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EFFECTS OF DIETARY FAT ON MUCOSAL CELL MEMBRANE LIPID COMPOSITION AND PEROXIDE LEVELS IN THE LARGE INTESTINE

by

MARCO ENRICO TURINI

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for the acceptance, a thesis entitled EFFECTS OF DIETARY FAT ON MUCOSAL CELL MEMBRANE LIPID COMPOSITION AND PEROXIDE LEVELS IN THE LARGE INTESTINE submitted by MARCO ENRICO TURINI in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE in EXPERIMENTAL MEDICINE

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ABSTRACT

It was hypothesized that dietary fat alters the membrane lipid composition and peroxidation of the so-called "non-proliferative" and "proliferative" cell populations in the rat large intestine. In this regard, Sprague-Dawley rats were fed diets providing 50% or 15% of energy as fat with polyunsaturated to saturated fatty acid ratio of 1.2 or 0.3 for a 25 day period. Cell populations were isolated and the effect of dietary fat on polyunsaturated fatty acid content and peroxide levels was determined.

Neither fat level, nor fatty acid composition of diets influenced total cholesterol, total phospholipid, and percentage of phospholipid classes. Increased polyunsaturated fatty acid content and increased unsaturation index of mucosal cell phospholipids occurred in animals fed high fat and/or high polyunsaturated to saturated fatty acid ratio diet. This increased in polyunsaturated fatty acid content was paralleled by a decrease in the monounsaturated fatty acid content of mucosal cell phospholipids in these animals. The total saturated fatty acid level was not significantly affected by diet treatment. Variation in phospholipid fatty acid composition between cell fractions - "non-proliferative" and "proliferative" - was also observed.

Lipid peroxide values obtained for mucosal cell lipid fractions were sensitive to dietary fat treatment. Groups fed high fat diets, compared to groups fed low fat diets, exhibited higher peroxide levels when expressing the results as nmole equivalents of cumen hydroperoxide/mg of protein. Higher peroxide levels were observed in the mucosal cell lipid fractions for rats fed high polyunsaturated to

saturated fatty acid ratio diets compared to rats fed low polyunsaturated to saturated fatty acid ratio diets when expressing lipid peroxide/nmole of phospholipids. Therefore, further research is required to delineate which dietary factors - fat level, polyunsaturated to saturated fatty acid ratio, or both - primarily influence lipid peroxidation.

In conclusion, changes in fat level and fatty acid composition of the diet altered the mucosal cell membrane lipid composition in the rat large intestine, and influenced the susceptibility of mucosal cell lipid to peroxidation.

THESIS ORGANISATION

Chapter 1 presents an extensive review on the large bowel cancer and its possible etiology. The theory on lipid peroxidation and free radicals, as well as the effects of diet and free radicals on colon cancer are covered. The review leads to the rationale and the hypothesis of this thesis research.

Chapter 2 reports and discusses the results of my study examining the effects of dietary fat treatment on the lipid composition and peroxide levels in the mucosal cells from the large intestine. An introduction recalls the rationale for this experiment, a general discussion covers the major findings and criticisms, and a proposal for future studies concludes the thesis.

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LITERATURE REVIEW AND RATIONALE

A. LARGE BOWEL CANCER, DIET, LIPID PEROXIDATION AND FREE RADICALS: A REVIEW

I. INTRODUCTION

According to the American Cancer Society, more than 123,000 new cases of colorectal cancer are diagnosed annually in the U.S.A. The etiological factors are numerous, but generally relate to dietary habits and to genetic factors. A great deal of research examines effects of diet on fecal constituents and on the incidence of large bowel cancer. However, little research has determined effects of dietary fat on membrane composition in the intestinal tract in relation to the rate of lipid peroxidation, generation of free radicals and possible implications for initiation of colonic carcinoma. This chapter reviews intestinal mucosal structure, main theories proposed for development of large bowel cancer, influence of dietary fat on membrane composition, and generation of lipid peroxides and free radicals in relation to physiological effects. A synopsis of this information leads to the rationale of this thesis research.

II. NORMAL HISTOLOGY OF THE COLORECTUM

The large bowel of mice can be divided into ascending, transverse, and descending colon (1). The rectum is differentiated from the descending colon by having transverse folds of mucosa. Oblique folds characterize the ascending colon, while transverse and descending portions are distinguished by longitudinal folds (1).

The wall of the colon and rectum are composed of discrete layers including mucosa, submucosa, muscularis propria and serosa (1,2). As

opposed to the small intestine, the large bowel does not have a villus (3,4). The mucosa contains simple straight tubular glands, known as crypts of Lieberkuhn (3-5). The lamina propria - loose aerolar connective tissue - separates the crypts (1,2,5). The lower third part of the crypt is the site of an intense proliferation of undifferentiated cells. During cell migration to the upper part of the crypt, these cells differentiate into absorptive cells, goblet cells. and, to a lesser extent, into endocrine cells to form the epithelium. When differentiated, these cells do not undergo cell division. Thus, initiation of neoplasia has a greater chance to occur in the highly proliferative portion of the crypts.

III. CELL CHARACTERIZATION

Plasma membranes of absorptive cells are divided into a luminal part, the brush border or microvillus membrane, and a basolateral region. Isolation and lipid characterization of microvillus and basolateral membrane (BLM) have been performed (6-16) mainly in rat and rabbit small intestinal epithelial cells. Interactions of lipids and proteins in enterocytes have been studied by differential scanning calorimetry, fluorescence polarization and Arrhenius kinetics (9-11). By these methods, important variations were found between proximal and distal portions of small or large intestine as well as within a cell between brush border and basolateral membranes(4,6,7,17,18). Basolateral membrane lipids exhibit a greater motional freedom compared with the microvillus. The lower fluidity observed in the brush border bilayer correlates with higher ratios of protein/lipid (w/w) and cholesterol/phospholipid (w/w) than in the basolateral membrane

(10,11). The variations observed in lipid dynamics may be important for membrane functions. However, the functional implications of maintaining compositional differences in these two types of membranes are not yet known (11). Only recently has information been collected on the nature of the membranes of colonocytes (17-21). Variations between proximal and distal rat colonic luminal membrane lipid fluidity and composition have been reported (18).

IV. LARGE BOWEL CANCER

The etiology of colorectal cancer may be attributed to two main factors: genetic and environmental (22). The environment is defined here as the content of the lumen of the intestinal tract, including diet, bile acids, and gut microflora. Racial, geographical or religious factors which influence life style may be related to differences in dietary habits. Consequently, attention is focused on the role of diet in large bowel cancer incidence and development.

a) Theories

i) The Fat-Fiber Theory

Both epidemiological and experimental studies support the theory of a tumor-promoting effect of high fat diets and a protective effect of dietary fiber (23-32). This theory emphasizes the role of secondary bile acids and gut microflora. Wynder et al (23), in an attempt to explain the relationship between dietary fat and colorectal cancer, postulated that concentrations of both bile acids and neutral sterols in the gut lumen and composition of the microflora are functions of fat

intake. They also hypothesized that gut bacteria, by degrading bile acids, may generate carcinogens. This is supported by a great deal of epidemiological research (27-29). When high risk populations - characterized by high fat, low fiber diets - are compared with low risk populations - whose diets are low fat, high fiber - important differences are observed in the constituents and microflora of feces. High risk groups such as the English (27,28), Scots (28) and North Americans (28) have been compared with low risk groups such as Ugandans (27,28), Japanese (28) and Indians (28). In the high risk populations, higher concentrations of secondary bile acids, deoxycholic and lithocholic acids - which have known cocarcinogenic or carcinogenic activity - and a much higher ratio of anaerobic to aerobic bacteria have been observed. Fiber, by its water-holding and organic acid- and cation-binding properties, has been considered protective in the etiology of large bowel cancer (26,29,31). When populations with similar fat intake but different amounts of fiber intake were compared, a decrease of colon cancer incidence was observed (29,31). It is hypothesized that as fiber has the properties to adsorb organic acids and be water-holding, with high fiber intake (16 mg/day), carcinogens and other toxic products will be "inactivated" and diluted. Fiber also enhances gastrointestinal activity, thus decreasing transit time in the gut. Therefore, by decreasing the chance of the carcinogenic event, fiber may play a protective role in the etiology of colon cancer. Trudel et al (26) demonstrated fat/fiber antagonism in rat dimethylhydrazine-induced carcinoma, supporting current epidemiological and experimental data. The influence of fat and fiber on incidence of large bowel cancer is well accepted, but the importance of type of fat

- saturated or unsaturated - still needs to be accurately delineated (26,33). Fibers vary in their effect; some types are antagonistic, others are protective (26,34,35).

ii) The Calcium Hypothesis

Newmark et al (36) recently elaborated a calcium hypothesis, complementing the theory for fiber's protective effect. Free fatty acids and fecal bile acids, by decreasing the pH in the gut lumen, may be irritating for large bowel epithelium. This may increase its permeability and sensitivity to carcinogens and other toxic agents. However, free fatty acids and fecal bile acids will react with calcium (Ca++) to form insoluble soaps which are excreted via feces. This increases the pH and decreases the incidence of carcinoma. The availability of calcium is diminished by phosphate, which competes with free fatty acids and fecal bile acids to form calcium phosphate. Newmark's hypothesis, based on the reaction of calcium to form insoluble soaps, suggests that dietary calcium should be increased from current intake of 0.93 g/day to 1.5-2.0 g/day, a quantity believed to be non-toxic. This theory needs further investigation.

It is noteworthy to mention that Ca^{++} administered to rats by the intraluminal route (gavage) decreased brush-border membrane fluidity, but not basolateral membrane fluidity, from mucosal cells of the small intestine (37). While no significant alteration in lipid or total phospholipid fatty acyl composition occurred, part of the observed effect could be explained by increased sphingomyelin content in this membrane type. On the other hand, Ca^{++} administered intraperitoneally did not affect fluidity, lipid composition or enzyme activity in

brush-border or basolateral membranes. In vitro experiments using membrane preparations from the small or large intestine showed decreased brush-border membrane fluidity, increased saturated fatty acid content of total phospholipids as well as increased saturation index of this membrane after Ca^{++} treatment (38). Different effects depending on sites along the gut were observed. Unfortunately, only data on the brush-border membrane from the small intestine was presented. Mechanisms by which Ca^{++} affects membrane structure and function is not yet understood. Ca^{++} is also a second messenger which is modulated by phosphatidylinositol turnover, and that regulates phospholipase A_2 activity (39). Prostaglandin metabolism is, in part, regulated by cytosolic Ca^{++} and phospholipase A_2. All these factors may play a significant role in developoment of neoplasia and carcinogenesis.

In summary, a decrease in incidence and development of colorectal cancer is correlated with a low fat, high fiber diet. A higher calcium intake may provide an additional protective factor. The postulated protective effect of selenium and vitamin E in large bowel cancer is discussed in a subsequent section.

b) Diseases Increasing Risk of Colon Carcinoma

Some diseases of the large bowel may lead to increased risk of colorectal carcinogenesis. Individuals at increased risk are those with familial polyposis, Gardner's syndrome, Turcot's syndrome, adenomatous or mixed adenomatous hyperplastic polyps and inflammatory bowel diseases including ulcerative colitis and Crohn's disease (40-42). The chance of mutation in these diseased bowels is higher

than in healthy intestinal tracts. The risk is a function of severity of disease and age of the patient.

c) **Carcinogenesis**

Chemically-induced tumours in animals and cell cultures (43,44) have been used to develop models for carcinogenesis. Two theories - the dysplasia-carcinoma sequence (42,44) and the "de novo" development of carcinoma (45) - have been proposed. The opponents of the latter theory claim that the originating adenoma is not distinguishable from normal tissue even under microscopic examination. More attention has been given to the adenoma-carcinoma sequence (Figure 1-1). This multistage development of cancer is characterized by initial and promotional stages. In the initiation, apparently normal proliferative cells in the lower third portion of the crypt of Lieberkuhn may be subject to mutations, leading to dysplastic tissue. By the action of a tumor promotor, adenoma (benign tumor) within dysplastic cells may be formed. In the promotional stage, some adenomatous cells may undergo further modifications leading to carcinoma (malignant tumor). Malignant tumors degrade adenomatous cells by secreting plasminogen, a proteolytic enzyme. Indeed, a cure is possible if all neoplastic tissue is removed before or up-to the early malignant transformations. The initiation of carcinogenesis is a rapid process which involves DNA damage. On the other hand, the promotional stage is a relatively slow and unknown phenomenon characterized by many physical changes and increased cell proliferation. Due to the rapidity of the initiation, the promotional stage has been considered crucial in prevention (43).

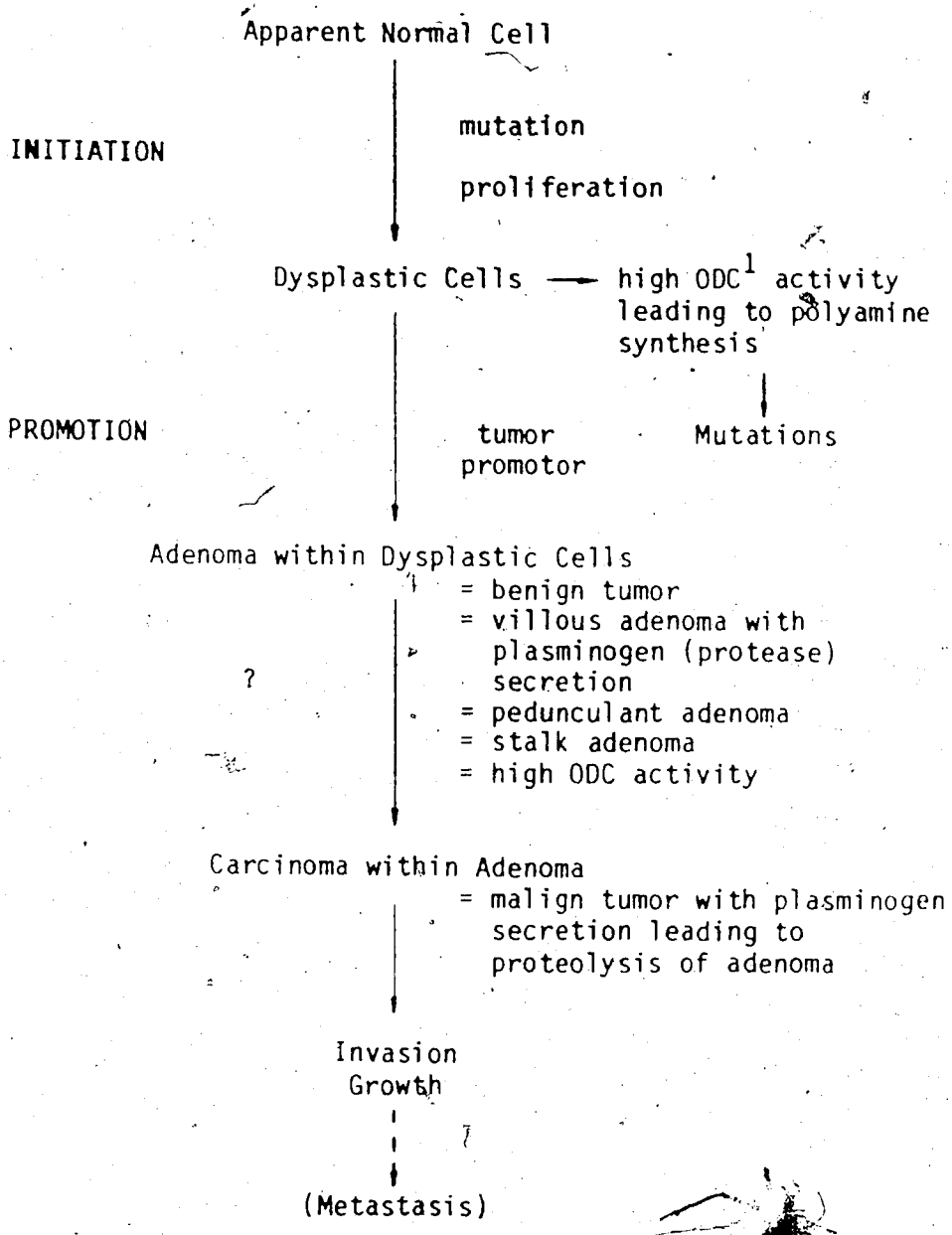

Figure 1-1. Carcinogenesis, initiation and promotion

[1]ODC, ornithine decarboxylase

V. INFLUENCE OF DIET ON CELL MEMBRANES

a) Mammalian Membrane: Structure and Composition

Singer and Nicolson (46) in 1972 proposed a fluid mosaic model of the membrane, emphasizing its dynamic aspect (Figure 1-2). In this model, lipids form a bilayer with their hydrophilic heads at the exterior and with their lipophilic tails at the interior of the membrane. Proteins can be either on the surface or embedded in the membrane. Other important components are carbohydrates, ions and water. Among the lipids, one can distinguish between polar lipids - such as phospholipids (the main class) or glycolipids - and non-polar lipids - such as mono-, di-, tri-glycerides, sterols and sterylesters. The specific composition of a membrane characterizes its physical and biological properties. Great variations in composition occur among tissues but also among cells within a tissue. Comparisons between the Arrhenius break-point in intact membranes and the transition temperature of the bulk lipids led to the new concept of the importance attributed to the microenvironment surrounding the enzyme or functional protein in the membrane (47).

b) Diet Influence on Membrane Composition and Function

Whether or not diet modifications in type and amount of fat can modulate membrane composition and consequently membrane physical and biological properties is of interest. "In vivo" and "in vitro" studies have been peformed as reviewed by Spector and Yorek (48). "In vivo", the effects of dietary fat on erythrocytes in humans (48) and on different membranes in diverse tissues of animals (49-55) have been

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Figure 1-2. The lipid-globular protein mosaic model [From Singer SJ and Nicolson GL, Science.1972;175:720-731]

The membrane consists of a lipid bilayer in which proteins (solid bodies with stippled surfaces) appear at the surface of the membrane or embedded in the lipid matrix. The lipid composition of the microdomains surrounding the proteins controls, in part, the protein motional freedom within the plane of the membrane.

investigated. "In vitro", the influence of various media on cells in culture, such as mammalian and Ehrlich ascites cells, fibroblasts and platelets, have been examined (48). It is now accepted that membrane lipid composition reflects, in part, dietary fat intake. Fluorescence polarization, electron spin resonance, and differential scanning calorimetry studies have shown relatively high membrane fluidity in membranes with a high unsaturated versus saturated fatty acid content (49,53). This fluidity is also affected by cholesterol. Furthermore, functional changes in membranes such as liver microsomes (50,51), hepatocytes (56) and mitochondria (57,58) have been observed when diet fat was modified. Thus, not only intrinsic, but also extrinsic influences control membrane lipid composition, affecting membrane-bound enzyme activities. For a review on the effect of diet fat on subcellular structure and function, see Clandinin et al.(59). The structure and composition of both brush border (9-11,16-18) and basolateral membranes (7,8,10,11,17) have been determined in rats (7-11,17,18) and in rabbits (16). Little, however, is known about the role of dietary fat on lipid composition of these membranes in both small and large intestines. Recently, Thomson et al. (61-63) studied effects of dietary fat manipulation on intestinal transport. In general, changes in intestinal function of healthy rats did not correlate with changes in the effective resistance of the intestinal unstirred water layer, the gut morphology, or with the brush-border membrane phospholipid or cholesterol content. However, in a previous study by this group (64) brush-border membrane phospholipid fatty acyl composition was altered by feeding a saturated fat diet. Thus, it is likely that changes in the fatty acyl chains of the membrane lipid is,

in part, responsible for changes in active and passive intestinal transport resulting from manipulation by dietary fat. The mechanism by which these changes occur seems complex and remains to be elucidated. Brasitus et al. (14) studied the effects of saturated and unsaturated triacylglycerol on the composition and fluidity of rat intestinal plasma membranes. In this experiment, proximal and distal portions of both small and large intestines were used. As observed in other tissues, diet influences enterocyte and colonocyte membrane composition. Marked variations between brush border and basolateral lipid composition and fluidity may underline individual functions of both these membranes. Further studies are needed to determine compositional differences exhibited and maintained by both membranes, dietary effects, and the physical and biological implication of changes in membrane composition.

VI. LIPID PEROXIDATION AND FREE RADICALS

a) Free Radicals

Free radicals are generated through a homolytic break (65). They are characterized by an impaired electron and thus a net spin. These characteristics are used to measure free radicals by Electron Spin Resonance (ESR) (66-68). The toxicity of free radicals is a function of their lifetime in relation to their reactivity (65,66). A radical with short lifetime and high reactivity will interact with a nearby substrate. The consequence of this interaction depends on the kind of substrate involved. Conversely, a radical with a long lifetime and lower reactivity will be able to diffuse from its site of origin to

another site where it may cause cell damage. Irradiation - ionizing radiation or light, or bond homolysis - uncatalysed or catalysed non-enzymatically or enzymatically, may generate free radicals (65). In vivo, free radicals are generally produced through a catalytic and complex pathway. Damage produced by free radicals are several. Reactive free radicals may cause DNA damage, destroy nucleotide coenzymes, generate lipid peroxides, injure membranes affecting their biological functions, and disturb SH-dependent enzymes (69).

b) Lipid Peroxides in Diet

Even if antioxidants are added to foods, lipid peroxides may be present in the diet (70). These peroxides are generated mainly by frying meat or heating oils to high temperatures.

Boigegrain et al (71) investigated the deperoxidation of hydroperoxylinoleic acid by glutathione peroxidase to its non-toxic product in the rat intestinal mucosa. Though the capacity of the intestine to reduce this lipid peroxide by glutathione peroxidase was 6-fold lower than in the liver, activity in the gut was not insignificant. This animal model needs to be applied to studies of humans.

The uptake of labelled secondary autooxidation products of linoleic acid was investigated in the rat (72). The dose was given intragastrically after a four hour fast, and approximately half of the orally fed secondary autooxidation products were excreted in the feces. From the other half that has been absorbed, approximatly 50% was discharged by urination and 25% through CO_2. The last 25% of secondary autooxidation products of linoleic acid were metabolized in the liver

with possible deleterious effects. Another group (73) performed the same experiment but fractionated the secondary autooxidation products into monomeric-polymeric and low molecular weight fractions. Interestingly, the low molecular weight compounds from methyl linoleate hydroperoxides were better absorbed. Only 3% of the radioactivity was recovered in the gastrointestinal tract and in the feces compared to 53% and 41% for the monomeric-polymeric fraction and methyl linoleate hydroperoxides, respectively. As can be expected, excretion through urine and CO_2 was much higher for the low molecular weight labelled compounds compared to the two other fractions.

Bergan and Draper (74) found that about 45% of the 1-^{14}C-methyl linoleate hydroperoxide, administered by intubation, still remained in the gastrointestinal tract of rats after 24 hours. It was found as intact peroxide, principally bound to the wall of the stomach. As no evidence was obtained for the absorption of unchanged peroxide, it is suggested that the hydroperoxide had undergone a reduction during absorption to the non-toxic hydroxy acid. A criticism to these experiments is that lipid peroxides tested were not mixed with food and levels were not physiological. This may affect the absorption process. On the other hand, it may be concluded that either long chain fatty acid hydroperoxides are not absorbed or epithelial cell membrane contains a defence system which transforms toxic lipid peroxides to non-toxic hydroxy acids. It may be more important that lipid peroxidation may occur intracellularly or in membrane.

c) Intracellular and Membrane Lipid Peroxidation

The process of lipid peroxidation is characterized by an

initiation, propagation and terminal sequence (Figure 1-3) (65). In the initiation stage, the decomposition of a substance such as an azocompound, generates a free radical. This free radical may react with oxygen to form a peroxyl radical. This unstable peroxyl radical may in turn interact with unsaturated lipid (the best substrate for peroxidation) and form a carbon-centred radical. In the propagation sequence, this new radical reacts with O_2 to again form a peroxyl radical, which in turn combines with another lipid. The end product of this reaction is a free radical and a hydroperoxide. Theoretically, these two steps are perpetuated until stopped by a termination reaction or until the substrate is used up. "In vivo", this propagation may not easily occur due to presence of antioxidants and absence of necessary substrates in proximity to the membrane (75). This termination reaction may occur when two radicals collide or by the presence of antioxidants such as vitamin E. The hydroperoxide may also undergo further reactions catalyzed by cytochrome P_{450}. These reactions generate break-down products, including unsaturated aldehydes or malondialdehydes, but also toxic free radicals such as hydroxide or superoxide (76,77). The action of iron (Fe^{++}) on hydroperoxide releases a free radical. By further degradation, this product leads to another free radical, which may enter the propagation sequence.

Some enzymatic reactions and drugs reduce oxygen to the toxic superoxide, hydroperoxide or hydroxyradical (77). The action of hydroquinones, catecholamines and flavins on H_2O also release superoxides. Another pathway, the iron-catalyzed Habe -Weiss reaction, also generates free radicals (77). Superoxide, hydroperoxide and Fe^{++}/Fe^{+++} are involved. Rates of intracellular radical production are

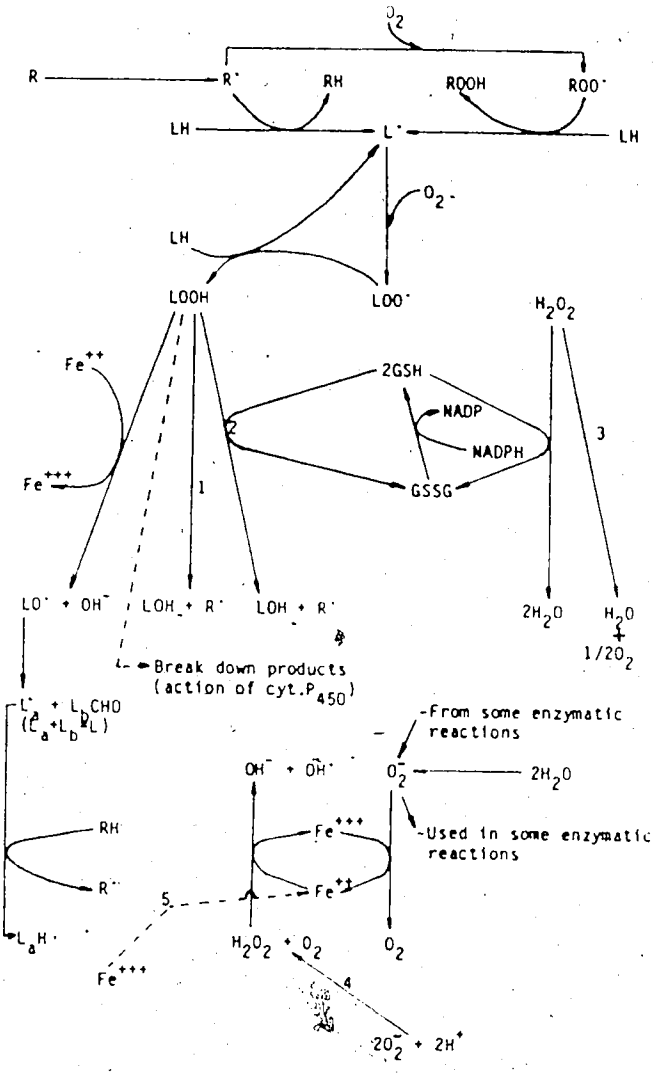

Figure 1-3. Lipid peroxidation and free radicals: pathways

The pathways described represent a summary from the literature, and does not keep count of the site specificities.

Legend:
- LH: unsaturated fatty acid
- R·, L·: free radicals
- ROO·, LOO·: peroxyl radicals
- ROOH, LOOH: hydroperoxides
- O_2: oxygen molecule
- O_2^-: superoxide
- OH^-: hydroxyl radical
- OH^-: hydroxide
- H_2O_2: hydroperoxide
- 1: heme-peroxidase
- 2: Se^{++}-dependent and non-Se^{++}-dependent glutathione-peroxidase
- 3: catalase
- 4: spontaneous or superoxide dismutase
- 5: ascorbate

affected by modulation of enzyme activities, cofactor availability, substrate concentrations,and oxygen tension (77). Furthermore, some conditions - such as hyperoxia, antibiotic and drug therapy, or ischemia - favour generation of free radicals and lipid peroxides (77). Free radicals not only have negative effects; they also play a protective role in phagocytosis against invading organisms (77), as well as in prostaglandin metabolism where small amounts of free radicals are needed for initiation of the cyclooxygenase reaction (78). Indeed, the cell must have a defence system against toxic lipid peroxides and free radicals. This defence system functions mainly via antioxidants such as vitamin E and the NADPH-dependent enzymes _ selenium- and non-selenium-dependent glutathione peroxidases _ which catalyse the reduction of hydroperoxides (70,79-81). Heme peroxidase has the same function, but releases potent free radicals. As vitamin E is the only known lipid soluble antioxidant, interactions with water soluble antioxidants of the cytoplasm might occur. In the past two years, a number of experiments have demonstrated interaction between glutathione, ascorbic acid and vitamin E (82-86). It was also shown that the cytosolic fraction is of prime importance for efficient defense against reduced oxygen species; protein may be involved (85,87).

A normal cell maintains a balance between production and degradation of free radicals (88). Displacement in this balance may lead to cell injury through structural and functional membrane damage or mutations. Extensive sub-cellular damage may result in cell death (89). On the other hand, cancer may originate from limited sub-cellular injuries. In a recent study, microsomes and plasma

membranes isolated from rat liver and hepatomas have been compared for lipid composition and antioxidant enzyme activities (90). Hepatoma membranes exhibited lower lipid peroxidation susceptibility and lower enzymatic protective activity against toxic reduced oxygen species compared to normal membranes. While vitamin E content of both hepatoma and rat liver membranes were similar, this surprising lower lipid peroxidation susceptibility in hepatocellular carcinoma may partly be explained by a protective action of increased membrane saturation index. Consequently, the vitamin E to polyunsaturated fatty acid ratio was higher in the tumor membranes and this observation is in agreement with results reported by others (91). Furthermore, normal membrane fractions, after treatment by oxy radicals, tend to exhibit similarities with tumor membranes(90). These observations led to the hypothesis that structural and functional alterations of tumor membranes is a consequence of previous suffering in vivo by oxy radicals. Lipid peroxides are known to decrease DNA synthesis, cell division, and tumor growth. Effects of free radicals on mutation and cancer initiation have been extensively reviewed (92).

Peroxidation has been studied mainly in smooth muscle cell cultures (93) erythrocytes (67), liver microsomes (76,94,95), hepatoma microsomes (94), hepatocytes (96) and Ehrlich ascites cells (97). Peroxidation in gut microsomal or plasma membrane fractions has only been studied to a limited extent.

VII. SELENIUM, VITAMIN E AND CANCER

A large number of studies related to vitamin E and selenium are found in the literature. It is well known that vitamin E limits lipid

peroxidation by free radicals, (reviewed by Tappel; 70). This antioxidative role functions in cooperation with selenium through Se-dependent glutathione peroxidase (98). In animals such as rats or chicks, a positive logarithmic correlation between GSH-peroxidase activity and selenium concentration has been found (70). A significant specific activity of the enzyme was measured in the stomach and small intestine.

The first animal experiments showing an anticarcinogenic effect of selenium was performed by Clayton and Baumann in 1949 (99). Many other studies have since shown this protective effect of selenium against cancer. The protective effect depends on the concentration used. Studies in animals with both spontaneous and chemically-induced tumours have been performed (100-103). The finding that growth of transplanted tumours was not prevented by selenium suggests its effect may be limited to aberrant cell populations in early phases of development.

Frequently, lower than normal concentrations of selenium in blood, serum or plasma were observed in cancer patients. Age-corrected cancer mortalities inversely correlated with calculated dietary selenium intakes for data of 27 countries (104). Regardless of statistical problems in epidemiological studies, the best correlation was found for breast ($r=-0.80$, $p<0.0001$), followed by colon cancers ($r=-0.74$, $p<0.0001$ for males and $r=-0.72$, $p<0.0001$ for females). A similar inverse correlation was found when selenium content of whole blood from healthy donors was examined, both for the 27 countries studied and for several states in the U.S. In conclusion, there is indication that selenium has a protective effect against cancer. Indeed, other antioxidants, including ascorbate and beta-carotene, have also been

correlated with decreased incidence of some forms of cancers (105).

A decreased incidence of mammary tumors in rats (106) and colonic tumors in mice (107) has been observed when diets were supplemented with vitamin E. Epidemiological data are not yet available on the possible protective effect of this vitamin in colorectal cancer. There are, however, two studies in progress - one in Canada (108) and the other in the U.S.A. (109).

VIII. SYNTHESIS OF TWO THEORIES ON COLON CANCER DEVELOPMENT: AN HYPOTHESIS

In light of the data presented both bile acids and free radicals may act in concert to promote neoplastic diseases. High polyunsaturated fatty acid intake, among all the parameters that may be influenced, increases membrane fluidity and membrane lipid peroxidation. Increased membrane fluidity may be associated with decreased membrane absorption selectivity leading to higher sensitivity to toxic products including carcinogens. Lipid peroxidation generates free radicals that may cause cell injuries such as membrane damage and DNA mutation. High fat intake has been associated with increased bile acid production from cholesterol. Secondary bile acids show carcinogenic activity, which may be enhanced in tissues with high membrane fluidity. Furthermore, dietary fat influences metabolism of prostaglandins, an important factor when considering neoplastic diseases. Direct effects of Ca^{++} or its action as a second messenger also has to be considered. It should be remembered that carcinogenesis is a dynamic process.

It is of interest to note that human colorectal cancer, as opposed

to rats or mice, develops only in the lower part of the large bowel, the site of bile acid absorption, while the whole large intestine is in contact with bile acids. Thus, it may be relevant why human large bowel cancer develops at the bile acid reabsorption site. Why are there differences between man and rodents in sites of large bowel cancer? How does dietary fat influence colorectal cancer development?

In conclusion, it seems that high fat diets of high polyunsaturated to saturated content may initiate and/or promote tumor development. Factors such as bile acids, membrane lipid fluidity and lipid peroxides may act in concert inducing and/or promoting neoplasia. Further investigations are needed to accurately delineate specific effects and mechanisms.

IX. SUMMARY

Colonic epithelial differentiated cells do not undergo division. Neoplasia and thus malignant tumours in this tissue likely originate from non-differentiated and proliferative cells located in the lower third portion of the crypt of Lieberkuhn. Factors such as fat, vitamin E, selenium, and other antioxidants may play an important role in the etiology of large bowel cancer. As observed in other tissues, a high polyunsaturated fatty acid diet should increase the content of this type of fatty acid in colonocyte membranes.

Polyunsaturated fatty acids are good substrates for lipid peroxidation and consequently free radical generation. These free radicals may cause cell damage, structural and functional injuries to membranes and mutations. Furthermore, polyunsaturated fatty acids (PUFAs) augment membrane fluidity, possibly leading to higher sensitivity to toxic

agents, including carcinogens. These factors increase risk of developing colorectal cancer by affecting proliferative cells. Vitamin E - by its antioxidant function - and selenium - as cofactor for glutathione peroxidase - play a significant protective role reducing incidence of some forms of cancer, such as mammary cancer. However, little is known about the influence of diet, first on large bowel cell membrane composition, and secondly on the role of lipid peroxidation in this tissue. Furthermore, little is known about the physiology of the proposed protective effect of vitamin E, selenium, and other antioxidative factors on colorectal carcinogenesis. In conclusion, as marked variations may occur from one tissue to another, further investigation is needed to determine the influence of these factors in the large bowel.

B. RATIONALE

Very little is known about the effect of dietary fat on the composition of the proliferative cell membranes in the crypt of the large bowel and on the extent of lipid peroxidation in this cell type. Rates of lipid peroxidation and free radical generation depend, in part, on the availability of polyunsaturated fatty acids in membranes, the main substrate for lipid peroxidation. Lipid peroxides and free radicals may cause cell damage and thus be important in development of large bowel cancer. Thus, understanding the relationship between dietary polyunsaturated fats and production of fatty acid peroxidation products in membranes is important to better understand the etiology of this disease.

C. OUTLINE OF THESIS

I. HYPOTHESIS

It is hypothesized that mucosal cell composition in the rat large intestine is influenced by diet. More specifically, it is hypothesized that:

1. The lipid composition of "non-proliferative" and "proliferative" mucosal cells in the large intestine reflects, in part, the fatty acid composition of the diet.

2. Lipid fractions of the mucosal cells from rats fed high (50% as energy) or low (15% as energy) fat diets with a high P/S ratio (P:S=1.2) exhibit more lipid peroxides than rats fed comparable high or low fat diets with a low P/S ratio (P:S=0.3)

3. A high fat diet (50% as energy) - either saturated or unsaturated - increases lipid peroxide production compared with a low fat diet (15% as energy) having the same P/S ratio (either P:S=1.2 or P:S=0.3).

II. STATEMENTS OF OBJECTIVES

The objective of the study was to investigate the effects of changes in the dietary fat level and fatty acid composition on the lipid composition and peroxidation of the mucosal cells in the rat large intestine. For this purpose, two cell populations, "non-proliferative" and "proliferative", were isolated. Lipids were

extracted, and phospholipid class separated to determine the total cholesterol and phospholipid content, as well as the percentage of each phospholipid class and the phospholipid fatty acid composition. Lipid peroxide levels were measured and correlated with the fatty acid unsaturation index of the cell phospholipids.

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CHAPTER 2

EFFECTS OF DIET TREATMENT ON LIPID COMPOSITION AND PEROXIDE LEVELS OF MUCOSAL CELLS IN THE LARGE INTESTINE

A. INTRODUCTION

Dietary fat has been correlated with increased incidence of some forms of cancers including skin, breast, and colon cancers (1-11). Colon cancer is the leading cause of death among all patients with malignancies in the United States. Previous studies have shown that dietary fat influences membrane structure and function (12-20). Other studies have also demonstrated that membranes with a high unsaturation index are more sensitive to peroxidation compared to membranes with a lower unsaturation index (21,22). This susceptibility to peroxidation is also influenced by free radical scavengers and antioxidants such as vitamin E and C, glutathione, and enzymes including superoxide dismutase, catalase, and Se^{++}- and non-Se^{++}-dependent glutathione peroxidases (23-29). As vitamin E is a membrane soluble antioxidant, interactions with cytosolic antioxidants occur (25,30-35). Lipid peroxides are involved in the regulation of DNA synthesis and cell division (36-38). Lipid peroxides are also thought to be involved in a number of diseases such as cancer and inflammation of the large bowel.

Several studies of mucosal cell membranes from small and large intestines have been performed (20,39-54). However, effect of dietary fat on colonocyte cell membrane lipid composition and peroxidation has not been reported. The objective of this study was first to determine whether or not dietary fat influences colonocyte cell membrane lipid and phospholipid fatty acid composition as observed in other tissues. Secondly, to determine whether or not dietary fat content of unsaturated fatty acids influences colonocyte cell lipid peroxide

levels. Colonocytes were fractionated into "non-proliferative" and "proliferative" cell populations to determine differences in the lipid composition between these cell types.

B. MATERIAL AND METHODS

I. ANIMALS AND DIETS

Four weanling-male Sprague-Dawley rats were obtained weekly for an eight week period from the University of Alberta Laboratory Animal Services. Animals were fed rat chow (Allied Mills, Inc., Speciality Feeds Department, Chicago, Ill.) for 3 or 5 days and then fed semi-purified diets (Table 2-1) for 25 days. Each week, four rats were distributed into four groups and fed one of the four diets (total n=8 for each group). Diets and water were supplied ad libitum to rats housed individually in a temperature (22°C) and light controlled room providing 12 hour light and dark periods. Diets were freshly prepared each week and animals fed on alternate days. Food and feed cups were changed at feeding time to avoid possible lipid peroxide intake from old food. The four semi-purified diets were: high fat or low fat with high (P/S=1.2) or low (P/S=0.3) polyunsaturated to saturated fatty acid ratios for each of the high and low fat diets (Table 2-2, figure 2-1). High fat diets provided 50% as energy from fat, while low fat diets provided 15% as energy from fat. Mixtures of safflower oil, hydrogenated beef tallow, and linseed oil were used to provide the dietary fat source. Linseed oil was included to provide the w-3 fatty acid source. Essential nutrient densities for non-fat components were

Table 2-1. Composition of Experimental Diets.

Fat Level	High		Low		
P/S Ratio[1]	High	Low	High	Low	Density (g/1000 kcal)
			(g)		
Fat mixture	267.9	266.7	65.3	64.9	55.9/55.6/16.7/16.7
High protein Casein (vit. free)	292.2	292.7	237.4	237.4	61
Cornstarch	81.5	81.6	406.9	406.9	17/17/104.5/104.5
Glucose	225.1	225.5	182.9	182.9	47
Non-nutritive cellulose	53.19	53.29	42.49	42.89	11.1/11.1/10.9/11.0
Vitamin mix[2] (Vit. E free)	11.0	11.0	8.9	8.9	2.3
Mineral mix[3]	55.3	55.4	44.9	44.9	11.55
Choline	4.3	4.3	3.5	3.5	0.9
Inositol	6.7	6.7	5.4	5.4	1.4
L-methionine	2.8	2.8	2.3	2.3	0.6
Dl-α -tocopheryl acetate (IU)	—	45.61	49.35	60.35	17.63 (IU/1000 kcal)
Energy (kcal)	4791	4799	3893	3893	

[1]P/S, polyunsaturated to saturated fatty acid ratio.

[2]Vitamin mix provided the following per kilogram of vitamin mixture: vitamin A, 2,000,000 IU; vitamin D, 200,000 IU; menadione, 0.5 g; choline, 200 g; p-aminobenzoic acid, 10 g; inositol, 10 g; niacin, 4 g; d-calcium pantothenate, 4 g; riboflavin, 0.8 g; thiamin-HCl, 0.5 g; pyridoxine-HCl, 0.5 g; folic acid, 0.2 g; biotin, 0.04 g; and vitamin B_{12}, 3.0 g.

[3]Bernhart-Tomarelli mineral mix (Teklad Test Diets, Madison, WI) provided the following per kilogram of mineral mixture: $CaCO_3$, 21.0 g; $CaHPO_4$, 735 g; MgO, 25.0 g; K_2HPO_4, 81.0 g; K_2SO_4, 68.0 g; NaCl, 30.6 g; Na_2HPO_4, 21.4 g; cupric citrate, 0.46 g; ferric citrate (16.7% Fe), 5.58 g; manganese citrate (13.9% Mn), 8.35 g; KI, 0.0072 g; zinc citrate, 1.33 g; and citric acid, 2.2728 g.

similar for the four diets on a per calorie basis. Vitamin E levels of the four diets were equalized to the vitamin E content of the high fat, high polyunsaturated to saturated fatty acid ratio, by addition of d-alpha-tocopheryl acetate to give a final vitamin E density of 17.63 IU/1000 kcal for each diet. Diets were prepared using essentially vitamin-free casein and a vitamin mix free of vitamin E. This vitamin mix has been prepared according to the composition of AOAC vitamin mix

Table 2-2. Fatty Acid Composition of Experimental Diets.

Fatty Acid	(%, w/w)			
	High Fat		Low Fat	
	High P/S[1]	Low P/S	High P/S	Low P/S
C14:0	2.0	3.5	1.9	3.7
C14:1	0.1	0.3	0.1	0.2
C15:0	0.3	0.5	0.3	0.5
C15:1	0.1	0.2	0.1	0.2
C16:0	16.5	24.1	16.4	24.8
C16:1	0.3	0.4	0.3	0.4
C17:0	0.9	1.7	0.9	1.7
C18:0	22.0	41.2	22.4	41.1
C18:1(9)	8.2	4.7	8.3	4.7
C18:2(6)	47.3	20.6	44.6	17.5
C18:3(6)	0.1	0.3	0.1	0.2
C18:3(3)	1.4	1.5	4.2	4.3
C20:0	0.3	0.5	0.1	0.5
C20:2(6)	0.2	0.2	0.1	0.1
C22:0	0.1	0.1	ND	ND
C22:5(3)	0.1	0.2	0.1	0.1
C22:6(3)	0.1	ND[4]	0.1	ND
Σsats[1]	42.1	71.6	42.0	72.3
Σmonounsats[1]	8.7	5.6	8.8	5.5
Σpolyunsats[1]	49.2	22.8	49.2	22.2
Σ(*w*-6)[1]	47.6	21.1	44.8	17.8
Σ(*w*-3)[1,2]	1.6	1.7	4.4	4.4
P/S[3]	1.17±0.05	0.32±0.01	1.17±0.03	0.31±0.02

[1]Abbreviations used are: P/S = polyunsaturated to saturated fatty acid ratio, Σsats = total saturated fatty acids, Σmonounsats = total monounsaturated fatty acids, Σpolyunsats = total polyunsaturated fatty acids, Σ(*w*-6) = total (*w*-6) fatty acids, Σ(*w*-3) = total (*w*-3) fatty acids.

[2]Densities (g/1000 kcal) of (*w*-3) fatty acids are similar for the four experimental diets.

[3]P/S, polyunsaturated to saturated fatty acid ratio; values are means ± SD of 11 freshly prepared diets.

[4]ND, not detected.

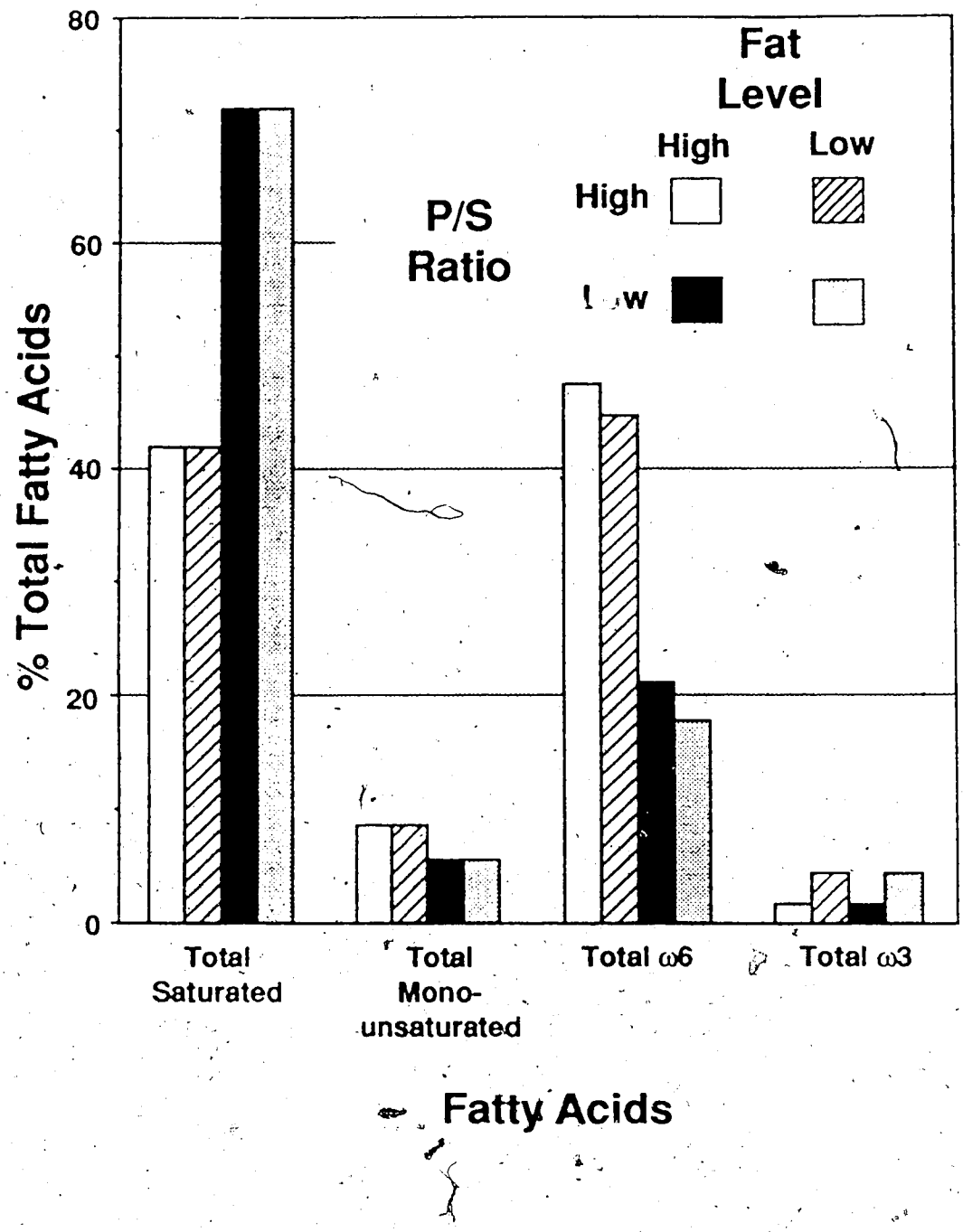

Figure 2-1. Fatty acid composition of diets

Four diets, high fat (50% as energy) and low fat (15% as energy) with high P/S (1.2) or low P/S (0.3) ratio, were fed to rats for 25 days. Safflower oil and hydrogenated beef tallow were used as fat source; linseed oil was added to provide w-3 fatty acid. The diets with similar fat levels differed in the amounts of saturated (primarily $C_{16:0}$) and w-6 (primarily $C_{18:2(6)}$) fatty acids.

(Teklad Test Diets, Madison, WI). Animals were weighed at delivery and at the beginning of dietary treatment then weekly, and at the time of killing. Rats were sacrificed by decapitation and their large intestine removed for mucosal cell isolation. Blood was collected into non-heparinized glass test tubes, and protected from light for serum vitamin E determination. Tubes were kept in the dark at room temperature for 30 minutes before being centrifuged at 500 g for 15 minutes at 10-15°C. The supernatants were collected (serum) and stored at -20°C (in the dark) until used.

II. CELL ISOLATION

The cell isolation procedure used has been adapted from methods described by Brasitus (55) and Weiser (49). The solutions used were: cold saline solution; solution A containing HCl (1.5 mM), NaCl (96.0 mM), sodium citrate (27.0 mM), KH_2PO_4 (8.0 mM), Na_2HPO_4 (5.6 mM), dithiothreitol (0.5 mM), pH 7.3; solution B consisting of Ca^{++} and Mg^{++} Free Hank's buffer containing triethanolamine-HCl (10.0 mM) and dithiothreitol (0.5 mM), pH 7.3; and standard buffer containing NaCl (139 mM), Na_2HPO_4 (10 mM), pH 7.3.

To eliminate gut bacterial contamination, solution A and B as well as cold saline solution were supplemented with Penicillin-G (k-salt, 660 U/ml) and streptomycin (1.6 mg/ml). Solution B was also supplemented with mycostatin (118.2 U/ml); phenylmethanesulphonyl fluoride (PMSF, 0.1 mM) was added to solution A and B just before use as were antibiotics to solution A. Ca^{++} and Mg^{++} Free Hank's buffer and antibiotics were only stable for one to two weeks, therefore solutions were freshly prepared each week, except for solution A which

was prepared as a stock solution for the whole experiment. All solutions were kept at 4°C in the dark to avoid antibiotic degradation.

The large intestine was excised, feces delicately removed by means of cold saline solution and flushed 3 times with 40 ml of the same cold saline solution. This tube was then tied at its proximal end, filled with solution A and closed at its other end with a hemostat. This slightly distended sac was placed in a 250 ml flask containing 125 ml of standard buffer solution (37°C), and incubated for 15 minutes at 37°C in a metabolic shaker waterbath (75 osc./min). The sac was then emptied, the solution discarded and replaced by solution B (pre-incubated at 37°C), and incubated. After each incubation, the solution was collected in a 15 ml Corex glass centrifuge tube and the sac refilled. Incubation times were 2 times 10 minutes followed by 10 times 15 minutes. The tubes (total=12) were kept on ice until centrifuged at 2000 rpm (484 g) at 4°C for 10 minutes in a Beckman model J2-21 centrifuge. Supernatants were discarded and pellets resuspended in 5 ml of standard buffer. At this stage, fractions 2 and 3, 4 and 5, and 6 and 7, as well as fractions 9 and 10, and 11 and 12 were pooled to give fractions I, II, III, IV, and V, respectively. These five tubes were centrifuged at 2000 rpm (484 g) at 4°C for 10 minutes. Supernatants were discarded, pellets I to III resuspended in standard buffer, filtered on 100 micrometer nylon mesh filter (Nitex) and pooled (A); fractions IV and V underwent the same process (B). Resuspending volume after filtration was 12 ml for both fractions (A) and (B). These two tubes were centrifuged at 2000 rpm (484 g) at 4°C for 10 minutes, supernatants discarded and pellets resuspended in 1 ml of standard buffer. Cell suspensions were homogenized by means of a

1/2" long #26 Gauge syringe needle. A similar process was conducted for sample #8 to get the protein released in this fraction. Aliquots for protein determination and 100 µl for thymidine kinase activity measurements were kept. The remainder to be used for lipid and lipid peroxide analysis was frozen in liquid nitrogen before storage. All tubes were kept at -70°C until used. The whole cell isolation procedure took 4 1/2 hours. Protein was determined by a modified method of Lowry (56) using bovine serum albumin (BSA) as a standard.

III. ^{3}H-THYMIDINE INCORPORATION

Preliminary experiments were performed to determine fractions containing proliferative cells using 2-Me-^{3}H-thymidine as a marker for DNA synthesis. Rats weighing 300-305 g were injected intraperitoneally 100 µCi (55) of 2-Me-^{3}H-thymidine 3 hours prior to sacrifice (49). Rats were killed by decapitation and cells isolated as previously described. However, fractions were filtered after the first centrifugation, and transferred by 3 times 1 ml of standard buffer into flint tubes (10mm x 75mm) after the second centrifugation. The cell fractions were not pooled. Flint tubes were centrifuged at 2000 rpm (484 g) at 4°C for 10 minutes, supernatants discarded and pellets resuspended at a volume of 500 µl with standard buffer. Cell fractions were homogenized with a 1/2" long #26 Gauge syringe needle and aliquots were taken for protein determination. After addition of 2 ml of cold 10% (w/v) TCA, containing 1 mg thymidine/4 ml, tubes were vortexed and kept overnight in the fridge (4°C). Tubes were then centrifuged at 5000 rpm (3020 g) at 4°C for 15 minutes, and supernatants discarded. Pellets were dissolved in 500 µl of Protosol, transferred by 3 times 2

ml of toluene base scintillation cocktail (100mg POPOP, 5g PPO per litre of toluene) into 7 ml counting vials, and counted for ^{3}H-thymidine incorporation. Counting was performed in a Beckman LS 5801 liquid scintillation system with an efficiency of 45%. Results were expressed as dpm/mg protein.

IV. THYMIDINE KINASE ASSAY

To differentiate "proliferative" from "non-proliferative" cells during the main experiment, thymidine kinase activity has been measured according to the method of Salser and Ballis (57). Reactions were carried out at 37°C in capped flint tubes (10mm x 75mm) in a waterbath. The reaction mixture containing 90-110 µg of homogenate protein (100 µl volume; adjusted if necessary by using cell resuspending standard buffer, pH 7.3), 12 µmol Tris-HCl (pH 8.0), 1.8 µmol $MgCl_2$, 1.8 µmol ATP, 1.5 µmol 3-phosphoglyceric acid, and 0.6 µmol NaF was pre-incubated for 10 minutes at 37°C. Reactions were initiated by addition of 50 µl of deoxythymidine-2-^{14}C (9 nmoles; 500,000 dpm), bringing the total volume to 250 µl, and incubated for 45 minutes. Reactions were stopped by immersing the tubes into boiling water for 3 minutes and then cooled in an ice bath until centrifuged (3020 g) at 10°C for 15 minutes. Aliquots (50 µl) were applied onto DEAE-cellulose filters (Whatman No. DE-81; 2cm diam.) and filters washed according to the method described by Bresnick and Karjala (58). The filters were quickly immersed in a beaker containing 30 ml of 0.001 M ammonium formate for 10 minutes. The discs were then washed in distilled H_2O and placed in a new beaker containing 30 ml of 0.001 M ammonium formate for 10 minutes. The discs were again washed with

distilled water, placed in distilled water for 5 minutes and finally immersed in 95% (v/v) ethanol before being dried at 80°C and placed in counting vials containing 5 ml of a toluene-based cocktail (5g PPO, 100mg POPOP per liter of toluene). This procedure removes the precursor deoxythymidine-2-^{14}C while retaining deoxythymidine-monophosphate-^{14}C. Counting was performed in a Beckman LS 5801 liquid scintillation system with an efficiency of 97%.

V. LIPID EXTRACTION

Cell lipids were extracted using a modified Folch procedure (59). Extractions were carried out in borosilicate glass test tubes (16mm x 125mm) and each of the solvents contained the antioxidant ethoxyquin (1 μg/l). Methanol (0.8 ml) was added to 650-800 μl of cell homogenate and the tubes vortexed for 20 seconds, 2 ml of chloroform-methanol (1:1, v/v) were then added, the tubes vortexed 20 seconds and 2.5 ml of chloroform-methanol (2:1, v/v) added before vortexing again for 20 seconds. Finally, 0.1 M KCl was added to make a 20% (v/v) aqueous solution and the tubes kept at 4°C for 1 hour. The lower phase was collected with a Pasteur pipette and transferred into a clean borosilicate glass test tube (13mm x 100mm). Lipids were re-extracted with 2.5 ml of chloroform, tubes vortexed for 20 seconds kept at 4°C for 30 minutes and the lower phase collected and added to the first extract. Chloroform was removed at 40°C under vacuum (Model RH 12-29 Speed Vac Concentrator Centrifuge; Savant Instruments Inc., Ont.) for 3 hours, the lipids resuspended in hexane, then transferred to 1.8 ml vials with teflon lined caps and stored at -70°C until used for lipid analysis.

VI. SEPARATION OF LIPIDS

Phospholipids were separated using the method of Touchstone et al. (60). Silica gel H plates (Analtech Silica Gel G, 20cm x 20cm, 250 microns; Analtech Inc., Newark, DE 1971) were activated at 110°C for 1 hour. Plates were then spotted with 50 µl of lipid extract and developed for 90 minutes in a tank containing 166 ml of chloroform: methanol: 2-propanol: 0.25% (w/v) KCl: triethylamine (30:9:25:6:18, by vol). Plates were air dried , sprayed with 0.03% 2,7-dichloro-fluoroscein in 0.01 N NaOH and phospholipids visualized under U.V. light. The spots were scraped into methylation tubes for fatty acid analysis.

VII. FATTY ACID ANALYSIS

Fatty acids were methylated using the boron-trifluoride (BF_3) technique of Metcalfe and Schmidtz (61). Distilled hexane (1 ml) and 1 ml of BF_3-methanol (14%, w/w) reagent were added to methylation tubes containing the phospholipids. The tubes were tightly capped and heated in a sand bath set at 100-110°C for 1 hour. Tubes containing the sphingomyelin were heated for 90 minutes. After the samples cooled (5 minutes), 1 ml of distilled H_2O was added, the tubes were vortexed for 30 seconds and left to stand for 20 minutes at room temperature. The hexane layer was transferred into 1.8 ml vials and fatty acid methyl esters re-extracted with 1 ml of distilled hexane. The second extracts were also added to the 1.8 ml vials. Methyl-esters were dried at 40°C under vacuum (Model RH 12-29 Speed Vac Concentrator Centrifuge; Savant Instruments Inc, Ont.) then the vials were flushed with N_2, capped with teflon lined caps and stored at -70°C until analysis by

capillary gas liquid chromatography.

Fatty acid methyl-esters were analyzed according to a method described by Hargreaves and Clandinin (62). Fatty acid methyl-ester separation was performed by automated gas-liquid chromotography (Vista 6000 GLC and Vista 654 data system; Varian Instruments, Georgetown, Ontario, Canada) using a fused silica BP20 capillary column (25m x 0.25mm i.d.; Varian, Georgetown, Ontario, Canada). Helium was used as the carrier gas at a flow rate of 1.8 ml/min using a splitless injection mode. Injector and detector temperatures were maintained at 250°C. The initial oven temperature of 90°C was increased to 172°C at 20C deg/min and held for 13.2 minutes, then increased again to 220°C at 3.5C deg/min for a total analysis of 45 min. Authentic standard mixtures of fatty acid methyl esters were injected to identify fatty acid methyl ester peaks.

VIII. TOTAL CHOLESTEROL ANALYSIS

Total cholesterol was determined according to the methods of Siedel et al. (63), Stahler et al. (64), and Trinder (65), using an enzymatic kit (Cholesterol C-system kit, Boehringer Mannheim gmbH Diagnostica). An aliquot of 50 µl of lipid sample (5-10 µg cholesterol) was transferred into a borosilicate glass test tube (13mm x 100mm) and dried under nitrogen. To the residue, resuspended in 50 µl of iso-propanol, were added 950 µl of cholesterol C-system reagent (Tris buffer: 100 mmol/l pH 7.7; magnesium aspartate: 50 mmol/l; 4 aminophenazone: 1 mmol/l; sodium cholate: 10 mmol/l; phenol: 6 mmol/l; 3,4-dichlorophenol: 4 mmol/l; hydroxypolyethoxy-n-alkanes: 0.3%; cholesterol esterase > 0.4 U/ml; cholesterol oxidase > 0.25 U/ml;

peroxidase > 0.2 U/ml). The reaction mixture was vortexed, incubated 20 minutes at room temperature, and sample absorbance read at 500 nm against a reagent blank. Cholesterol concentrations were determined using a calibration curve (0-40 μg cholesterol) and results expressed as μg total cholesterol per mg of protein.

IX. TOTAL AND RELATIVE PERCENTAGE OF PHOSPHOLIPIDS

a) Total Phospholipids

Total phospholipids were determined using a modified method described by Raheja et al. (66). To 50 μl of lipid sample, dried down under N_2 in borosilicate glass test tubes (13mm x 100mm), were added 0.4 ml of chloroform and 0.1 ml of chromogenic solution. The tubes were placed in a sand bath at 100°C for 1 minute and cooled to room temperature. Chloroform (1 ml) was added to each tube and absorbance at 810 nm was determined using a Bausch and Lomb Spectronic 21 spectrophotometer. The blank contained everything except lipids and concentrations were calculated using sphingomyelin as a standard. The chromogenic reagent was prepared as follows: 4 g of ammonium molybdate were dissolved in 30 ml of distilled H_2O to give solution I. To 10 ml of concentrated HCl were added 2.5 ml of mercury and 20 ml of solution I to give solution II. To the remainder of solution I were carefully added 50 ml of H_2SO_4 as well as solution II to give the chromogenic stock solution. Finally, 5 ml of chromogenic stock solution, 9 ml of methanol, 1 ml of chloroform and 4 ml of distilled H_2O were mixed to give the working chromogenic solution.

b) Relative Percentage of Phospholipids

The relative percentage of total phospholipids were determined using a densitometry method described by Gassbaro (67). Samples (9.9 or 13.2 µl derived from 100-180 µg of cell protein) were spotted on high performance thin layer chromotography plates (Whatman, HP-K high performance silica gel plates, 10cm x 10cm). The plates were allowed to develop for approximatly 40 minutes (up to 1 cm of the top of the plate) in small development tanks containing 42 ml of chloroform: methanol: 2-propanol: triethylamine: 0.25% (w/v) KCl (15:4.5:12.5: 6.5:3.5, by vol). The plates were air dried and stained carefully by immersing them in Touchstone solution, blotted with #1 Whatman filter paper and charred at 200°C for 10-15 minutes (phospholipids are brown bands on white background) before scanning them on a Beckman Appraise densitometer. The Touchstone cupric acetate stain was prepared as follows: 3 g of cupric acetate were dissolved in 8 ml of 85% phosphoric acid and 50 ml of deionized H_2O; once dissolved, the volume was brought to 100 ml with deionized H_2O. This stain is very stable and can be prepared in bulk to be stored at room temperature.

X. LIPID PEROXIDE DETERMINATION

Lipid peroxides were determined using the new assay kit "Determiner LPO" (Kyowa medics Co., Tokyo, Japan) with some modifications to increase test sensitivity. Tests were performed in borosilicate glass test tubes (13mm x 100mm). To 100 µl of lipid extract (1.0-1.5 mg protein equivalent) in methanol were added 150 µl of the first reagent (30 U/ml of ascorbic oxidase, Good buffer - 100 mM MOPS = 3-[N-morpholino]propanesulfonic acid pH 5.8, stabilizer); tubes were vortexed and incubated 5 minutes at 30°C in a shaking water bath

before adding 300 µl of the second reagent (52.7 M of MCDP = 10N-methyl-carbamoyl-3,7-dimethylamine-10H-phenothiazine, Good buffer - 100 mM MOPS pH 5.8, 68 mg/l of hemoglobin, surface active reagent, chelating agent and stabilizer), vortexing and incubating for 10 more minutes. Absorbance was determined at 675 nm in a Perkin-Elmer model Lambda 3b spectrophotometer. Sample concentrations were calculated using a standard curve in the range of 0-1.0 nmole of cumen hydroperoxide. Blank absorbance changing with time, all sample absorbancies were determined against water. The test was performed in a dark room with red light; lipid peroxide determination was performed on batches of one blank and five samples starting the reaction in each tube at 1 minute intervals.

XI. VITAMIN E DETERMINATION

a) Diet

Oil samples for vitamin E determination have been prepared using a method described by Carpenter, Jr. (68). Safflower oil (5 g) and 1 g of safflower oil supplemented with d-alpha-tocopheryl acetate were dissolved in 100 ml volumetric flasks with distilled hexane and brought to volume. 100 µl of these solutions were injected onto the HPLC column.

b) Serum

Serum samples were prepared using a slight modification of the simplified clarification method described by Nierenberg and Lester (69). To 500 µl of thawed serum transferred to 1.5 ml polypropylene microcentrifuge tubes were added 50 µl of d-alpha-tocopheryl acetate

(100 µg/ml) in ethanol and 50 µl of 0.125% (w/v) BHT in ethanol to give the internal standard and initiate protein precipitation. After vortexing for 15 seconds, 300 µl of 0.025% (w/v) of butylated hydroxytoluene (BHT) in butanol-ethyl acetate (1:1, v/v) were added and tube vortexed for 60 seconds. An aqueous solution (150 µl) of K_2HPO_4 (1 g/ml) was added before vortexing for 30 seconds and centrifuging in a MSE microcentrifuge at 13,000 rpm for 1 minute at 4°C. The organic upper layer (250 µl) was transferred by automatic pipette into a borosilicate glass test tube (10mm x 75mm), and dried in 5 to 10 minutes at 75°C under N_2. No loss occurred during this process as checked with a standard mixture of dl-alpha-tocopherol and d-alpha-tocopheryl acetate. The residue was resuspended in 250 µl of distilled hexane transferred to a 0.4 ml polypropylene microcentrifuge tube and centrifuged at 13,000 rpm for 1 minute at 4°C. The clarified distilled hexane (100 µl) was injected onto the HPLC column.

Tocopherols were analyzed using a method described by Buttris and Diplock (70). Dl-alpha-tocopherol and d-alpha-tocopheryl acetate determination was performed by HPLC chromatography (5500 LC and Vista 402 data system, Varian Instruments, Georgetown, Ontario, Canada), using a Micropack Si-5 column (4.5mm i.d. x 15cm length). Absorbance was determined using an ultraviolet absorption detector set at 294 nm. The mobile phase was 8% (v/v) HPLC grade methyl t-butyl ether in distilled hexane at a flow rate of 2 ml/minute. Solvents were filtered and degassed by N_2 prior use. The system was standardized by injection of 100 µl mixture containing 50 µg/ml of dl-alpha- tocopherol (Sigma Chemical Co., St.Louis, MO, USA) and d-alpha- tocopheryl acetate (Veris, La Grange, Ill., USA) in hexane after being checked for

linearity in the range of 5-100 μg/ml. (See appendix 2).

XII. STATISTICAL ANALYSIS

Before analyzing for effect of diet treatment and differences between cell populations, effect of day was examined by two way analysis of variance considering the 16 days of experimentation as 16 blocks. If no block effect was observed, the effect of diet treatment and differences between "non-proliferative" and "proliferative" cell fractions were examined by Least Square ANOVA. The program was conceived for unbalanced data; however, the mean values reported in this thesis are the observed ones, and not the corrected ones. Effect of diet treatment on total protein released, serum vitamin E concentrations, and lipid peroxide levels were examined by two way analysis of variance procedures after analysis for block effect. ^{3}H-thymidine incorporation into DNA of "non-proliferative" and "proliferative" cells was analyzed by a Student's t-test (71).

C. RESULTS

I. EFFECT OF DIET TREATMENT ON BODY WEIGHT

The mean body weights of animals between groups prior to or after diet treatments were not significantly different. Due to the difficulty of obtaining rats at a given weight every week , there was a significant difference ($p<0.002$) in mean body weight from one delivered batch to another at initiation of treatments . The overall mean body weights (mean±SD) were 77.6 ± 4.5 g (n=29) and 252.7 ± 20.8 (n=29) at

initiation of treatments and at the time of sacrifice, respectively. No significant difference in weight gain was observed during the period of diet treatments when considering the effects of week and day of experiments, fat level or polyunsaturated to saturated fatty acid ratio.

II. EFFECT OF DIET TREATMENT ON MUCOSAL CELL ISOLATION AND THYMIDINE KINASE ACTIVITY

Prior to initiation of experimental procedure, rats fed rat chow were injected with ^{3}H-thymidine to localize "proliferative" cell versus "non-proliferative" cell fractions. As a first step, a few experiments were performed without pooling the collected fractions (data not shown). From these preliminary experiments, fractions #2-7 and #9-12 were pooled and considered as "non-proliferative" and "proliferative" cell fractions respectively. Two more experiments were performed showing a ^{3}H-thymidine count of 3050 ± 107 dpm/mg protein (mean±SD) and 7550 ± 257 dpm/mg protein for "non-proliferative and "proliferative" cell fractions, respectively (Table 2-3). These values were significantly different ($p<0.002$) as determined by Student's t-test.

a) Cell Isolation

Cell recovery, when expressing the results as mg of protein, was not affected by day of experimentation. Diet fatty acid composition, but not diet fat level, had a highly significant effect ($p<0.005$) on cell isolation (Table 2-4).

The mean percentage of protein, whatever the diet treatment, distributed between "non-proliferative" and "proliferative" cell

Table 2-3. ^{3}H-thymidine incorporation into DNA of mucosal cells from the rat large intestine.

Cell Fraction	dpm/mg protein	Total protein (μg)	Protein (%, w/w)
Non-proliferative[1]	3050 ± 107[3]	5740 ± 1230	51.1 ± 3.0[4]
Proliferative[2]	7550 ± 257	5390 ± 523	48.9 ± 3.0
Fractions #2-12		11100 ± 1750	100.0

Student's t-test: Difference of ^{3}H-thymidine incorporation between "non-proliferative" and "proliferative" cell fractions was significant at $p<0.002$.

[1]Represents pooled fractions #2-7.

[2]Represents pooled fractions #9-12.

[3]Values are means ± SD.

[4]Total and percentage of protein calculated from fractions #2-8.

Table 2-4. Effect of diet treatment on mucosal cell released from the rat large intestine (total μg of protein[1]).

Fat Level	High		Low		Effect of Treatment[3]		
P/S Ratio[2]	High	Low	High	Low	Fat Level	P/S Ratio	Int
	4780 ± 1440 (n=7)	8890 ± 2660 (n=6)	6120 ± 1220 (n=5)	8050 ± 3270 (n=6)	NS	0.005	NS

[1]Sum of protein (mg) from "non-proliferative" cells (pooled fractions #2-7), "proliferative" cells (pooled fractions #9-12) and fraction #8.

[2]P/S Ratio, polyunsaturated to saturated fatty acid ratio.

[3]Significant effects ($p<$) by two-way analysis of variance procedures are indicated. Int, interaction between effects of fat level and polyunsaturated to saturated fatty acid ratio; NS, not significantly different.

fractions was 34.26% and 65.74%, respectively. Fat level and polyunsaturated to saturated fatty acid ratio in the diet had no effect on percent of protein released when considering both fractions independently (Table 2-5).

b) Thymidine Kinase Activity

Linearity between time and product formed has been observed up to 45 minutes (data not shown). No effect of experimental day or diet treatment on thymidine kinase activity was observed. Enzyme specific activity was 41.1% higher ($p<0.05$) in the "proliferative" cell fraction compared to the "non-proliferative" cell population (Table 2-6). A significant interaction ($p<0.05$) between effects of cell fraction and polyunsaturated to saturated fatty acid ratio was observed.

III. EFFECT OF DIET TREATMENT ON LIPID COMPOSITION OF MUCOSAL CELLS

Experimental day, amount of fat or polyunsaturated to saturated fatty acid diet ratio did not affect mucosal cell total cholesterol content (nmol/mg protein). The "non-proliferative" cell population exhibited a 12% higher total cholesterol level compared to the "proliferative" cell fraction ($p<0.05$; Table 2-7).

Day of experiment, fat level, polyunsaturated to saturated fatty acid ratio in the diet, or cell fraction tested did not significantly affect the total phospholipid content of mucosal cells in the large intestine (Table 2-7). There was, however, a significant interaction ($p<0.05$) between the effect of polyunsaturated to saturated fatty acid diet ratio and cell fraction isolated.

Cholesterol to phospholipid ratio (nmol/nmol) was not affected by

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Table 2-5 Yield of "non-proliferative" and "proliferative" mucosal cells from the rat large intestine (%, w/w of protein[1]).

Fat Level	High				Low			
P/S Ratio[2]	High		Low		High		Low	
Cell Fraction	Non-Prolif.[3]	Prolif.[3]	Non-Prolif.	Prolif.	Non-Prolif.	Prolif.	Non-Prolif.	Prolif.
%	35.4±7.7[4]	64.6±7.7	31.2±4.6	68.8±4.6	34.0±7.0	66.0±7.0	36.5±7.5	63.5±7.5
n[2]	7	7	6	6	5	5	6	6

[1]Percentage of protein in each fraction calculated from a total protein (100%) = protein from ("non-proliferative" cells + "proliferative cells" + fraction #8); percentage of protein in "non-proliferative" cells includes fraction #8.

[2]Abbreviations used: P/S Ratio, polyunsaturated to saturated fatty acid diet ratio; n, number of replicates.

[3]Non-Prolif. = "non-proliferative" cell fraction, Prolif. = "proliferative" cell fraction.

[4]Values are means ± SD; no statistical analysis performed.

Table 2 6. Effect of diet treatment on thymidine kinase activity of mucosal cells from the rat large intestine.

Fat Level	High				Low			
P/S Ratio[1]	High		Low		High		Low	
Cell Fraction	Non-Prolif.[2]	Prolif.[2]	Non-Prolif.	Prolif.	Non-Prolif.	Prolif.	Non-Prolif.	Prolif.
Specific Activity (nmole/mg protein[1])	3.2±1.2[3]	4.1±0.9	3.2±0.4	5.4±2.5	3.2±1.3	2.5±1.1	2.1±1.2	4.5±1.8
% Activity[4]	43±12	57±12	40±12	60±12	56±15	44±15	40±15	60±15
n[1]	6	6	5	5	5	5	6	6

[1]Abbreviations used are:-P/S Ratio, polyunsaturated to saturated fatty acid ratio; n, number of replicates.

[2]Non-Prolif. = "non-proliferative" cell fraction; Prolif. = "proliferative" cell fraction.

[3]Values are means ± SD

[4]% Activity with Σ ("non-proliferative" cells + "proliferative" cells) thymidine kinase activity = 100%.

[5]Effect of treatment on thymidine kinase specific activity was determined by least square analysis of variance procedures. Significant differences between cell fractions ($p<0.05$), as well as an interaction between effects of dietary P/S ratio and cell fraction ($p<0.05$) were observed. No significant effect of fat level, P/S ratio, or other interactions were identified.

Table 2-7. Effect of diet treatment on total cholesterol, total phospholipids, and cholesterol to phospholipid ratio of mucosal cells from the rat large intestine.

Fat Level	High				Low				Effect of Treatment[1]						
P/S Ratio[1]	High		Low		High		Low				Fat Level * P/S Ratio		Fat Level * Cell Fr.	P/S Ratio * Cell Fr.	Fat Level * P/S Ratio * Cell Fr.
Cell Fraction	Non-Prolif.[2]	Prolif.[2]	Non-Prolif.	Prolif.	Non-Prolif.	Prolif.	Non-Prolif.	Prolif.	Fat Level	P/S Ratio		Cell Fr.			
Cholesterol (nmole/mg protein)[4]															
	70.3±11.1[3]	60.1±7.1	64.4±12.8	59.1±7.3	68.9±14.5	60.9±14.5	66.4±15.9	61.0±9.4	NS	NS	NS	.05	NS	NS	NS
n[1]	6	7	6	6	5	5	5	6							
Phospholipids (nmole/mg protein)[4]															
	147±40[3]	122±48	150±47	171±34	142±40	106±26	149±46	142±9	NS	NS	NS	NS	NS	.05	NS
n	6	7	5	6	5	5	6	6							
Cholesterol/Phospholipids (nmole/nmole)															
	0.51±0.14[3]	0.60±0.37	0.45±0.11	0.36±0.08	0.50±0.10	0.59±0.14	0.42±0.12	0.43±0.05	NS	.05	NS	NS	NS	NS	NS
n	6	7	5	6	5	5	5	6							

[1]Abbreviations used are: P/S Ratio, polyunsaturated to saturated fatty acid ratio; n, number of replicates.

[2]Non Prolif. cells = "non-prolifeative" cell fraction; Prolif. cells = "proliferative" cell fraction.

[3]Values are means ± SD.

[4]Cholesterol Mw = 386.7; Phospholipids Mw = 730.

[5]Significant effects by least square analysis of variance procedures are indicated ($p<$); Cell Fr., cell fraction; *, interaction; NS, not significantly different.

day of cell isolation or fat level. Cell fractions, "non-proliferative" and "proliferative", exhibited similar ratios of cholesterol to phospholipid. Groups fed diets providing a high polyunsaturated to saturated fatty acid ratio (P/S=1.2) showed a 32.5% increase ($p<0.05$) in cholesterol to phospholipid ratio compared to groups fed diets containing a low polyunsaturated to saturated fatty acid ratio (P/S=0.3); (Table 2-7).

Main phospholipid classes observed were phosphatidylethanolamine and phosphatidylcholine representing greater than 50% and 22% of total mucosal cell phospholipid, respectively (Table 2-8). Minor phospholipids consisted of phosphatidylinositol, phosphatidic acid plus phosphatidylserine, and sphingomyelin representing more than 6%, 5%, and 5% of total mucosal cell phospholipid, respectively. Mucosal "proliferative" cells exhibited a 44.9% greater content of sphingomyelin ($p<0.05$) compared to the "non-proliferative" cell population. Interactions between the effect of fat level and cell fraction ($p<0.05$), and between the effect of diet fatty acid composition and cell fraction ($p<0.005$) on sphingomyelin content were observed. Phosphatidylcholine content of total phospholipids in the "proliferative" cell fraction was 20.8% lower ($p<0.05$) compared to the "non-proliferative" cell population. Interactions between the effect of diet fatty acid composition and cell fraction ($p<0.05$) for phosphatidylcholine, and between the effect of fat level and diet fatty acid composition ($p<0.01$) for phosphatidylinositol were observed.

Phospholipid content, expressed as mg per mg of protein, showed significant differences ($p<0.05$) between cell fractions for phosphatidylcholine and phophatidylinositol (Table 2-9). Mucosal cell

Table 2.8. Effect of diet treatment on relative percentage of total phospholipid of "non-proliferative" and "proliferative" mucosal cells from the rat large intestine.

Fat Level	Phospholipids (%, w/w)								Effect of Treatment[4]						
	High				Low						Fat Level •		Fat Level •	P/S Ratio •	Fat Level Ratio •
P/S Ratio[1]	High		Low		High		Low								
Cell Fraction	Non-Prolif.[2]	Prolif.[2]	Non-Prolif.	Prolif.	Non Prolif	Prolif.	Non-Prolif.	Prolif.	Fat Level	P/S Ratio	P/S Ratio	Cell Fr.	Cell Fr.	Cell Fr	Cell Fr
Sphingomyelin	3.8±0.5[3]	8.7±4.8	4.5±0.6	6.7±3.3	5.2±0.8	5.4±3.0	6.1±2.0	7.6±3.1	NS	NS	NS	.05	.05	.05	NS
Phosphatidylcholine	32.7±4.6	26.4±11.3	31.6±5.3	27.3±7.8	28.7±3.2	16.2±7.2	28.3±4.4	26.2±8.5	NS	NS	NS	.05	NS	.05	NS
Phosphatidic acid + Phosphatidylserine	6.8±0.9	5.8±4.8	5.9±3.8	5.5±3.9	4.9±2.1	8.6±3.1	5.3±0.6	7.2±3.9	NS	NS	NS	NS	NS	NS	NS
Phosphatidylinositol	8.4±1.7	4.4±2.4	10.0±3.4	8.9±2.3	8.9±1.5	8.7±1.1	8.1±3.2	7.2±1.5	NS	NS	.01	NS	NS	NS	NS
Phosphatidylethanolamine	48.2±4.2	53.4±3.6	49.1±5.6	51.6±9.3	52.4±5.0	61.0±7.0	57.7±4.6	51.8±9.7	NS	NS	NS	NS	NS	NS	NS
n[5]	3	4	5	6	4	5	3	6							

[1]Abbreviations used: P/S Ratio, polyunsaturated to saturated fatty acid ratio; n, number of replicates.

[2]Non-Prolif. cells = "non-proliferative" cell fraction; Prolif. cells = "proliferative" cell fraction.

[3]Values are means ± SD.

[4]Significant effects by least square analysis of variance procedures are indicated ($p<$). Cell Fr., cell fraction; •, interaction; NS, not significantly different.

Table 2-9. Effect of diet treatment on phospholipid content of "non proliferative" and "proliferative" mucosal cells from the rat large intestine.

	Phospholipids (μg/mg protein)								Effect of Treatment*						
Fat Level	High				Low						Fat Level •		Fat Level •	P/S Ratio •	Fat Level Ratio •
P/S Ratio[1]	High		Low		High		Low								
Cell Fraction	Non-Prolif.[2]	Prolif.[2]	Non-Prolif.	Prolif	Non Prolif.	Prolif.	Non-Prolif.	Prolif.	Fat Level	P/S Ratio	P/S Ratio	Cell Fr.	Cell Fr	Cell Fr	Cell Fr.
Sphingomyelin	3.5±1.0[3]	6.2±2.7	4.8±2.0	8.2±4.5	5.8±2.4	4.1±2.8	7.2±2.8	8.1±3.8	NS	.05	NS	NS	NS	NS	NS
Phosphatidylcholine	30.6±12.4	20.1±15.0	31.1±12.0	33.1±9.2	31.2±8.6	12.5±6.6	32.5±5.8	27.0±9.7	NS	NS	NS	.05	NS	NS	NS
Phosphatidic acid + Phosphatidylserine	6.4±2.7	3.6±3.8	5.5±3.1	6.1±3.4	4.9±1.1	6.6±3.0	6.2±1.5	7.6±4.6	NS	NS	NS	NS	NS	NS	NS
Phosphatidylinositol	7.5±1.4	5.4±6.6	9,8±1.3	11.3±4.6	9.5±2.1	6.7±2.0	9.1±3.0	6.7±2.0	NS	NS	NS	.05	NS	NS	NS
Phosphatidylethanolamine	44.7±15.0	48.0±25.9	50.0±21.4	65.8±23.5	57.7±19.2	46.9±13.2	60.4±16.1	52.9±10.6	NS	NS	NS	NS	NS	NS	NS
Total Phospholipids (TPL)	107±29	91±38	109±35	125±25	104±29	77±19	109±33	103±13							
n[1] (TPL)	6	6	5	6	5	5	6	6							
n (others)	3	4	4	6	4	5	3	6							

[1]Abbreviations used: P/S Ratio, polyunsaturated to saturated fatty acid ratio; n , number of replicates.

[2]Non-Prolif. cells = "non-proliferative" cell fraction; Prolif. cells = "proliferative" cell fraction.

[3]Values are means ± SD.

*Significant effects by least square analysis of variance procedures are indicated ($p<$); Cell Fr., cell fraction; •, interaction; NS, not significantly different.

sphingomyelin content for rats fed diets providing a polyunsaturated to saturated fatty acid ratio of 1.2 was 30.7% lower ($p<0.05$) compared to rats fed diets with polyunsaturated to saturated fatty acid ratio of 0.3.

IV. EFFECT OF DIET TREATMENT ON PHOSPHOLIPID FATTY ACID COMPOSITION OF MUCOSAL CELLS

a) Phosphatidylethanolamine

Major fatty acids in phosphatidylethanolamine were $C_{20:4(6)}$, $C_{18:0}$, $C_{18:1(9)}$, and $C_{18:2(6)}$ representing 32.8%, 18.9%, 13.3%, and 9.1%, respectively, and thus accounted for more than 70% of the total fatty acid content (Table 2-10). Groups fed high fat diets increased ($p<0.005$) phosphatidylethanolamine $C_{18:2(6)}$, total polyunsaturated, and w-6 fatty acid content as well as the unsaturation index ($p<0.01$), and decreased ($p<0.05$) the content of $C_{16:1}$, $C_{18:1(9)}$, $C_{18:1(5+7)}$, total monounsaturated fatty acids and total w-3 fatty acid series compared to groups fed low fat diets. The phosphatidylethanolamine of rats fed diets with a low polyunsaturated fatty acid content exhibited a significantly ($p<0.005$) higher level of $C_{18:1(9)}$, $C_{18:1(5+7)}$, and total monounsaturated fatty acids compared to rats fed diets high in polyunsaturated fatty acid content. On the other hand, amounts of $C_{18:2(6)}$, total polyunsaturated fatty acids, total w-6 fatty acids, and the unsaturation index were significantly lower ($p<0.01$) in rats fed diets providing the low polyunsaturated to saturated fatty acid ratio compared to rats fed diets providing the high polyunsaturated fatty acid to saturated ratio. The following fatty acids, $C_{20:4(6)}$, $C_{16:1}$, $C_{16:0}$ and $C_{18:0}$, as well as the total saturated fatty acid content of

Table 2-10. Effect of diet treatment on phosphatidylethanolamine fatty acid composition of "non-proliferative" and "proliferative" mucosal cells from the rat large intestine

	Fatty Acid Composition (%, w/w)								Effect of Treatment[1]						
Fat Level	High				Low						Fat Level •		Fat Level •	P/S Ratio •	Fat Level Ratio •
P/S Ratio	High		Low		High		Low								
Cell Fraction	Non-Prolif.[2]	Prolif.[2]	Non-Prolif.	Prolif.	Non-Prolif.	Prolif.	Non-Prolif.	Prolif.	Fat Level	P/S Ratio	P/S Ratio	Cell Fr.	Cell Fr.	Cell Fr.	Cell Fr.
C16:0	8.7±1.7[4]	7.9±1.3	8.0±1.0	6.4±0.3	8.3±0.7	7.1±0.3	11.2±1.3	7.1±0.2			++	+++		+	
C16:1	1.7±0.5	1.4±0.2	1.2±0.8	1.3±0.2	1.7±0.2	1.8±0.4	2.2±0.7	2.1±0.2	+++						
C18:0	21.4±0.6	19.0±0.7	20.5±2.1	18.3±1.5	17.9±1.3	17.7±1.7	20.3±4.0	15.7±0.5				+++			
C18:1(9)	10.0±0.9	9.5±0.8	12.1±1.0	13.3±1.3	12.7±1.4	14.0±1.4	15.9±2.1	18.7±0.8	+++	+++		+++		+	
C18:1(5) + (7)	1.3±0.3	0.9±0.4	1.3±0.2	1.6±0.4	2.1±0.3	2.1±0.2	2.5±0.5	3.0±0.3	+++	+++					
C18:2(6)	12.5±2.0	11.6±1.1	10.2±0.8	9.4±1.6	7.6±0.7	7.4±0.7	7.4±1.3	7.0±0.4	+++	++	+				
C20:4(6)	30.9±3.7	33.1±2.8	33.5±3.3	35.5±1.6	35.3±1.7	35.7±2.2	26.5±3.3	31.6±1.5			+++	+			
Σsats[3]	33.5±2.5	29.5±2.3	32.7±2.9	27.3±1.3	29.8±1.2	27.2±1.6	35.8±4.6	26.6±1.1			+	+++			
Σmonounsats[3]	13.7±1.1	12.4±0.4	15.2±0.7	16.7±1.9	17.4±1.4	18.5±1.8	21.2±2.7	24.3±0.9	+++	+++		+		+	
Σpolyunsats[3]	52.8±3.5	58.0±2.3	53.1±3.7	55.9±1.4	53.0±1.9	54.2±2.9	42.9±4.0	49.0±1.4	+++	+++	+++	+++			
Σ(w-6)[3]	49.6±3.7	54.3±2.3	49.7±2.9	51.9±1.9	48.9±1.8	50.0±2.7	39.2±3.6	44.8±1.4	+++	+++	+++	+++			
Σ(w-3)[3]	2.4±0.7	2.9±1.0	2.9±1.8	3.6±0.7	3.6±0.9	3.7±0.5	3.1±0.8	3.8±0.4	+						
UI[5]	1.83±0.15	2.07±0.10	1.89±0.17	2.03±0.05	1.96±0.09	2.01±0.12	1.55±0.15	1.80±0.06	++	+++	+++	+++			
n[3]	7	6	5	5	5	5	6	5							

[1]Abbreviations used are: P/S Ratio, polyunsaturated to saturated fatty acid ratio; Σsats = total saturated fatty acids, Σmonounsats = total monounsaturated fatty acids; Σpolyunsats = total polyunsaturated fatty acids; Σ(w-6) = total (w-6) fatty acids; Σ(w-3) = total (w-3) fatty acids; n = number of replicates.

[2]Non-Prolif. = "non-proliferative" cell fraction; Prolif. = "proliferative" cell fraction.

[3]UI = unsaturation index defined as the sum of fatty acids with 2 or more double bonds multiplied by their relative percentage, and the result divided by 100.

[4]Values are means ± SD for major fatty acids.

[5]Significant effects by least square analysis of variance procedures are indicated (+ $p<0.05$, ++ $p<0.01$, +++ $p<0.005$); Cell Fr., cell fraction; •, interaction effects.

phosphatidylethanolamine were not sensitive to the fatty acid composition of the diet.

Cell fractions, "non-proliferative" and "proliferative", also exhibited differences in the fatty acid profile. The "proliferative" cells exhibited a lower ($p<0.005$) phosphatidylethanolamine fatty acid content of total monounsaturated fatty acid, $C_{16:0}$ and $C_{18:0}$ compared to the "non-proliferative" cells. Increased levels ($p<0.05$) of total monounsaturated fatty acids, $C_{18:1(9)}$, and polyunsaturated fatty acids, w-6 series and $C_{20:4(6)}$ as well as an increased unsaturation index ($p<0.005$) in phosphatidylethanolamine of the "proliferative" cell population compared to the "non-proliferative" cell population were observed. Finally, no significant difference between cell fractions for $C_{18:1(5+7)}$, $C_{18:2(6)}$ and the sum of w-3 fatty acids was observed. Some interactions occurred, mainly between the effect of dietary fat level and dietary fatty acid composition (Table 2-10).

b) Phosphatidylcholine

Diets altered major fatty acyl constituents of phosphatidylcholine in both "non-proliferative" and "proliferative" cell fractions (Table 2-11). A significant decrease ($p<0.005$) in $C_{16:1}$, $C_{18:1(9)}$, and $C_{18:1(5+7)}$ accounted for the lower total monounsaturated fatty acyl content of phosphatidylcholine in the groups fed high fat diets compared to the groups fed low fat diets. On the other hand, higher levels ($p<0.005$) of $C_{18:2(6)}$, $C_{20:4(6)}$, and the sum of polyunsaturated fatty acids occurred in animals fed the high fat diets. While the total w-6 fatty acid content of phosphatidylcholine increased ($p<0.005$) in rats fed the high fat diets, neither the total polyunsaturated w-3,

Table 2-11. Effect of diet treatment on phosphatidylcholine fatty acid composition of "non-proliferative" and "proliferative" mucosal cells from the rat large intestine.

Fatty Acid Composition (%, w/w)									Effect of Treatment[1]						
Fat Level	High				Low						Fat Level • P/S Ratio		Fat Level • Cell Fr.	P/S Ratio • Cell Fr.	Fat Level • P/S Ratio • Cell Fr.
P/S Ratio[1]	High		Low		High		Low								
Cell Fraction	Non-Prolif.[2]	Prolif.[2]	Non-Prolif.	Prolif.	Non-Prolif.	Prolif.	Non-Prolif.	Prolif.	Fat Level	P/S Ratio					
C16:0	23.2±2.0[4]	25.5±3.8	24.0±1.0	25.5±1.2	25.3±1.2	29.3±2.6	24.1±1.2	27.0±0.9				+ + +			
C16:1	1.0±0.2	0.8±0.2	1.1±0.4	1.7±0.2	1.2±0.6	2.2±0.7	2.00±0.9	3.4±0.4	+ + +	+ + +		+ + +	+ + +	+	
C18:0	12.9±0.9	12.4±0.9	10.4±0.5	10.4±1.4	10.5±1.8	9.9±0.6	10.5±2.9	8.1±0.7	+ + +	+ + +					
C18:1(9)	10.0±1.7	10.3±0.6	15.3±2.2	15.2±1.8	16.5±2.6	15.7±2.6	22.1±1.2	20.7±2.4	+ + +	+ + +					
C18:1(5) + (7)	1.8±0.3	1.7±0.4	2.5±0.2	2.5±0.3	4.8±0.6	5.4±1.1	5.7±0.5	7.6±1.2	+ + +	+ + +		+ +	+ + +		
C18:2(6)	24.0±1.8	23.6±1.5	19.5±1.2	18.3±0.6	15.2±1.1	13.7±1.0	12.0±0.9	11.2±1.5	+ + +	+ +	+	+			
C20:4(6)	14.1±1.4	13.3±1.7	14.2±1.7	13.1±1.7	14.1±1.5	12.6±1.5	11.2±1.2	9.7±0.6	+ +	+	+	+ + +			
Σsats[1]	41.1±2.7	42.2±4.7	39.7±1.4	40.1±1.7	40.9±1.1	43.6±2.1	40.0±1.9	40.6±0.8				+			
Σmonounsats[1]	13.7±1.1	13.6±0.9	20.5±2.0	21.1±1.8	24.0±2.3	24.8±2.5	31.4±2.0	33.6±1.9	+ + +	+ + +		+ +	+	+	
Σpolyunsats[1]	43.4±2.4	42.6±3.7	38.9±2.7	37.4±2.4	34.2±2.0	30.8±2.1	27.8±1.5	25.5±1.9	+ + +	+ + +		+			
Σ(w-6)[1]	42.7±2.1	42.0±3.6	38.0±2.7	36.6±2.2	33.2±2.0	29.8±2.3	27.1±1.2	24.5±1.7	+ + +	+ + +		+ +			
Σ(w-3)[1]	0.7±0.3	0.6±0.2	0.9±0.2	0.8±0.3	1.1±0.4	0.9±0.4	0.7±0.4	1.0±0.2							
UI[3]	1.28±0.07	1.25±0.13	1.17±0.09	1.13±0.09	1.09±0.07	0.96±0.08	0.87±0.06	0.79±0.05	+ + +	+ + +		+ +			
n[1]	6	6	5	5	5	5	6	6							

[1]Abbreviations used are: P/S Ratio, polyunsaturated to saturated fatty acid ratio; Σsats = total saturated fatty acids, Σmonounsats = total monounsaturated fatty acids; Σpolyunsats = total polyunsaturated fatty acids; Σ(w-6) = total (w-6) fatty acids; Σ(w-3) = total (w-3) fatty acids; n = number of replicates.

[2]Non-Prolif. = "non-proliferative cell fraction; Prolif. = "proliferative cell fraction

[3]UI = unsaturation index defined as the sum of fatty acids with 2 or more double bonds multiplied by their relative percentage, and the result divided by 100.

[4]Values are means ± SD for major fatty acids.

[5]Significant effects by least square analysis of variance procedures are indicated (+ $p<0.05$, + + $p<0.01$, + + + $p<0.005$); Cell Fr., cell fraction; •, interaction effects.

nor the total saturated fatty acid content was significantly altered. $C_{16:0}$ increased ($p<0.05$) and $C_{18:0}$ decreased ($p<0.005$) in animals fed low fat diets. The unsaturation index decreased ($p<0.005$) for animals fed diets low in fat.

Diet fatty acid composition also influenced phosphatidylcholine fatty acyl chains with trends similar to those observed by dietary fat level (Table 2-11). The fatty acids $C_{16:1}$, $C_{18:1(9)}$, $C_{18:1(5+7)}$ as well as the sum of monounsaturated fatty acids were significantly lower ($p<0.005$) for rats fed diets having a high polyunsaturated fatty acid content. Increased levels ($p<0.005$) of total monounsaturated fatty acids and total polyunsaturated w-6, including $C_{18:2(6)}$ and $C_{20:4(6)}$, but not w-3 series, were observed in groups fed diets of high polyunsaturated to saturated fatty acid ratio. $C_{16:0}$ and total polyunsaturated fatty acids were not significantly different between groups fed diets varying in fatty acid composition; however, phosphatidylcholine $C_{18:0}$ content was lower (16.0%, $p<0.005$) in animals fed diets with low polyunsaturated to saturated fatty acid ratio. Variations observed in these fatty acids led to significant alteration of the unsaturation index with a 13.5% decrease in mucosal cell phosphatidylcholine for rats fed the low fat diets compared to rats fed high fat diets.

Phosphatidylcholine fatty acid composition varies between cell populations (Table 2-11). The fatty acid content of $C_{16:0}$, $C_{16:1}$, and $C_{18:1(5+7)}$ were higher by 11.1% ($p<0.005$), 64.2% ($p<0.005$) and 16.2% ($p<0.01$) in the phosphatidylcholine of the "proliferative" cells compared to the "non-proliferative" cells, respectively. The polyunsaturated fatty acid level of $C_{18:2(6)}$ and $C_{20:4(6)}$ was lower by

5.5% ($p<0.05$) and 9.1% ($p<0.005$) in the "proliferative" cells. $C_{18:0}$ and $C_{18:1(9)}$ content was not significantly different between both cell fractions. Higher total saturated ($p<0.05$) and total monounsaturated ($p<0.01$) fatty acids, and lower total polyunsaturated ($p<0.05$) and total w-6 ($p<0.01$) fatty acids in the "proliferative" cell population compared to the "non-proliferative" cells were observed. No significant difference in phosphatidylcholine total polyunsaturated w-3 fatty acids between cell fractions occurred. The unsaturation index was higher by 6.8% in "proliferative" cells compared to "non-proliferative" cells.

c) Phosphatidylinositol

Dietary treatment altered the fatty acid composition of phosphatidylinositol (Table 2-12). High fat diet fed groups compared to low fat diet fed groups exhibited a decreased content ($p<0.05$) of $C_{16:1(5+7)}$, $C_{18:1(9)}$, and $C_{18:1(5+7)}$, accounting for the decrease of total monounsaturated fatty acid level. On the other hand, the phosphatidylinositol content of $C_{18:2(6)}$, total polyunsaturated and sum of w-6 fatty acids as well as the total saturated fatty acids were increased ($p<0.05$) in rats fed high fat diets. Surprisingly, $C_{20:4(6)}$ level was decreased in groups fed high fat diets compared to groups fed low fat diets. Neither $C_{16:0}$, $C_{18:0}$, the sum of w-3 series, nor the unsaturation index were affected by dietary fat level.

Similar effects of dietary polyunsaturated to saturated fatty acid ratio on phosphatidylinositol fatty acid composition were observed (Table 2-12). Feeding rats high fat diets increased ($p<0.05$) the content of $C_{18:0}$, $C_{18:2(6)}$, total polyunsaturated and sum of w-6 fatty

Table 2-12. Effect of diet treatment on phosphatidylinositol fatty acid composition of "non proliferative" and "proliferative" mucosal cells from the rat large intestine

Fat Level	Fatty Acid Composition (%, w/w)								Effect of Treatment[1]							
	High				Low						Fat Level * P/S Ratio		Fat Level * Cell Fr.	Fat Level * Cell Fr.	P/S Ratio * Cell Fr	Fat Level Ratio * Cell Fr
P/S Ratio[1]	High		Low		High		Low									
Cell Fraction	Non-Prolif.[2]	Prolif.[2]	Non-Prolif.	Prolif.	Non-Prolif.	Prolif.	Non-Prolif.	Prolif.	Fat Level	P/S Ratio	P/S Ratio	Cell Fr.	Cell Fr.	Cell Fr	Cell Fr
C16:0	20.4±1.3[3]	17.3±3.3	27.6±5.1	15.7±3.3	21.4±4.3	15.2±1.0	24.1±5.6	14.7±1.9				+ + +		+	
C16:1	1.2±0.2	1.4±0.5	1.3±0.5	1.0±0.6	1.7±0.5	1.2±0.2	2.2±0.7	1.6±0.2	+						
C18:0	32.0±2.3	31.6±1.9	27.1±3.2	32.3±1.8	29.7±1.2	32.0±0.4	26.1±3.1	31.0±1.2		+		+ + +		+	
C18:1(9)	4.6±0.3	6.0±1.5	6.1±0.4	7.2±0.7	7.8±1.7	8.1±0.5	10.8±1.0	11.8±0.4	+ + +	+ + +	+ +	+ + +			
C18:1(5)+(7)	2.2±0.1	1.8±0.4	3.0±0.3	2.7±0.2	4.1±0.9	4.6±0.9	6.0±0.4	6.6±0.7	+ + +	+ + +	+		+ + +		
C18:2(6)	16.4±1.2	16.7±3.2	13.6±1.8	14.8±1.0	10.9±0.6	11.3±0.4	8.0±0.8	8.8±0.7	+ + +	+ +					
C20:4(6)	12.9±1.1	12.5±3.2	10.9±1.6	15.3±2.9	14.2±3.0	16.8±1.8	11.0±2.8	14.5±0.6				+ +			
Σsats[1]	57.1±1.9	52.9±3.0	60.6±3.2	51.0±2.5	55.7±3.8	50.4±1.3	55.0±3.7	49.0±2.3	+			+ + +			
Σmonounsats[1]	8.6±0.5	10.6±2.0	10.8±1.0	12.1±1.2	14.6±1.2	14.4±0.9	19.7±1.3	20.7±1.3	+ + +	+ + +	+ + +				
Σpolyunsats[1]	34.3±2.0	36.9±4.1	28.5±3.8	37.5±4.1	30.0±4.5	35.4±1.4	25.1±3.7	30.8±1.3	+	+		+ + +			
Σ(w-6)[1]	33.4±1.9	35.0±4.1	28.0±3.7	35.6±3.8	29.0±3.9	33.3±1.0	23.7±3.4	28.4±1.0	+ +	+ + +		+ + +			
Σ(w-3)[1]	0.9±0.6	0.8±0.5	0.5±0.6	1.0±0.3	0.7±0.4	1.1±0.4	1.3±0.5	1.7±0.3							
UI[4]	1.01±0.07	1.08±0.15	0.85±0.11	1.15±0.16	0.96±0.16	1.15±0.07	0.83±0.12	1.02±0.05		+		+ + +			
n[1]	5	6	5	6	5	5	4	6		*					

[1]Abbreviations used are: P/S Ratio, polyunsaturated to saturated fatty acid ratio; Σsats = total saturated fatty acids; Σmonounsats = total monounsaturated fatty acids; Σpolyunsats = total polyunsaturated fatty acids; Σ(w-6) = total (w-6) fatty acids; Σ(w-3) = total (w-3) fatty acids; n = number of replicates

[2]Non-Prolif. = "non-proliferative" cell fraction; Prolif. = "proliferative" cell fraction

[4]UI = unsaturation index defined as the sum of fatty acids with 2 or more double bonds multiplied by their relative percentage, and the result divided by 100.

[3]Values are means ± SD for major fatty acids.

[1]Significant effects by least square analysis of variance procedures are indicated (+ $p<0.05$, + + $p<0.01$, + + + $p<0.005$); Cell Fr., cell fraction; *, interaction effects.

acids, while decreasing ($p<0.05$) the level of $C_{18:1(9)}$, $C_{18:1(5+7)}$, and total polyunsaturated fatty acids. The unsaturation index was higher ($p<0.05$) in animals fed diets a high polyunsaturated to saturated fatty acid ratio compared to animals fed diets with a low polyunsaturated to saturated fatty acid ratio. Varying the fatty acid composition of the diets did not alter the levels of either $C_{16:0}$, $C_{16:1(5+7)}$, $C_{20:4(6)}$, total saturated fatty acids, or the sum of w-3 fatty acid series.

Cell fractions, "non-proliferative" and "proliferatve", exhibited differences in the phosphatidylinositol fatty acid profile (Table 2-12). Higher content ($p<0.005$) of $C_{16:0}$ and total saturated fatty acid, and lower content ($p<0.01$) of $C_{18:0}$, C18:1(9) and $C_{20:4(6)}$, as well as total polyunsaturated and sum of w-6 fatty acids were observed in the "non-proliferative" cell population compared to the "proliferative" cells. The level of w-3 fatty acids was not different between cell populations, while the unsaturation index was significantly higher ($p<0.005$) in the "proliferative" cells.

d) Phosphatidylserine

As for other phospholipids, phosphatidylserine fatty acid composition was modulated by dietary fat treatment (Table 2-13). Groups fed low fat diets exhibited lower levels ($p<0.005$) of $C_{18:2(6)}$ and the sum of the w-6 series accounting for the lower content ($p<0.005$) of total polyunsaturated fatty acid. These same groups also showed a lower content of $C_{18:0}$ ($p<0.005$) compared to groups fed high fat diets. Higher content ($p<0.005$) of $C_{16:1(5+7)}$, $C_{18:1(9)}$, $C_{18:1(5+7)}$, and total monounsaturated fatty acid in phosphatidylserine of animals fed low fat diets were observed. Fat level did not affect

Table 2.13. Effect of diet treatment on phosphatidylserine fatty acid composition of "non-proliferative" and "proliferative" mucosal cells from the rat large intestine.

	Fatty Acid Composition (%, w/w)								Effect of Treatment[2]							
Fat Level	High				Low											
P/S Ratio[1]	High		Low		High		Low									
Cell Fraction	Non-Prolif.[2]	Prolif.[3]	Non-Prolif.	Prolif.	Non-Prolif.	Prolif.	Non-Prolif.	Prolif.	Fat Level	P/S Ratio	Fat Level • P/S Ratio	Cell Fr.	Fat Level • Cell Fr	P/S Ratio • Cell Fr	Fat Level Ratio • Cell Fr	
C16:0	19.6±4.3	9.4±1.7	17.6±6.6	6.6±1.8	18.3±5.9	8.9±1.6	17.1±2.0	5.4±0.5				+ + +				
C16:1	2.6±0.9	1.8±0.3	1.8±0.4	1.2±0.5	1.9±0.7	2.6±1.4	3.1±0.7	2.3±0.2	+ +		+					
C18:0	31.0±2.4	34.4±2.2	31.8±1.5	35.0±1.9	30.2±2.6	32.5±1.8	27.7±1.1	30.9±2.0	+ + +		+	+ + +				
C18:1(9)	23.5±2.1	28.6±1.2	25.5±3.0	33.0±2.9	27.5±3.4	36.6±2.3	28.6±3.0	38.3±1.2	+ + +	+ + +		+ + +				
C18:1(5)+(7)	0.6±0.3	0.5±0.3	0.6±0.3	0.8±0.3	1.3±0.2	1.5±0.3	1.7±0.4	2.4±0.2	+ + +	+ + +		+ + +				
C18:2(6)	7.2±1.4	8.3±2.0	6.1±0.7	7.0±0.9	4.5±0.6	3.2±1.1	3.6±0.5	3.4±0.5	+ + +	+						
C20:4(6)	3.0±0.5	3.0±0.6	3.2±0.9	3.2±1.1	3.1±0.7	1.9±1.3	3.5±1.1	2.8±0.5								
Σsats[4]	58.4±2.3	51.3±2.2	58.1±4.0	47.7±1.1	55.6±4.2	47.9±3.5	53.8±2.0	44.8±1.4	+	+		+ + +				
Σmonounsats[5]	27.6±2.4	33.6±3.8	28.7±2.7	37.7±3.9	32.4±3.1	41.3±3.8	35.1±2.6	45.3±2.1	+ + +	+ + +		+ + +				
Σpolyunsats[6]	14.0±0.8	16.4±2.7	12.8±2.1	14.1±3.4	11.9±1.8	13.7±2.5	10.9±1.6	9.9±1.7	+ + +	+						
Σ(w-6)[7]	12.9±0.7	15.0±2.7	12.4±2.0	12.6±2.4	9.9±1.7	12.2±2.0	9.7±1.4	9.1±1.5	+ + +							
Σ(w-3)[8]	1.1±0.4	1.4±0.3	0.7±0.3	1.42±1.11	2.0±0.2	1.5±0.6	1.3±0.5	0.9±0.4		+				+		
UI[9]	0.42±0.05	0.47±0.07	0.39±0.07	0.46±0.10	0.40±0.05	0.44±0.09	0.35±0.06	0.33±0.05	+			+				
n[10]	6	5	5	5	4	5	5	5								

[1]Abbreviations used are: P/S Ratio, polyunsaturated to saturated fatty acid ratio; Σsats = total saturated fatty acids, Σmonounsats = total monounsaturated fatty acids; Σpolyunsats = total polyunsaturated fatty acids; Σ(w-6) = total (w-6) fatty acids; Σ(w-3) = total (w-3) fatty acids; n = number of replicates

[2]Non-Prolif. = "non-proliferative" cell fraction; Prolif. = "proliferative" cell fraction.

[9]UI = unsaturation index defined as the sum of fatty acids with 2 or more double bonds multiplied by their relative percentage, and the result divided by 100

[3]Values are means ± SD for major fatty acids.

[2]Significant effects by least-square analysis of variance procedures are indicated (+ $p<0.05$, + + $p<0.01$, + + + $p<0.005$); Cell Fr., cell fraction; •, interaction effects

the w-3 fatty acid series, and the unsaturation index was significantly higher ($p<0.05$) in phosphatidylserine of the high fat diet fed groups.

The fatty acid composition of the diets also altered the fatty acid composition of phosphatidylserine (Table 2-13). A decrease ($p<0.005$) in $C_{18:1(9)}$ and $C_{18:1(7+5)}$ fatty acid content occurred in animals fed diets with a high polyunsaturated to saturated fatty acid ratio, accounting for the lower level ($p<0.005$) of total monounsaturated fatty acids. The content of $C_{18:2(6)}$, total saturated and polyunsaturated fatty acids, as well as the sum of w-3 series were significantly higher ($p<0.05$) in groups having a high polyunsaturated fatty acid intake compared to groups having a low polyunsaturated fatty acid intake. Finally, the fatty acid composition of the diet did not affect either $C_{16:0}$, $C_{16:1(5+7)}$, $C_{18:0}$, $C_{20:4(6)}$, the sum of w-6 fatty acid series, or the unsaturation index of phosphatidylserine.

The cell populations exhibited differences in the fatty acyl chain composition of phosphatidylserine (Table 2-13). While the levels of $C_{16:0}$ and total saturated fatty acids were higher ($p<0.005$) in the "non-proliferative" cell population compared to the "proliferative" cells, levels of $C_{18:0}$, $C_{18:1(9)}$, $C_{18:1(5+7)}$, and total monounsaturated fatty acids were lower ($p<0.005$) in the "non-proliferative" cells. The unsaturation index of phosphatidylserine was significantly higher ($p<0.05$) in the "proliferative" cell fraction. The fatty acids $C_{16:1(5+7)}$, $C_{18:2(6)}$, $C_{20:4(6)}$, and the sum of w-6 and w-3 fatty acid series did not show level differences between cell populations.

V. EFFECT OF DIET TREATMENT ON SERUM VITAMIN E LEVELS AND ON LIPID PEROXIDE LEVELS IN MUCOSAL CELL LIPID

a) Serum Vitamin E Levels

Neither experimental day, fat level, nor fatty acid composition of the diet had an effect on serum vitamin E levels when determined as alpha-tocopherol (Table 2-14). Although not significant, serum vitamin E concentration of the low fat diet fed groups was 17% higher when compared to the high fat diet fed groups.

b) "Proliferative" Mucosal Cell Lipid Peroxide Levels

The standard curve of cumen hydroperoxide in the range of 0.1-1.0 nmole is shown in figure 2-2. Color spectra comparison between cumen hydroperoxide and peroxide of mucosal cell lipid fraction demonstrated

Table 2-14. Effect of fat diets containing similar Vitamin E densities (IU/1000 kcal) on rat serum Vitamin E levels (μg/dl).

Fat Level	High		Low		Effect of Treatment[4]		
P/S Ratio[1]	High	Low	High	Low	Fat Level	P/S Ratio	Int
	1980	1390	1770	1430	NS	NS	NS
	± 309[2]	± 319	± 505[3]	± 298			
	(n=7)	(n=6)	(n=4)	(n=6)			

[1]Abbreviations used: P/S Ratio, polyunsaturated to saturated fatty acid ratio.

[2]Values are means ± SD.

[3]This mean ± SD value tends to be higher compared to others due to one rat showing a much higher serum Vitamin E level (2480 μg/dl).

[4]Effect of treatment determined by two-way analysis of variance procedures; Int, interaction between effects of fat level and polyunsaturated to saturated fatty acid ratio; NS, not significantly different.

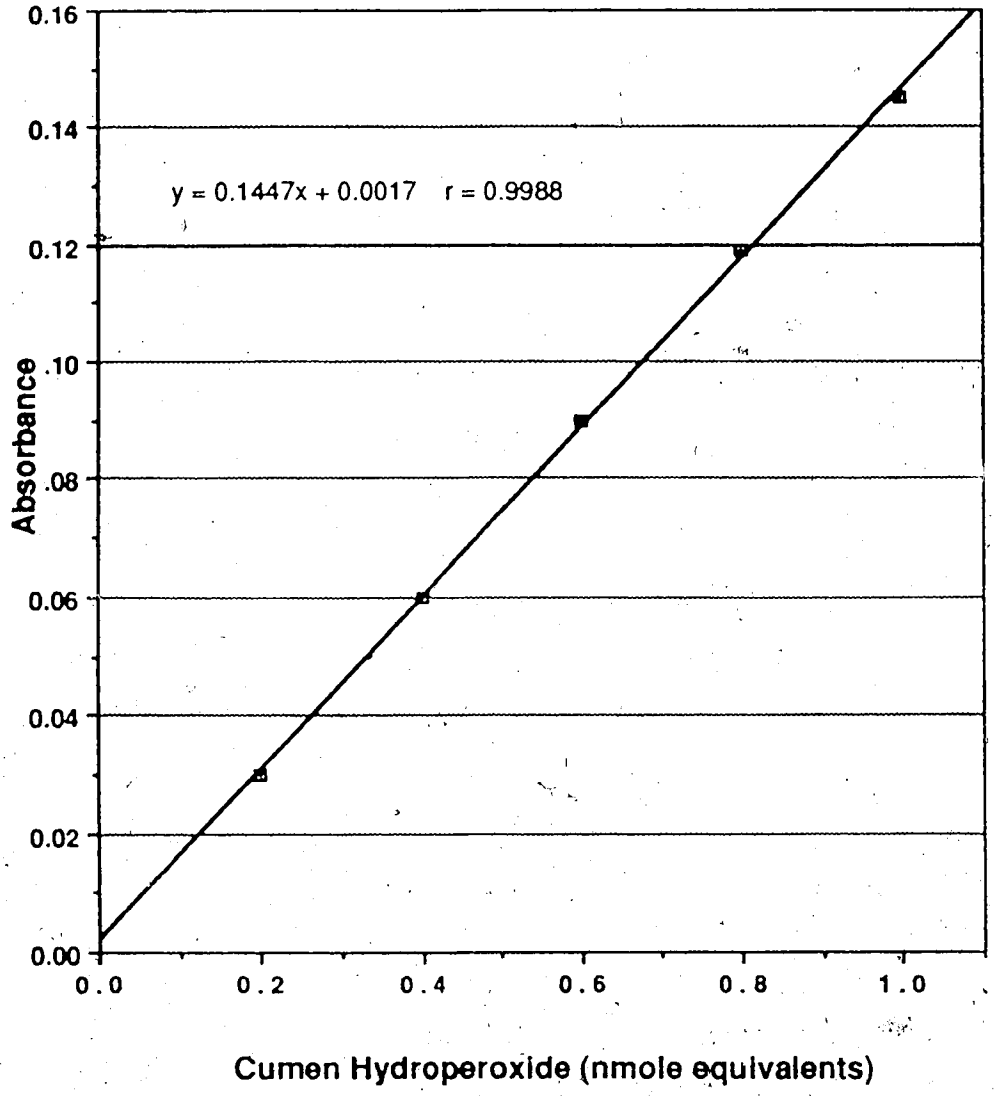

Figure 2-2. Standard curve for cumen hydroperoxide

Cumen hydroperoxide (0-1.0 nmoles) were determined as described in Material and Methods. (⊡) represent mean values of 2 or 3 replicates.

no interference due to turbidity or other opponent factors (figure 2-3).

Experimental day had no effect on lipid peroxide levels. Fat level, but not dietary fatty acid composition, affected lipid peroxide values when expressed as nmol equivalent of cumen hydroperoxide/mg protein. Feeding high fat diets increased "proliferative" cell lipid peroxide values by 64% ($p<0.05$) compared to groups fed low fat diets (Table 2-15).

When results are expressed as nmole of cumen hydroperoxide equivalent per mg (or mol) of phospholipids, no effect of fat was observed. However, groups fed a polyunsaturated to saturated fatty acid diet ratio of 1.1 exhibited a 73% increase ($p<0.006$) in lipid peroxide content compared to groups fed a polyunsaturated to saturated fatty acid diet ratio of 0.3 (Table 2-15).

To correlate lipid peroxidation with the phospholipid fatty acid composition and the unsaturation index, statistical analysis for effects of diet treatment on phosphatidylethanolamine and phosphatidylcholine fatty acids of the "proliferative" mucosal cells in the large intestine was performed and is summarized (Table 2-16). Except for $C_{20:4(6)}$ fatty acid of phosphatidylethanolamine, both phospholipids were significantly affected in their fatty acid composition, including $C_{18:2(6)}$, $C_{20:4(6)}$, total polyunsaturated and w-6 series fatty acids, by fat level and fatty acid composition of the diets. An increase in all these fatty acids were observed in groups fed diets with a high fat content (fat effect) and in groups fed diets with a high polyunsaturated to saturated fatty acid ratio (P/S effect). Both fat

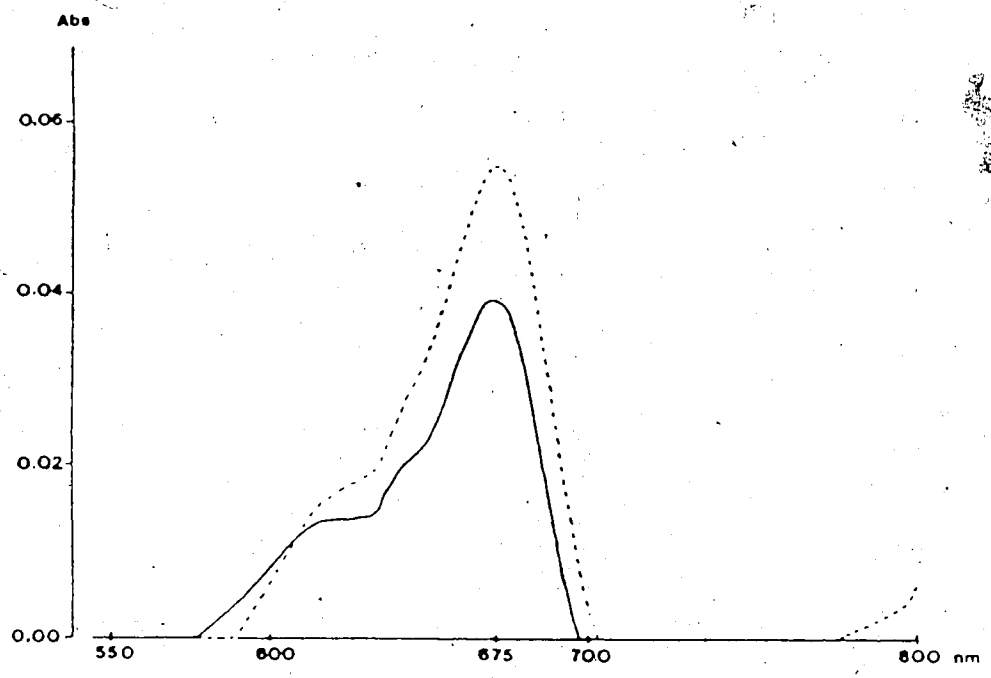

Figure 2-3. Absorption spectra of cumen hydroperoxide and of lipid hydroperoxide from rat colonic cell lipid determined by the Hb-MB test

Cumen hydroperoxide (----- , 0.2 nmoles) and 100 µl of rat colonic cell lipid fraction (———) have been treated as described in Material and Methods. Zero absorbance was adjusted at 800nm with H_2O against H_2O. (chart speed: 10nm/cm, scan rate: 1nm/sec, pen: 0-0.1 Abs).

Table 2-15. Effect of diet treatment on lipid peroxidation of "proliferative" mucosal cell lipid fraction from the rat large intestine.

Fat Level	Lipid Peroxide Values[1]				Effect of Treatment[4]		
	High		Low		Fat	P/S	
P/S Ratio[2]	High	Low	High	Low	Level	Ratio	Int
nmole/ mg protein	0.11[3] ±0.02	0.07 ±0.04	0.05 ±0.01	0.06 ±0.01	0.05	NS	NS
nmole/ mg Phospho-lipids	1.09 ±0.30	0.53 ±0.28	0.90 ±0.33	0.62 ±0.14	NS	0.006	NS
nmole/ μmole Phospho-lipids	0.80 ±0.22	0.38 ±0.21	0.66 ±0.24	0.44 ±0.11	NS	0.006	NS
n[3]	5	5	2	6			

[1]Lipid peroxide values are expressed as nmole of cumen hydroperoxide (multiply by factor 2 to obtain results in nequ.).

[2]Abbreviations used: P/S Ratio, polyunsaturated to saturated fatty acid ratio; n, number of replicates.

[3]Values are means ± SD.

[4]Significant effects ($p<$) by two-way analysis of variance procedures are indicated; Int, interaction between effects of fat level and polyunsaturated to saturated fatty acid ratio; NS, not significantly different.

level and fatty acid composition of the diets also affected the unsaturation index in the phospholipids.

Table 2 [illegible] Effect of diet treatment on phosphatidylethanolamine and phosphatidylcholine fatty acid composition of "proliferative" mucosal cells from the rat large intestine

Fat Level	Fatty Acid Composition (%, w/w)				Effect of Treatment[a]		
	High		Low				
P/S Ratio	High	Low	High	Low	Fat Level	P/S Ratio	Int
Phosphatidylethanolamine							
C18:2(6)	11.6±1.1[b]	9.4±1.6	7.4±0.7	7.0±0.4	0.001	0.02	NS
C20:4(6)	33.1±2.8	35.5±1.6	35.7±2.2	31.6±1.5	NS	NS	0.01
Σsats	58.0±2.3	55.9±1.4	54.2±2.9	49.0±1.4	0.001	0.003	NS
Σ(ω-6)	54.3±2.3	51.9±1.9	50.0±2.7	44.8±1.4	0.001	0.003	NS
Σ(ω-3)	2.9±1.0	3.6±0.7	3.7±0.5	3.8±0.4	NS	NS	NS
UI	2.07±0.10	2.03±0.05	2.01±0.12	1.80±0.06	0.0005	0.01	NS
Phosphatidylcholine							
C18:2(6)	23.6±1.5	18.3±0.6	13.7±1.0	11.2±1.5	0.001	0.001	0.03
C20:4(6)	13.3±1.7	13.1±1.7	12.6±1.5	9.7±0.7	0.01	NS	0.05
Σpolyunsats	42.6±3.7	38.9±2.7	30.8±2.1	25.5±1.9	0.001	0.001	NS
Σ(ω-6)	42.0±3.6	38.0±2.7	29.8±2.3	24.5±1.7	0.001	0.001	NS
Σ(ω-3)	0.58±0.17	0.89±0.24	0.91±0.35	0.95±0.19	NS	NS	NS
UI	1.25±0.13	1.17±0.09	0.96±0.08	0.79±0.05	0.001	0.01	NS

[a] Abbreviations used are: P/S Ratio, polyunsaturated to saturated fatty acid ratio; Σsats = total polyunsaturated fatty acids; Σ(ω-6) = total (ω-6) fatty acids; Σ(ω-3) = total (ω-3) fatty acids.

UI = unsaturation index defined as the sum of fatty acids with 2 or more double bonds multiplied by their relative percentage, and the result divided by 100.

[b] Values are means ± SD.

[c] Significant effects ($p<$) by two-way analysis of variance procedures are indicated. Int, interaction between effects of fat level and polyunsaturated to saturated fatty acid ratio; NS, not significantly different.

D. DISCUSSION

Dietary fats have a variety of effects on cell membranes. Previous studies have demonstrated that dietary fat affects membrane lipid content and phospholipid fatty acyl composition (13,15,16,18-20). Other studies also reported increased membrane lipid peroxide levels with increased membrane polyunsaturated fatty acid content and membrane unsaturation index (21,22). Experiments examining brush-border membrane and basolateral membrane in the small and large intestine have been performed and demonstrated modulation of structure and function by fat diet treatment (20,41,47). The experiment presented in this thesis extends current knowledge by examining the hypothesis that changes in dietary fat level and fatty acid composition alter colonocyte membrane composition which in turn may affect lipid peroxide levels in this cell type.

I. EFFECT OF DIET TREATMENT ON MUCOSAL CELL ISOLATION AND THYMIDINE KINASE ACTIVITY

a) Cell Isolation

"Non-proliferative" and "proliferative" cell populations from the small intestine have been successfully isolated (49,50,51,53). Methods to isolate these cell types from the large intestine have also been reported (54,55,72). The procedure used in the present experiment is a modification of the methods described by Brasitus (55) for the cecum and proximal colon and by Weiser (49) for the small intestine. The procedure has been modified for ease and better control of the cell integrity as the isolation period in the present study is rather long.

Ca^{++} and Mg^{++} Free Hank's buffer containing triethanolamine-HCl and dithiothreitol was used as the main isolating solution due to its properties of controlling constant pH and osmolarity, while supplying vital elements for cell metabolism. The disadvantage of the method is a long isolation time, resulting from collection of twelve fractions. This is balanced by the advantage that the method is "smooth" and almost no clumped cells are observed in the fractions as opposed to the Brasitus' procedure (personal communication). Clumped cells and cell sheets are not desired as they are likely to represent not only epithelial cells but a mixture of epithelial and non-differentiated cells. Clumped cells, debris and mucus-like material were removed by filtration. Although cells from "non-proliferative" and "proliferative" cell fractions were well pelleted, fluffy material, perhaps mucus,could occasionally be observed around the pellet of the "non-proliferative" cell fraction. This "non-proliferative" cell population consists of columnar epithelial cells, goblet cells, some endocrine cells and lymphocytes (55). Each fraction was stained with hematoxylin and eosin, periodic acid-Schiff (PAS), Wright's, and mucicarmine stains. Unfortunately, cell fixation was performed by air drying and this process does not allow for cell type recognition.

As observed by others (52) there was a high variability of total material released (mg of protein) between animals (Table 2-4). Unexpectedly, the diet treatment exhibited an effect on the cell isolation procedure, with a 55% higher amount of collected material in groups fed diets with low polyunsaturated to saturated fatty acid ratio compared to groups fed diets with high polyunsaturated to saturated fatty acid ratio (Table 2-4). This effect is not due to the procedure

as the percentage of protein distributed between "non-proliferative" and "proliferative" cell populations was apparently similar for each dietary group (Table 2-5). However, it is possible that cell cohesion and mucus properties have been altered by diet treatment. Change in the effectiveness of the unstirred water layer may have also occurred. Further investigation is needed to support these suggestions. Based on the results of ^{3}H-thymidine incorporation into DNA (Table 2-3), a better distribution would have been 50-60% and 40-50% of protein in the "non-proliferative" and "proliferative" cell populations, respectively, with approximately 12 mg of total cell protein.

Comparisons with other studies are difficult to make as the proc[redacted]sue sites used are varied. For instance, Brasitus [redacted]ecum and proximal colon while Craven and DeRubertis [redacted]stal colon from the colonic flexure to 1 cm above [redacted]n, the latter study, cells from two colons were [redacted]-total protein were collected. It is noteworthy to [redacted]that no filtration step was included, thus contamination with mucus was likely. On the other hand, 10% (w/v) of heat-treated fetal bovine serum was used to breakdown the mucus, and antibiotics added to the solution to prevent bacterial growth.

In the future, some modifications to improve the method as well as some additional controls should be performed. First, a 15 minute bath with 10% fetal calf serum in 1.6% Jolik's modified minimum essential medium should be included (55). This solution has the property to degrade the mucus and would ease cell release increasing material collected. Secondly, cells should be fixed adequately and stained for cell identification. Furthermore, cells and intestinal tissue should

be prepared for microscopic observation at different stages of the procedure.

b) Thymidine Kinase Activity

Thyimidine kinase activity has been measured in regenerating rat livers (73-76), in leukocytes (58), and in small (57) and large (55,57) intestines of rats (55,57) and humans (57). Differences in tissues examined, enzyme purity, and way of expressing results make it difficult to compare the data of the current study with the literature. Both ^{3}H-thymidine (73-75) and ^{14}C-thymidine (55,57,58,76) as enzyme substrates have been used. Important variations in amount (from $1x10^{5}$ to $2x10^{6}$ dpm) of radioactive substrate added to the reaction mixture occurred among studies (55,57,58,73). Two groups (57,58) expressed their results as mol of product/min/mg of protein. According to the test conditions, these reported results are practically impossible. Due to a low thymidine kinase activity in the samples, 500,000 dpm/reaction mixture with a reaction time of 45 minutes have been used to measure the enzyme activity. As the test is subject to variations from one assay to another, batches of 2 blanks and 4 samples, including each time the "non-proliferative" and "proliferative" cell fractions of the same animal, were processed to decrease possible errors.

Thymidine kinase specific activity was 41% higher ($p<0.05$) in the "proliferative" cell population compared to "non-proliferative" cells. Therefore, it seems that these fractions exhibit metabolic differences. However, the activities obtained are lower than those observed by Brasitus (55) in cecum plus proximal rat colon and Salser and Balis (57) in rat colon. Brasitus (55) separated, by gradient

centrifugation, cell fractions with increasing thymidine kinase activity from 5.7 to 19.0 pmol/min/mg of protein with an average of 11.3 pmol/min/mg of protein for the four isolated fractions. On the other hand, Salser and Balis (57) obtained a mean value of 6.5 pmol/min/mg of protein in rat colon. Average thymidine kinase activity obtained in this study for the "proliferative" cell fraction was 4.1 pmol/min/mg of protein. The differences can be explained in part by differences in methodology and segment of tissue examined, and/or could be due to the small amount of material collected. Unexpectedly, the group fed a diet with a low fat and low polyunsaturated to saturated fatty acid ratio exhibited lower thymidine kinase activity in the so-called "proliferative" cell population compared to "non-proliferative" cells (Table 2-6).

II. EFFECT OF DIET TREATMENT ON LIPID COMPOSITION OF MUCOSAL CELL

Cholesterol and phospholipid content of the mucosal cells in the large intestine were found to be lower compared to those obtained for microsomes of the small intestine (77). In this regard, dissimilarities between cholesterol and phospholipid content of jejunum and ileum have been observed (77). These differences may also be explained by variation in diet, and/or dilution effect by protein when using the whole cell homogenate for analysis compared to a purified membrane fraction. Cholesterol and phospholipid values found were also lower than those reported in brush-border membranes of rat small intestine (43,78,79). These observations are in agreement with another study (77) in which microsomes from rat small intestine exhibited lower content of both these lipid classes. Cholesterol to phospholipid

ratios in brush-border membrane of rat small (20,43,78-81) and large (47,82) intestine are generally higher than those obtained in this study for mucosal cells from the large bowel. The results found are consistent with those of Thomson et al. (43,79) suggesting no alteration of the bulk lipid composition of the cell membranes by dietary fat manipulation. On the other hand, this group did not report a significant effect of dietary fat on the molar ratio of these lipids in the brush-border membrane from the small intestine as others (20) or as in this study for mucosal cells in the large intestine. No such effect was observed in the basolateral membrane of either the proximal and distal small intestine, or the proximal and distal colon (20). Although not significant, the mucosal cell total phospholipid content of animals fed diets with a low polyunsaturated to saturated fatty acid ratio was higher compared to animals fed diets with a high polyunsaturated to saturated fatty acid ratio (153 vs 129 g/mg of protein). This may account for the significant effect ($p<0.005$) of dietary fatty acid composition observed on the molar ratio of cholesterol to phospholipid (Table 2-7).

Unfortunately, it is not possible to further compare data of this study with those obtained by Brasitus et al. (20,44,47,80-84) since this group reported their results as a percentage of the total lipid. Higher cholesterol ($p<0.005$) content was found in the "non-proliferative" cell populations compared to "proliferative" cells. This result needs further investigation to delineate if it is an artifact or a constant observation. In the latter case, reasons and mechanisms for the difference observed will need further study.

III. EFFECT OF DIET TREATMENT ON PHOSPHOLIPID CONTENT OF MUCOSAL CELLS

Morphological, functional and structural changes in the mucosa of the intestinal tract occur with age and disease such as diabetes, and can be influenced by external factors including irradiation, chronic ethanol exposure and nutrient intake (20,39-43,78,79,85-87). Although dietary fat induces changes in the brush-border membrane lipid composition and the phospholipid distribution in diabetic rats, generally less or no significant changes are observed in healthy animals (41,43,79). On the other hand, Brasitus et al. (20) found that dietary fat significantly altered the lipid composition and phospholipid distribution of rat small intestinal microvillus, while the basolateral membrane was altered to a lesser extent. These discrepancies between studies may in part be due to differences in animal strain, diet composition and the period of feeding. Furthermore, the modulation of membrane structure and function by dietary fat manipulation involves complex mechanisms that cannot be explained only on the basis of altered membrane lipid composition (79). In the present study, and in agreement with the results obtained by others in brush-border membrane of the small intestine (41,79), no significant effect of fat level or diet fatty acid composition on the phospholipid class content was observed (Table 2-8). The slight discrepancies in the significance of the statistical test depending on the unit used to express the results (Tables 2-8 and 2-9) may be explained by the difference of one observation between both sets of data, an important factor for statistical analysis.

In agreement with the findings of Garg et al. (77) on the microsomal fraction of rat small intestine, the colonocytes exhibited a

higher percentage of phosphatidylethanolamine than phosphatidylcholine. This result differs from a number of membranes where the phosphatidylcholine content is higher than phosphatidylethanolamine. Phosphatidylcholine may be synthesized via three different pathways. In the first pathway, phosphatidylcholine is formed by acylation of lysophosphatidylcholine (88). In the second, or so called "de-novo" pathway, phosphatidylcholine is synthesized by reaction of diacylglycerol with CDP choline (89). The third pathway involves the phosphatidylethanolamine methyltransferase enzyme to convert phosphatidylethanolamine to phosphatidylcholine (90). While the first two pathways have been identified in rat small intestinal epithelial cells (91), failure to detect phosphatidylethanolamine methyltransferase activity in the microsomal fraction of this cell type has been reported (90). Recently Dudeja et al. (92,93) found such activity not only in the rat colonic brush-border (92) and basolateral membranes (94), but also in various membranes from the small intestine (93). The transmethylase activities in the different subcellular fractions of the rat small intestinal epithelial cells ranged from 20-60% those of the plasma membrane (93). Unfortunately, only the data for the brush-border membrane were presented. Although the reasons and mechanisms for the differences in the phospholipid profile among the various membranes of a cell are not yet known, a lower activity or content of phosphatidylethanolamine methyltransferase in microsomes compared to the plasma membrane may partly explain the higher level of phosphatidylethanolamine than phosphatidylcholine in the colonocytes and in the microsomes of the rat small intestine. Modulation of this enzyme by dietary fat has been reported (62). Further research is

needed in this area.

IV. EFFECTS OF DIET TREATMENT ON PHOSPHOLIPID FATTY ACID COMPOSITION OF MUCOSAL CELLS

Dietary fat altered the phospholipid fatty acid composition and the unsaturation index of the "non-proliferative" and "proliferative" mucosal cells from the rat large intestine; the effects varied from one phospholipid class to another (Tables 2-10 to 2-13). Since all the essential nutrients for non-fat components remained constant, the effects observed are primarily due to dietary fat manipulation. Feeding either high fat diets (50% as energy) or high polyunsaturated fatty acid content diets (P/S=1.17) decreased the total monounsaturated fatty acid levels, and increased the total polyunsaturated fatty acid contents of the colonic cell phospholipids. Alterations in phospholipid polyunsaturated fatty acids by changing diet fat content was reflected in changes of the w-6 (phosphatidylethanolamine, phosphatidylcholine, phosphatidylinositol, phosphatidylserine) and w-3 (phosphatidyl- ethanolamine) fatty acid series. Increasing the dietary polyunsaturated to saturated fatty acid ratio altered the w-6 fatty acid series in phospholipids but did not change w-3 fatty acids, except for phosphatidylserine. Diet variations did not influence saturated fatty acid content of the major phospholipids (phosphatidylethanolamine phosphatidylcholine). Fatty acid composition of sphingomyelin could not be determined due to the small relative percentage of this phospholipid (av. 6 %,w/w of total phospholipid). Therefore, the finding that colonic cell phospholipid fatty acid composition reflects, in part, dietary fat manipulation is consistent with previous studies

(13,15,16,18-20,95).

Rats are homeotherms maintaining a constant body temperature despite environmental temperature variations. Similarly, organisms tend to keep a constant cell membrane fluidity despite external stress, such as diet, by modulating their membrane phospholipid fatty acid compositions and cholesterol levels - a process called "homeoviscous adaptation" (96-98).

Changes in the membrane lipid composition alter membrane functions (16,18,19,99-101). For instance, activities of acylcoenzyme A: cholesterol acyl transferase in rat liver microsomes (99), ATPase in rat heart mitochondria (19,100,101), glucagon-stimulated adenylate cyclase in rat liver plasma membrane (16), and NTPase in mouse liver nuclear envelope (18) were all influenced by dietary fat manipulation via alteration in membrane lipid composition. For a review on the effects of dietary fat on the membrane structure and function, see Clandinin et al. (15,17). Although it is possible to predict how dietary fat will alter the membrane lipid composition, it is yet not possible to predict how membrane functions will be affected (102).

Increased membrane polyunsaturated fatty acid content and increased membrane cholesterol content have generally been correlated with enhanced membrane fluidity (20,82,103). Discrepancies in this finding may be partly explained if not only the membrane phospholipid fatty acid or membrane cholesterol content, but also the interactions of both these factors are considered to understand regulation of the modulation of membrane fluidity. Changes in the membrane fluidity by dietary fat treatment have been reported in both the rat small and large intestine, as well as within intestinal cell membranes -

brush-border and basolateral membranes (20). Although not measured in this study, it is likely that diet treatment altered mucosal cell membrane fluidity in the large intestine.

An opposite trend in dietary fat effects for phosphatidylcholine and phosphatidylethanolamine fatty acyl chain composition between both cell fractions was observed. Phosphatidylcholine from the "proliferative" cell population tended to exhibit higher saturated fatty acid and lower mono- and polyunsaturated fatty acid levels compared to "non-proliferative" cells. A reverse effect, except for the monounsaturated fatty acids, occurred in phosphatidylethanolamine. No significant variations in the w-3 fatty acid series were observed. Further research is needed to delineate biological reasons and mechanisms for such differences.

In summary, the present experiment, in agreement with others (13,15,16,18-20,95), extended the current knowledge that dietary fat altered both the phospholipid fatty acid composition and the unsaturation index of mucosal cells from the rat large intestine. The effects varied between cell populations, "non-proliferative" and "proliferative", as well as among phospholipid classes within a cell type. Further research is needed to better understand the effects observed.

V. EFFECT OF DIET TREATMENT ON SERUM VITAMIN E LEVELS AND ON LIPID PEROXIDE LEVELS IN MUCOSAL CELL LIPID

a) Serum Vitamin E Levels

Methods to analyse serum vitamin E generally suggest hexane as the extracting solvent (104-106). Using the procedure described by Chow

and Omaye (106), low dl-alpha-tocopherol and d-alpha-tocopheryl acetate recoveries (20-25%) have been obtained. Rat serum alpha-tocopherol recoveries were 34% and 109% in absence or presence of the antioxidant butyl-hydroxy-toluene (BHT), respectively; while in human serum this antioxidant did not have such an effect (106). However, d-alpha-tocopherol added as internal standard to their sample was 0.07 g, of which less than half has been injected onto the column. This low amount of standard used does not allow calculation of recovery. To avoid the saponification step, which may be accompanied by loss of vitamin E, a slight modification of the clarification method described by Nierenberg and Lester was used (69). Recoveries for dl-alpha-tocopherol and d-alpha-tocopheryl acetate were 105% and 123%, respectively. The latter high recovery can be explained by peak interference as compounds tend to be eluted at similar times at the beginning of the chromatogram. A better internal standard would have been gamma-tocopherol, which is expensive, or tocol, which is not available on the market. Both these compounds would be eluted at a longer retention time on a portion of the chromatogram where no peak is detected. Good reproducibility between duplicates was obtained. As in humans, the rat serum levels reported in the literature showed great variations (140-1560 μg/dl); (107-113). The variations may be due to differences in diet vitamin E and fat content, period of feeding, and/or animal age and strain; methodological problems (recovery) should also be considered. The serum alpha-tocopherol values found in this study (av. 1500 μg/dl) are in the upper range of those reported.

A non-significant difference in serum alpha-tocopherol status of the animals reflects a similar vitamin E density intake (IU/kcal)

between each group and/or a well controlled diet. Although not significant, and disregarding the abnormally high serum alpha-tocopherol concentration of one rat (2480 µg/dl) fed the low fat high polyunsaturated to saturated fatty acid ratio diet, groups fed the low fat diets tended to exhibit a higher mean vitamin E value. This difference may be partly explained, by slight differences in vitamin E content among diets, by slight differences in food intake among groups, and by increased vitamin E mobilization in rats with higher polyunsaturated fatty acid intake. There was no direct relationship between polyunsaturated fatty acid intake and serum vitamin E levels. Serum vitamin E may not reflect vitamin E status in tissues; however, liver microsomes did not exhibit differences of alpha-tocopherol content in rats fed vitamin E controlled diet (114). Therefore it is likely that vitamin E status in the "proliferative" cells of the rat large intestine among dietary groups was similar.

b) Lipid Peroxide

Since diets were prepared fresh once a week, peroxide levels in the diets have not been determined. According to Reddy and Tanaka (115), no significant levels of peroxides were detected in freshly prepared and one week old diets kept at -4°C.

Lipid peroxides and free radicals are known to induce cell membrane damage and DNA mutations. A great deal of experiments shown increased lipid peroxidation under various conditions including septic (116) and vitamin E deficient animals (117) as well as after irradiation with gamma-rays (118) and UV light (21) or after injection of some types of carcinogens (119). Several methods for lipid peroxide

determination are available (see ref. 120). These methods include measurement of O_2 consumption, measurement of heptane released in breath samples, erythrocyte susceptibility to hemolysis, detection of conjugated dienes, loss of polyunsaturated fatty acid in membrane phospholipids, fluorescence analysis of lipid peroxidation products, chemiluminescence detection, and the thiobarbituric acid reactive substance (TBA-RS) assay. The TBA-RS procedure is the most widely used. However, this method does not measure lipid peroxide per se, but the end product, malondialdehyde (MDA), resulting from the breakdown of lipid peroxides and some other non-lipidic compounds. The procedure, sensitive to parameters such as temperature and pH, involves an acid-heating step which may initiate lipid peroxidation, thus increasing TBA-RS. It is also of importance to differentiate results obtained under non-induced lipid peroxidation systems (121-123) from those measuring susceptibility of sample to lipid peroxidation induced by ascorbate-iron or NADPH-iron (114,124,125). The lack of a standardized procedure may partly explain conflicting results. Recently, Ohishi et al. (126) described a new methylene blue derivative for use in a colorimetric method (Hb-MB method) which detects specifically hydroperoxides, endoperoxides and peroxy radicals. This assay is now available as a kit which was used in this experiment. Prior to measurements of lipid peroxides in coloncytes, a few tests were performed. First, as previously reported (127), an equimolar relationship between nanoequivalents of cumen hydroperoxide detected, and the peroxide value measured by the iodometric method was confirmed using canola oil (data not shown). Secondly, turbidity and cloudiness in the reaction system were observed under the conditions described

previously and as reported by Kanazawa et al. (128) when using rat liver microsomal fractions (data not shown). In the present experiment, lipid peroxides were determined in the lipid fraction from the "non-proliferative" mucosal cells in the rat large intestine, and as can be seen in figure 5, no turbidity or interference occurred. Although ethoxyquin, a yellow-brown antioxidant added to the lipid extraction solvents, did not influence the assay at the concentration used, butyl-hydroxy-toluene would be a better choice for future studies as this antioxidant is colorless and does not influence the test (129).

In the present study, lipid peroxides were assayed only in the "proliferative" cell fraction. Although rats fed physiologically relevant diets exhibited very low levels of equivalent cumen hydroperoxides, changes in fat level and fatty acid composition of the diets significantly altered lipid peroxidation level (Table 2-15).

Results in the literature are generally expressed as lipid peroxide levels per mg of protein. This allows comparisons between similar type of membranes or tissues, but does not allow correlations between different types of materials as the amount of substrate for peroxidation per unit of protein varies. For instance, rat liver microsomal fractions contain 400-600 µg of phospholipid/mg of protein, while the rat large intestinal cell homogenates hold approximately 100 µg of phospholipid/mg of protein. For comparison, the results should also be expressed as amount of lipid peroxides per mg or moles of phospholipids.

Discrepancies among published data besides experimental variations, may partly be explained by differences in the physiological states of the animals as lipid peroxidation is not only dependent on

substrate available (polyunsaturated fatty acids) but also on the antioxidant status, the pool and activity of various enzymes (129) as well as on other factors (age, diseases) of the tissue or membrane studied. Peroxidation is often measured under stressed conditions such as vitamin E deficient (117) or iron-overloaded (130) animals, situations that increase the sensitivity to attack by reduced oxygen species. Since in the present experiment animals were fed physiologically relevant diets, comparisons with other studies for lipid peroxide should be performed with control values. However, since the end products measured are different, comparisons are difficult to make and data will be presented for information. Values (nmole of MDA/mg of protein) for the TBA-RS test, under non-induced lipid peroxidation conditions, reported for control groups were 0.7, 2.0, 1.5, and 0.8 in the homogenates of rat liver, brain, and kidney, respectively (116). Others reported 0.2-0.45 nmoles MDA/mg of protein in rat liver microsomes (114), and 0.9-2.0 nmoles MDA/mg of protein in rat gastric mucosa (131). As the leuco-methylene blue derivative for use in this colorimetric method is recent, published data are practically non-existent. Kanazawa et al. (128), using this assay, reported lipid peroxide levels of 1.31 to 1.58 nanoequivalents of linoleic acid hydroperoxide per mg of protein in rat liver microsomes. The causes for the lower values found in this study compared to those reported may be due to differences in the tissue studied, to variations in the phospholipid content (microsomes contain about 5 times more phospholipid/mg of protein compared to cell homogenate; dilution effect) and the pool of polyunsaturated fatty acids available for peroxidation. Furthermore, variations in the experimental conditions

including diets as well as animal age and strain have also to be considered. Lipid peroxides determined in control and alcohol-treated rats by both methods (the TBA-RS test and the Hb-MB assay) led to conflicting results (128). This further underlines the lack of specificity with the TBA-RS procedure, which demonstrated increased malondialdehyde (MDA) detected in alcoholic rats compared to non-treated animals, while increase of hydroperoxylinoleate did not occur.

A radical scavenger or the collision of two free radicals is needed to stop the propagation of lipid peroxidation. An environment with high polyunsaturated fatty acid levels will facilitate the propagation step compared to an environment with lower polyunsaturated fatty acid levels, assuming both contain similar free radical defense systems. The present experiment supports the hypothesis of an increased membrane polyunsaturated fatty acid content in groups fed high fat or high polyunsaturated to saturated fatty acid ratio diets compared groups fed low fat or low polyunsaturated to saturated fatty acid ratio diets (Table 2-10 to 2-13), which in turn increases the lipid peroxide levels in these membranes (Table 2-15). However, it is yet not possible to determine which of the dietary factors - fat level, polyunsaturated to saturated fatty acid ratio, or both - primarily affect lipid peroxidation as first, both factors influence substrates available for peroxidation and secondly, both factors also affect the peroxide levels depending on the units used to express the results. These findings challenge those from Bringham (132) who reported an effect of diet vitamin E content, but not of diet fatty acid composition (P/S ratio), on lipid peroxidation in rats. However, the

methods used to determine lipid peroxides were different from the one used in this study, and some discrepancies in the results occurred depending on the procedure used.

In conclusion, the biological significance of the difference in the lipid peroxide levels observed between fat diet treated groups is not yet known. Further studies are needed to determine the extent of possible cell damage by such small amounts of peroxides, and to delineate more precisely the relationship between dietary fat and membrane cell injury by reduced oxygen species.

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F. CONCLUSIONS AND GENERAL DISCUSSION

Dietary fat influences the membrane structure and function (1,2). Peroxidation of lipids and reduced oxygen species may cause cell injury including membrane damage and DNA mutations (3).

In this thesis, it was hypothesized that the mucosal cell membrane composition is influenced by diet, more specifically, it was hypothesized that:

1. The lipid composition of "non-proliferative" and "proliferative" mucosal cells in the large intestine reflects, in part, the fatty acid composition of the diet.

2. Lipid fractions of the mucosal cells from rats fed high (50% as energy) or low (15% as energy) fat diets with high P/S ratio (P/S=1.2) exhibit more lipid peroxides than rats fed comparable high or low fat diets with a low P/S ratio (P/S=0.3).

3. A high fat diet (50% as energy) either - saturated or unsaturated - increases lipid peroxide production compared with a low fat diet (15% as energy) having the same P/S ratio (either P/S=1.2 or P/S=0.3).

The hypothesis has been verified as follows:

Hypothesis 1.

Both fat level and fatty acid composition of the diets influenced the mucosal cell membrane composition in the rat large intestine. A higher monounsaturated fatty acid content in the mucosal cell lipids

from rats fed low fat or low P/S diets and a higher polyunsaturated fatty acid content in the mucosal cell lipids from rats fed high fat or high P/S diets occurred. Although diets differed in their saturated fatty acid levels, no significant changes in the saturated fatty acid levels of the phospholipids in the mucosal cells were observed.

Hypothesis 2 and 3.

Both dietary fat level and dietary fatty acid composition influenced the mucosal cell lipid peroxidation in the rat large intestine. However, the effects of diet treatment depended on the units used to express the results. High fat diets increased the lipid peroxide values expressed as nmole equivalents of cumen hydroperoxide/mg of protein, while high P/S ratio increased lipid peroxide levels expressed as nmole equivalents of cumen hydroperoxide/mg or mole of phospholipids. Therefore, it is still to be determined which dietary factors - fat level, fatty acid composition, or both of them - are of primary importance. The effects observed are unlikely to be due to differences in vitamin E status as alpha- tocopherol levels were not significantly different between groups. In addition, a previous study showed no alpha-tocopherol differences in the liver microsomes of rats fed vitamin E controlled diets (4). Since the lipid peroxide levels measured were low, biological significance of the differences observed by dietary fat manipulations should be determined.

Although not hypothesized, cell fractions, "non-proliferative" and "proliferative", exhibited differences in the phospholipid fatty acid composition. Due to lack of material, lipid peroxide levels in the "non-proliferative" cell population were not measured.

Experimental procedures have been adjusted for the purpose of this thesis. Although consistency in the percentage of cell distribution between both cell populations, whatever the diets, occurred, wide variations in absolute amounts of cell released, among and within groups, were observed. Consequently, the "non-proliferative" cell fraction in a group with "high" absolute amount of total protein may correspond to the "non-proliferative" plus "proliferative" cell fractions of a group with low cell release (overlapping). This, besides the length of the experiment, may account for the statistical interactions observed. For example, thymidine kinase activities from rats which released more than 8 mg of total mucosal cell protein were compared to those from rats which released less than 6 mg of total mucosal cell protein. The "high" protein released group exhibited thymidine kinase activities of 2.76 + 0.29 and 5.79 + 0.56 (mean + SE, $p<0.01$) nmole of product/min/mg of protein in the "non-proliferative" and "proliferative" cell fractions, respectively. On the other hand, the group with "low" cell release exhibited thymidine kinase activities of 3.04 + 0.37 and 3.31 + 0.51 (mean + SE) nmoles of product/min/mg of protein in "non-proliferative" and "proliferative" cell populations, respectively. Indeed, these latter activities were not significantly different. These comparisons between fractions, whatever the diet treatment, were possible as no dietary effects on thymidine kinase were observed. Possible solutions to avoid dietary effects and differences in amount of material collected is discussed below.

Although no data on the stained cells are available, contamination by mucus and bacteria were small or unlikely. Mucus, fluffy material, sediments around the cell pellet and was observed only occasionally in

the "non-proliferative" cells. Bacterial membranes are characterized by branch-chain fatty acids; no such fatty acids were detected during the fatty acid analysis by capillary gas chromatography. However, procedures to verify purity of the cell preparations should still be undertaken.

As mentioned in the Discussion, some work should be performed to validate the method. This involves the addition of a pre-incubation bath of the gut with a solution of 10% (w/v) fetal calf serum in 1.6% Jolik's minimum essential medium to breakdown the mucus (5), and microscopic controls for purity of the cell fractions. Intestinal preparations for microscopic observations should also be performed.

The present study is the first report on dietary fat effects on lipid composition and peroxidation in two types of mucosal cells in the rat large intestine. It is also the first time that higher content of the phosphatidylethanolamine than phosphatidylcholine has been observed in this tissue. This is in agreement with the result of a recent study on the epithelial cell microsomes in the rat small intestine (6). Brush-border and basolateral membranes from epithelial cells in the rat small and large intestine contain higher levels of phosphatidylcholine than phosphatidylethanolamine (7-9).

In conclusion, data presented in this thesis are in agreement with other studies demonstrating dietary fat effects on epithelial cell plasma membranes in the rat small and large intestine (7-9). As previously reported for other tissues, increased membrane susceptibility to lipid peroxidation correlated with increased membrane phospholipid polyunsaturated fatty acid levels. Further research is needed to better understand the effects of dietary fat manipulations on

the gut and the possible medical applications of such manipulations.

G. FUTURE STUDIES

Several studies using the methodology described in this thesis could be performed. This includes the following:

validation of the cell isolation procedure by first, treating the gut with a pre-incubation solution containing 10% (w/v) fetal calf serum in 1.6% Jolik's minimum essential medium, secondly adjusting triethanolamine-HCL concentration in the solution of the cell isolation if necessary. The cells should be counted and stained for differentiation, and gut preparations observed microscopically. Specific staining of bacteria and mucus should also be performed;

for comparisons, peroxides should be measured in freshly prepared and stored cell lipid samples. The biological relevance of small lipid peroxide values, such as those reported in this study, should be investigated;

effects of various fat diets (fish oil or vegetable oil) with controlled vitamin E levels (and perhaps other antioxidants) on both membrane lipid composition and peroxidation in these cell types should be looked at. Site variations, i.e. along the large bowel - proximal vs. distal portion - and within cells - microsomes, brush-border and basolateral membranes - should also be considered;

biological reasons for the lower phosphatidylcholine than phosphatidylethanolamine in microsomes compared to plasma membranes as well as pathways of synthesis (membrane inter-relationships) should be investigated.

Some experiments related to colon cancer could also be considered. For instance, changes in premalignant brush-border membrane, but not

basolateral membrane, by 1,2-DMH-induced colon cancer in the rat have been reported (10-12). Of interest would be to determine what are the premalignant transformations in both "non-proliferative" and "proliferative" mucosal cells as well as in different types of membranes within a cell population. The effects of dietary fat on this model should be investigated. One should also pay attention to diseases of the large intestine that are influenced by dietary fat intake and likely to be related to deleterious effects of lipid peroxides and free radicals. Finally, another aspect that could be considered is the effects of diet treatment on the mucus composition, on the effective resistance of the unstirred water layer, and on the mucosal cell cohesion in the large intestine.

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I. APPENDICES

APPENDIX 1: Vitamin E Content of Safflower Oil and Supplemented Safflower Oil as Determined by HPLC

To keep similar vitamin E intake per caloric basis between each group, diets have been adjusted for vitamin E content. Safflower oil, after determining its vitamin E concentration, was supplemented with d-alpha-tocopheryl acetate to give a stock solution of 10 IU/g of oil. Using this solution, the vitamin E levels of the four diets were equalized to the vitamin E content of the high fat, high polyunsaturated to saturated fatty acid ratio diet which had the highest vitamin E content without supplement. Alpha-tocopherol and d-alpha-tocopheryl acetate were determined by HPLC as described in the material and methods. Results were as follows:

Source	alpha-tocopherol (IU/100 g of oil)	d-alpha-tocopheryl acetate (IU/g of oil)
Safflower oil	52.88 + 0.73[1]	
Supplemented Safflower Oil		
Theoretical		10
Practical		10.50 + 0.01[2]

[1]Value is mean + SD of 2 replicates injected 3 times

[2]Value is mean + SD of 2 replicates

APPENDIX 2: HPLC separation of rat serum alpha-tocopherol

Abbreviations: A-TOCO.AC., alpha-tocopheryl acetate (internal standard); A-TOCO., alpha-tocopherol.