	C 16:1	C 18:0	C 18:1	C 18:2
0.1% Ca	1.0 ± 0.1 [c]	44.6 ± 1.4 [b]	0.7 ± 0.0 [c]	17.4 ± 0.4 [c]	13.0 ± 0.4 [c]	5.5 ± 0.2 [c]
0.5% Ca	1.4 ± 0.1 [b]	46.4 ± 1.7 [b]	1.1 ± 0.0 [b]	33.3 ± 1.7 [b]	6.8 ± 0.2 [c]	2.7 ± 0.1 [c]
1.0% Ca	0.8 ± 0.1 [c]	34.5 ± 5.3 [c]	0.5 ± 0.1 [c]	15.0 ± 2.2 [c]	25.7 ± 5.8 [b]	11.1 ± 2.9 [b]
2.0% Ca	0.5 ± 0.1 [d]	49.8 ± 1.9 [b]	1.1 ± 0.2 [b]	29.6 ± 3.1 [b]	7.0 ± 0.5 [c]	3.0 ± 0.2 [c]

	C 18:3	C 20:1	C 20:3	C 20:4	C 20:5
0.1% Ca	0.2 ± 0.0 [b]	0.6 ± 0.1 [c]	1.1 ± 0.1	15.3 ± 0.8 [b]	0.5 ± 0.1 [d]
0.5% Ca	1.2 ± 0.1 [b]	1.2 ± 0.1	1.3 ± 0.2 [b]	3.7 ± 0.2 [d]	0.9 ± 0.1 [bc]
1.0% Ca	0.1 ± 0.0 [b]	0.8 ± 0.2 [c]	0.9 ± 0.1 [c]	10.0 ± 1.3 [c]	0.7 ± 0.0 [cd]
2.0% Ca	1.0 ± 0.0 [c]	1.6 ± 0.4 [b]	1.0 ± 0.1	4.1 ± 0.4 [d]	1.2 ± 0.1 [b]

	Saturated	Monounsaturated	Polyunsaturated
0.1% Ca	59.5 ± 2.0 [c]	13.4 ± 0.5 [c]	27.1 ± 1.8 [c]
0.5% Ca	51.6 ± 1.7 [c]	5.7 ± 0.3 [c]	42.7 ± 1.9 [b]
1.0% Ca	46.3 ± 8.9 [c]	28.2 ± 7.1 [b]	25.5 ± 1.8 [c]
2.0% Ca	78.1 ± 1.9 [b]	9.6 ± 1.1 [c]	12.3 ± 0.8 [d]

[a] Values represent means (% of total) ± S.E.

[b-d]: Means within a column with different superscripts differ at $p \leq 0.05$.

Table B: The Effect of Varying Levels of Dietary Calcium on the Phosphatidylethanolamine Percent Fatty Acid Composition in the Colonic Mucosa of Tumor Bearing Rats. [a]

	C 14:0	C 16:0	C 16:1	C 18:0	C 18:1	C 18:2
0.1% Ca	2.2 ± 0.5 [b]	13.2 ± 1.6	3.2 ± 0.6 [b]	15.8 ± 2.0 [c]	25.3 ± 6.2	7.4 ± 1.8 [b]
0.5% Ca	1.3 ± 0.6	12.8 ± 2.1	1.8 ± 0.3 [c]	16.4 ± 1.0 [c]	27.1 ± 2.4 [b]	9.2 ± 0.7 [b]
1.0% Ca	0.5 ± 0.1 [c]	11.4 ± 2.0	1.2 ± 0.1 [c]	21.1 ± 1.9 [c]	14.7 ± 2.2 [c]	7.2 ± 1.4 [b]
2.0% Ca	1.0 ± 0.2	13.8 ± 2.8	2.1 ± 0.4	37.0 ± 4.1 [b]	19.3 ± 2.1	3.2 ± 0.7 [c]

	C 18:3	C 20:1	C 20:3	C 20:4	C 20:5
0.1% Ca	0.7 ± 0.3	0.8 ± 0.2 [c]	1.5 ± 0.3	29.5 ± 6.0 [b]	0.5 ± 0.1 [c]
0.5% Ca	0.3 ± 0.0 [c]	0.7 ± 0.1 [c]	1.3 ± 0.2 [c]	27.7 ± 3.8	1.3 ± 0.7 [c]
1.0% Ca	0.5 ± 0.1	0.6 ± 0.0 [c]	2.2 ± 0.2 [b]	39.8 ± 3.3 [b]	0.7 ± 0.1 [c]
2.0% Ca	1.0 ± 0.2 [b]	2.4 ± 0.7 [b]	1.5 ± 0.3	13.9 ± 5.9 [c]	4.8 ± 1.2 [b]

	Saturated	Monounsaturated	Polyunsaturated
0.1% Ca	26.4 ± 1.4 [c]	25.6 ± 6.1	48.0 ± 5.6
0.5% Ca	26.4 ± 2.2 [c]	25.8 ± 3.5	47.8 ± 4.7
1.0% Ca	26.6 ± 0.9 [c]	13.4 ± 2.7	60.0 ± 3.3 [b]
2.0% Ca	45.9 ± 5.2 [b]	21.1 ± 1.9	33.0 ± 6.0 [c]

[a] Values represent means (% of total) ± S.E.

[b-c] : Means within a column with different superscripts differ at $p \leq 0.05$.

Table C: The Effect of Varying Levels of Dietary Calcium on the Phosphatidylserine Percent Fatty Acid Composition in the Colonic Mucosa of Tumor Bearing Rats. [a]

	C 14:0	C 16:0	C 16:1	C 18:0	C 18:1
0.1% Ca	1.8 ± 0.4 [b]	20.4 ± 4.1 [c]	2.2 ± 0.5	39.1 ± 4.0 [b]	13.2 ± 0.8
0.5% Ca	0.7 ± 0.1 [c]	40.7 ± 2.2 [b]	0.5 ± 0.1 [c]	18.2 ± 0.9 [d]	10.9 ± 0.6
1.0% Ca	1.8 ± 0.1 [b]	25.1 ± 2.3 [c]	3.9 ± 1.5 [b]	26.0 ± 1.7 [c]	16.5 ± 3.3
2.0% Ca	1.0 ± 0.1 [c]	46.4 ± 1.1 [b]	0.6 ± 0.0 [c]	17.2 ± 1.5 [d]	12.1 ± 0.9

	C 18:2	C 20:1	C 20:3	C 20:4	C 20:5
0.1% Ca	2.9 ± 1.1 [c]	2.8 ± 0.6 [b]	1.9 ± 0.2 [bc]	15.1 ± 1.2	0.5 ± 0.1 [b]
0.5% Ca	4.5 ± 0.2	0.9 ± 0.1 [c]	2.2 ± 0.4 [b]	20.8 ± 1.7	0.5 ± 0.0 [b]
1.0% Ca	3.7 ± 1.1	2.0 ± 0.1 [b]	1.0 ± 0.1 [d]	19.8 ± 3.7	0.2 ± 0.0 [c]
2.0% Ca	5.7 ± 0.7 [b]	0.8 ± 0.2 [c]	1.4 ± 0.1 [cd]	14.5 ± 1.5	0.4 ± 0.1

	Saturated	Monounsaturated	Polyunsaturated
0.1% Ca	61.3 ± 1.2 [bc]	17.8 ± 0.7 [bc]	20.9 ± 2.2
0.5% Ca	59.6 ± 1.4 [c]	11.8 ± 0.7 [d]	28.6 ± 1.7
1.0% Ca	52.8 ± 1.9 [d]	22.0 ± 3.2 [b]	25.2 ± 2.9
2.0% Ca	64.5 ± 1.0 [b]	13.0 ± 1.0 [cd]	22.5 ± 0.1

[a] Values represent means (% of total) ± S.E.

[b-d] : Means within a column with different superscripts differ at $p \leq 0.05$.

Table D: The Effect of Varying Levels of Dietary Calcium on the Phosphatidylinositol Percent Fatty Acid Composition in the Colonic Mucosa of Tumor Bearing Rats. [a]

	C 14:0	C 16:0	C 16:1	C 18:0	C 18:1
0.1% Ca	1.7 ± 0.3	11.8 ± 1.3	1.4 ± 0.3	43.0 ± 2.1 [b]	10.9 ± 2.1 [c]
0.5% Ca	1.5 ± 0.4	10.1 ± 0.7	1.2 ± 0.4	38.5 ± 3.6 [b]	18.9 ± 0.5 [b]
1.0% Ca	1.2 ± 0.2	9.1 ± 0.5	1.2 ± 0.2	41.4 ± 2.1 [b]	13.5 ± 0.6 [c]
2.0% Ca	2.2 ± 0.7	11.9 ± 1.9	2.0 ± 0.4	20.9 ± 1.3 [c]	14.3 ± 1.7 [c]

	C 18:2	C 20:1	C 20:3	C 20:4	C 20:5
0.1% Ca	2.4 ± 0.4	2.3 ± 0.4	1.5 ± 0.2	24.8 ± 2.3 [c]	0.3 ± 0.0
0.5% Ca	4.5 ± 1.2	3.3 ± 0.5 [b]	1.5 ± 0.3	19.9 ± 2.6 [c]	0.7 ± 0.2
1.0% Ca	3.2 ± 0.1	2.4 ± 0.2 [b]	2.0 ± 0.1	25.8 ± 2.4 [c]	0.3 ± 0.0
2.0% Ca	5.0 ± 1.0	1.1 ± 0.5 [c]	2.6 ± 0.8	38.9 ± 3.6 [b]	1.1 ± 0.6

	Saturated	Monounsaturated	Polyunsaturated
0.1% Ca	49.5 ± 2.0 [b]	13.2 ± 1.5 [c]	37.3 ± 1.6 [c]
0.5% Ca	41.9 ± 3.1	23.0 ± 1.5 [b]	35.1 ± 1.6 [c]
1.0% Ca	50.0 ± 2.8 [b]	16.6 ± 0.6 [c]	33.4 ± 2.6 [c]
2.0% Ca	34.6 ± 4.6 [c]	17.0 ± 2.1 [c]	48.5 ± 6.1 [b]

[a] Values represent means (% of total) ± S.E.

[b-c] : Means within a column with different superscripts differ at $p \leq 0.05$.

Appendix III

- Figure A: Diagrammatic representation of the response of PKC and ACF to varying levels of calcium in the presence or absence of high fat.
- Figure B: Diagrammatic representation of the correlation between the biomarkers PKC and ACF.

Figure A: Diagrammatic representation of the response of PKC and ACF to varying levels of calcium in the presence or absence of high fat

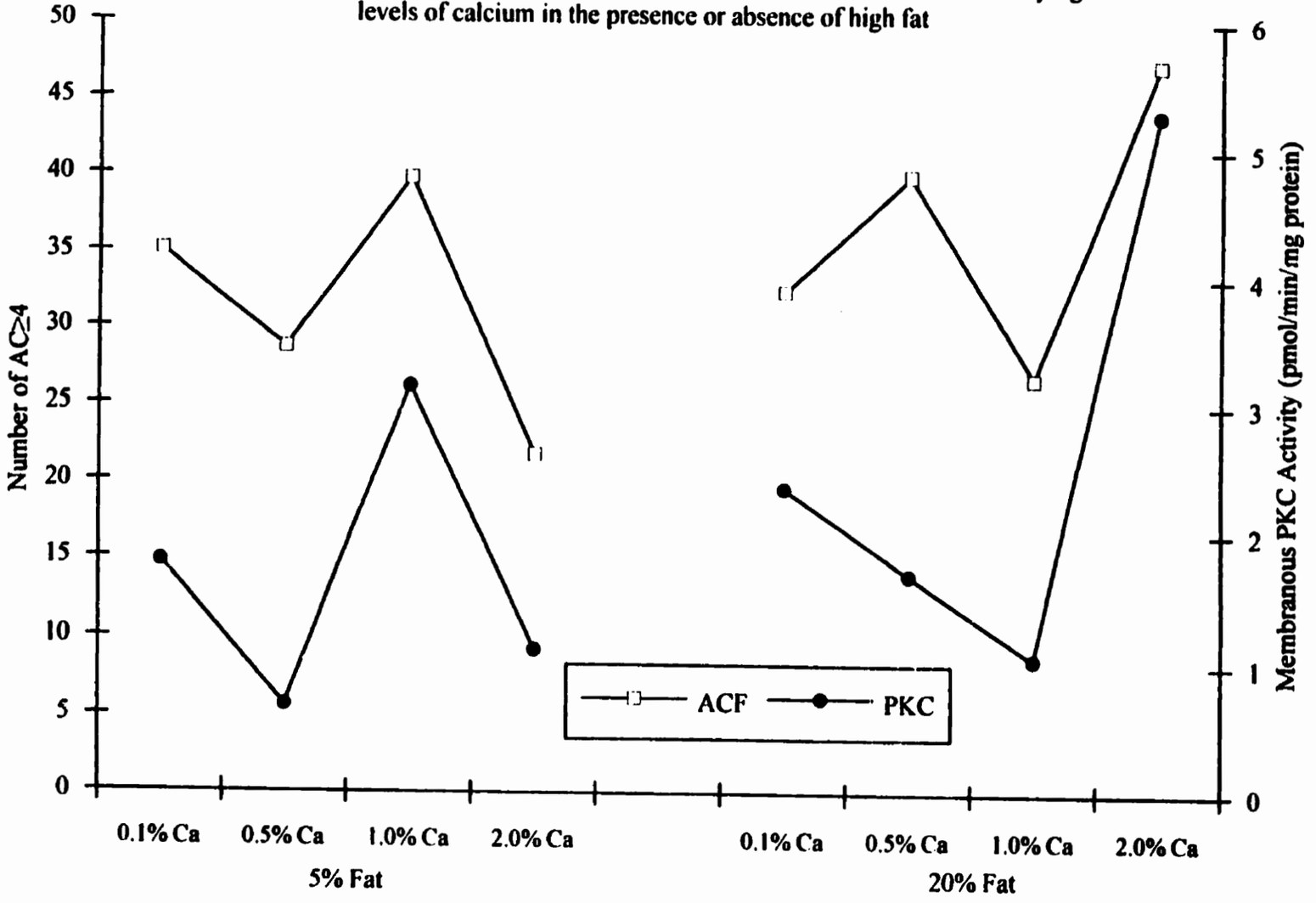

Figure B: Diagramatic representation of the correlation between the biomarkers PKC and ACF