DIETARY CALCIUM AS A POSSIBLE ANTI-PROMOTER OF COLON CARCINOGENESIS

Promotor: Dr J.H. Koeman, hoogleraar in de Toxicologie

Co-promotor: Dr R. van der Meer, hoofd Afdeling Voedingsfysiologie, Nederlands Instituut voor Zuivelonderzoek (NIZO), Ede

John Arthur Lapré

DIETARY CALCIUM AS A POSSIBLE ANTI-PROMOTER OF COLON CARCINOGENESIS

Proefschrift
ter verkrijging van de graad van doctor
in de landbouw- en milieuwetenschappen
op gezag van de rector magnificus,
dr H.C. van der Plas,
in het openbaar te verdedigen
op dinsdag 2 juni 1992
des namiddags te vier uur in de Aula
van de Landbouwuniversiteit te Wageningen.

ISN: 558279

'Science should be made as simple as possible, but not simpler'

(A. Einstein)

Aan mijn ouders

Stellingen

1. Suppletie van de voeding met calcium kan het risico voor colonkanker verlagen door luminale binding van cytotoxische galzouten en vetzuren.
   Dit proefschrift

2. Galzouten en vetzuren in concentraties onder hun individuele kritisch micellaire concentratie zijn samen in staat om gemengde micellen te vormen die sterk cytotoxisch zijn.
   Dit proefschrift

3. Niet de totale, maar slechts de oplosbare fractie galzouten en vetzuren is bepalend voor de cytotoxiciteit van de darminhoud, darmepitheelbeschadiging en proliferatie van darmepitheelcellen.
   Dit proefschrift

4. De conclusie van Appleton *et al.* dat galzouten in de darm niet binden aan calcium omdat de galzoutexcretie niet wordt gestimuleerd, is onjuist.
   Appleton GVN, Owen RW, Wheeler EE, Challacombe DN, Williamson RCN. *Gut* 1991;32:1374-1377
   Dit proefschrift

5. Het colon is niet geschikt om vet te bevatten.

6. Onderzoek naar de promotie van darmkanker door suikerpolyesters is van groot belang voor de beoordeling van de toelating van deze vetvervangers als voedingsbestanddeel.

7. Het weergeven van celproliferatie in mucosale 'scrapings' van de darm als quotiënt dpm $^{3}H/\mu g$ DNA is alleen relevant wanneer slechts de hoeveelheid dpm ^{3}H de variërende factor is en de hoeveelheid DNA per 'scraping' niet verschilt tussen de experimentele groepen.

8. Intrarectale instillatie van calcium, fosfaat en lipiden bij proefdieren om de in vivo interactie tussen deze stoffen te onderzoeken brengt ons ver van de werkelijkheid en heeft geen fysiologische relevantie.
   Hu PJ, Baer AR, Wargovich MJ. *Nutr Res* 1989;9:545-553

9. Het dioxinerisico voor biologische mengsels van dioxines en PCB's zoals aangetroffen in voedingsmiddelen wordt voornamelijk bepaald door co-planaire polychloorbifenylen (PCB's) en niet door dioxines. Derhalve dienen de toxische effecten van PCB's een belangrijk aandachtsgebied voor onderzoek te vormen.
Liem A.K.D. et al., RIVM-rapport 730501.034, 1991

10. Gezien de centrale rol die aan stikstofmonooxyde (NO) wordt toegekend in vele biologische processen, is het verbazingwekkend dat deze stof pas in 1987 werd geïdentificeerd.
Moncada S, Palmer RMJ, Higgs EA. *Pharm Rev* 1991;43:101-142

11. De recente hypothese dat peroxydatie van LDL de eerste stap is in het ontstaan van een atherosclerotische plaque, kan 'radicale' gevolgen hebben voor het denken over atherosclerose.

12. Om de toxische effecten van retinol en retinylesters bij gebruik van vitaminepreparaten te vermijden, verdient het aanbeveling retinol en retinylesters te vervangen door het minder toxische ß-caroteen.

13. Omdat het N-terminale RNA-bindende deel van het manteleiwit van Cowpea Chlorotic Mottle Virus zich in evenwicht kan bevinden tussen een random-coil en een α-helixconformatie, is het vrijwel onmogelijk om de structuur van dit peptide met behulp van twee-dimensionale kernspinresonantie te bepalen.
Marinette ván der Graaf. Proefschrift Landbouwuniversiteit Wageningen, te verdedigen op dinsdag 2 juni 1992

14. Het verloop van de reorganisaties in de zuivelbedrijfstak is niet in overeenstemming met de slagvaardigheid die van 'Joris Driepinter' verwacht mag worden.

15. Een beoefenaar van aikido denkt in cirkels, een wetenschapper denkt rechtlijnig; toch sluit het één het ander niet uit.

Stellingen behorend bij het proefschrift: 'Dietary calcium as a possible anti-promoter of colon carcinogenesis.' John Lapré, Wageningen 2 juni 1992.

VOORWOORD

Zoals bekend is een proefschrift meestal niet een solo van degene die op de omslag staat, maar is het eerder het resultaat van een symfonie-orkest. Evenals bij een dergelijk orkest zijn bij het tot stand komen van dit proefschrift vele verschillende 'instrumenten' betrokken geweest. Enkele van de belangrijkste mensen wil ik hier met name noemen, maar dat wil niet zeggen dat de anderen niet belangrijk waren.

Jan Koeman wil ik bedanken voor het feit dat hij bereid is geweest als promotor op te treden ondanks het feit dat het onderzoek zich op het schemergebied tussen voeding en toxicologie heeft gericht.

Roelof, zonder jouw flitsende ideeën zou er veel minder een allegro in dit proefschrift zijn ontstaan. Jouw nimmer aflatende enthousiasme voor onderzoek en onze frequente duetten over het werk hebben mij zeer gestimuleerd. Eens hoop ik hetzelfde vermogen te krijgen om in onderzoek onmiddellijk de vinger op de minder zuivere noten te leggen. Ik hoop dat we in de toekomst op dezelfde, uiterst genoeglijke, wijze kunnen blijven samenwerken.

Hielke, het bereiden van voeders op het Centrum Kleine Proefdieren en onze daaropvolgende lunches bij 'Le Pistolet' vormden altijd een uitstekend intro op de dierproeven. Zonder jouw 'gouden handjes' tijdens de faecale analyses zouden de proeven ongetwijfeld minder geslaagd zijn geweest. Ik hoop voor jou dat FC Groningen nog eens kampioen wordt!

Ook Denise en Mirjam die het colonkankergroepje completeren, en de anderen van de afdeling Voedingsfysiologie wil ik bedanken voor hun inbreng en kritische noten. Daarnaast was en is het natuurlijk ook gewoon gezellig op de afdeling. Ik hoop dat de diverse stagiaires van de HLO en LUW op de afdeling een leuke tijd hebben gehad en veel hebben opgestoken; ik heb in ieder geval wel veel plezier van/met jullie gehad en veel van jullie geleerd. Joop, Simon, Henk en Jacques ben ik erkentelijk voor het feit dat er altijd tijd vrij kon worden gemaakt als ik weer eens op het laatste moment figuren en dia's nodig had.

De bijeenkomsten met de 'Groningers' hebben er zeker toe bijgedragen dat ik de verschillende invalshoeken (zoals de klinische kant) van het darmkankeronderzoek begon in te zien. Bert Groen van het AMC wil ik bedanken voor de snelle improvisatie m.b.t. de CaCo-2 proeven. Natuurlijk ben ik de medewerkers van het C.K.P. en met name Jo, Gerrit en Maria zeer erkentelijk voor de uitstekende wijze waarop de dierproeven zijn uitgevoerd.

Marinette, het feit dat we tegelijk bezig waren met ons proefschrift had als voordeel dat we het beiden druk hadden. Gelukkig staan vanaf nu 'de proefschriften' niet meer centraal in onze weekeinden!

CONTENTS

The studies reported in this thesis were carried out at the Department of Nutrition, Netherlands Institute for Dairy Research (NIZO), Ede, The Netherlands.
Two research grants from the European Community are gratefully acknowledged (Grants 663/88-1.7 and 1001/90-32.1).

CHAPTER 1 INTRODUCTION

Partly based on:
- R. Van der Meer, J.A. Lapré. *IDF-bulletin* 1991;255:55-59
- R. Van der Meer, J.H. Kleibeuker, J.A. Lapré. *Eur J Cancer Prev* 1991;1(suppl.2):55-62

General introduction

The work described in this thesis has been carried out at the Netherlands Institute for Dairy Research (NIZO) as a part of the project 'Calcium and colon cancer'. The possible protective effects of calcium with regard to colon cancer are important for the dairy industry because about 70% of the calcium-intake in the Netherlands is derived from dairy products. Therefore, the relationship between calcium and colon cancer is an important research topic at the department of Nutrition of NIZO.

This introducing chapter provides an overview which highlights the role of dietary fat and the interactions with two modulating factors, fibre and calcium, in the etiology of colon cancer. Fibre has extensively been studied as a promising modulating factor of colon carcinogenesis. The role of calcium has been less well studied (since approximately a decade) as a protective factor of colon carcinogenesis and its mechanism of action and relative importance are not clear. It should be mentioned that this introduction does not pretend to cover and summarize all the research in the field of colorectal cancer, but it provides the necessary background of the hypothesis how calcium could decrease the risk of colon cancer.

Epidemiology of colorectal cancer

In Western societies, colorectal cancer is the second most common malignancy in both males and females (IARC, 1982). The high incidence of colorectal cancer in the general population is strongly related to age (Bresalier *et al.*, 1988): about 90% of these cancers occur in patients 50 years of age or older. Migration studies have repeatedly shown that people migrating from low-risk countries to high-risk countries acquire the increased risk of the host country within decades (Haenszel & Kurihara, 1968; Ziegler *et al.*, 1986). This indicates that environmental factors play an important role in the incidence of colon cancer. It is generally assumed that the occurrence of colon cancer is associated with affluence, of which diet seems to be the most important one (Doll and Peto, 1981; Willett, 1989). Several types of epidemiological studies showed positive correlations with the intake of dietary fat (e.g. Jain *et al.*, 1980; Miller *et al.*, 1983; Willett *et al.*, 1990) and negative associations with the intake of cereals and vegetables (e.g. Bjelke, 1974; Phillips, 1975; Tuyns *et al.*, 1987). In multivariate analyses the strongest associations were found with the intake of saturated fat. Negative correlations were found for calcium and the incidence of colorectal cancer (review: Sorenson, 1988; Garland *et al.*, 1989). These

epidemiological studies should be used as a basis for experimental research concerning the etiology and modulation of colon cancer. In the next paragraph the genetic and phenotypic alterations associated with colon cancer are briefly discussed.

Histological and genetical development of colorectal cancer

About 10% of the colon cancer cases are patients with a genetic predisposition for colon cancer such as hereditary non-polyposis colonic carcinoma (HNPCC or Lynch syndrome) and familial adenomatous polyposis (FAP). Histological examinations of (pre)neoplastic changes of the colonic mucosa resulted in the now generally accepted adenoma-carcinoma sequence for the development of colorectal cancer (Lipkin, 1974). This multistage development of colorectal cancer can be summarized as follows: first in normal mucosa, cellular replication becomes stimulated. Subsequently these hyperproliferative cells may accumulate and transform to adenomatous polyps. Eventually these adenomas transform to malignancy.

With the development of the molecular biology and the discovery of the (proto)oncogenes a giant step in the understanding of the genetic background of this adenoma-carcinoma sequence has been achieved. The research group of Vogelstein has developed an attractive model for the genetic alterations associated with the multi-stage development of colorectal cancer (Fearon *et al.*, 1987; Vogelstein *et al.*, 1988). The phenotypical changes described by Lipkin (1974) probably reflect mutations of oncogenes and tumor-suppressor genes as found by Vogelstein *et al.* (1988) (Figure 1). These histological and genetic changes in colonic epithelial cells must form the backbone of the research for the causes of colorectal cancer. The different phases in the development of colorectal cancer may offer several opportunities for intervention.

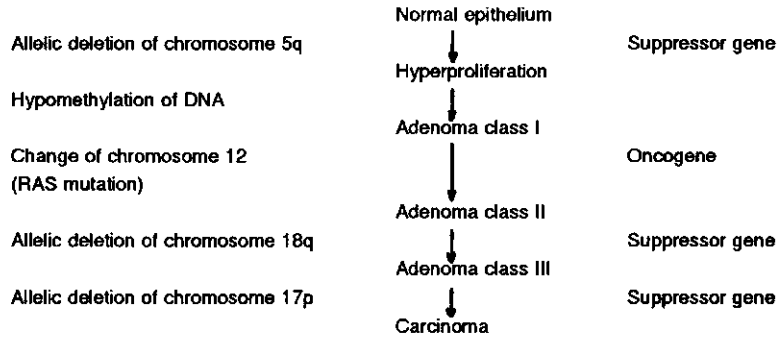


Figure 1 Genotypical and associated phenotypical changes according to Vogelstein *et al.* (1988)

The concept of environmental carcinogenesis

Present knowledge of environmental carcinogenesis is mainly based upon the induction of (pre)cancerous lesions by chemicals. The relationship between environmental factors and colon cancer may be largely explained by the influence of dietary components on carcinogenesis, because the role of other environmental factors seems to be of less importance. Therefore, the concept of chemical carcinogenesis is an important basis in the search for environmental etiology of colon cancer.

The mechanism of chemical carcinogenesis is essentially to be divided into two parts: initiation and promotion. Initiation consists of a genotypic alteration which results in a preneoplastic cell. Subsequently during the promotion phase a preneoplastic cell develops phenotypically in a malignant one. Initiating substances interact with DNA and result in mutations. Promoters may have a variety of targets (Boutwell, 1974). For instance, specific binding to cellular receptors or inhibition of intercellular communication have been reported as tumor promoting activities. One important mechanism of tumor promotion is stimulation of cell-proliferation (Preston-Martin *et al.*, 1990). An increased proliferation increases the growth of (pre)malignant cells thus stimulating the fixation of genotypic alterations. Proliferation may be induced by specific mitogenic stimuli or by cell-killing resulting in a compensatory hyperproliferation. Promotion is a process which in its early stages seems to be reversable (Verma & Boutwell, 1980). In addition, a promoter must be present during a prolonged period after initiation to lead to phenotypical changes associated with tumor formation. Tumor promotion is therefore directly and mechanistically linked to the pathogenesis of tumor formation. On the other hand, cocarcinogenicity is a process that creates those circumstances in which carcinogenesis may become stimulated. Cocarcinogens are not necessarily linked to the mechanism and pathogenesis of tumor formation. For instance, an induction of the hepatic cytochrome P450 system resulting in an increased progression of precarcinogens to ultimate carcinogens can be considered as a cocarcinogenic action. An increased cell-proliferation which makes cells more susceptible for mutagens can be considered as a cocarcinogenic event, but proliferation of initiated cells as a tumor promoting activity. The promotion phase may be an important target for modulation of colon carcinogenesis. For instance, diet may be related to colon cancer mainly by influencing tumor promotion in the large intestine (Weisburger & Wynder, 1987).

Figure 2 summarizes some hypothetical pathways for the relationship between diet and colon carcinogenesis by a crude subdivision in initiation and promotion associated relationships.

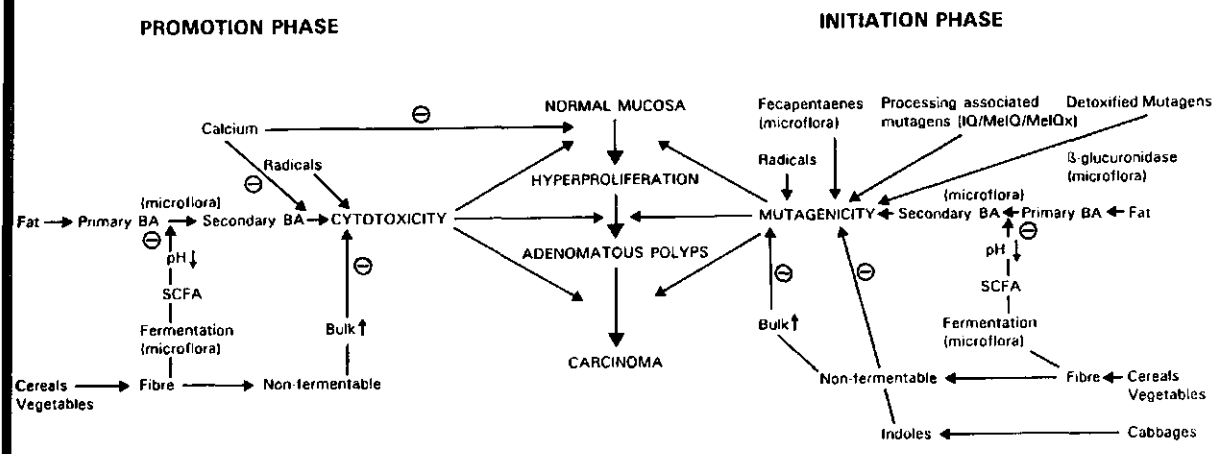


Figure 2 Hypothetical pathways for the relationship between diet and colon cancer which are currently under investigation. SCFA: short-chain fatty acids; BA: bile acids; (Me)IQ: (methyl) imidazoquinolines; MeIQx: methyl imidazoquinoxalines.

Dietary fat and bile acids

Because of the relationship between dietary fat and the incidence of colon cancer observed in epidemiological studies, most research has focussed on the possible role of dietary fat in colon carcinogenesis. Therefore, much attention has been paid to bile acids and their enterohepatic circulation. Bile acids are surface-active compounds which are synthesized in the liver from cholesterol. Because of their surface-active properties bile acids solubilize lipids in the small intestine and are thus essential to the digestion and absorption of dietary fat. More than 90% of the bile acids are reabsorbed in the terminal ileum and transported to the liver. This process is the so-called enterohepatic circulation (EHC) of bile acids. However, this EHC is not a completely closed loop (Van der Meer, 1983), approximately 10% of the bile acids

escape reabsorption and are metabolized by the colonic flora. The primary bile acids cholate (C) and chenodeoxycholate (CDC) conjugated with either glycine or taurine, are deconjugated and dehydroxylated (7α-dehydroxylation) and thus form the secondary bile acids deoxycholate (DC) (from cholate) and lithocholate (LC) (from chenodeoxycholate) (Figure 3). Subsequently, the bile acids are excreted in the feces.

It has been reported that groups at high risk for colon cancer have higher concentrations of bile acids in their feces compared to controls (Reddy & Wynder, 1977; Hill *et al.*, 1987). However, other investigators did not find a significant association between fecal bile acid concentration and the incidence of colorectal cancer (Breuer & Goebell, 1985). Several studies have shown that a high-fat diet increases the output and fecal concentration of bile acids compared to a low-fat diet (Cummings *et al.*, 1978; Reddy, 1981). In animal experiments using tumor-induction models dietary fat increased the incidence of colorectal tumors (Nigro *et al.*, 1975; Reddy, 1975, 1981; Bird & Bruce, 1986) and the excretion of bile acids in a dose-dependent way (Reddy, 1975, 1981). These data indicate that a high-fat intake, bile acids and colon cancer may be causally related.

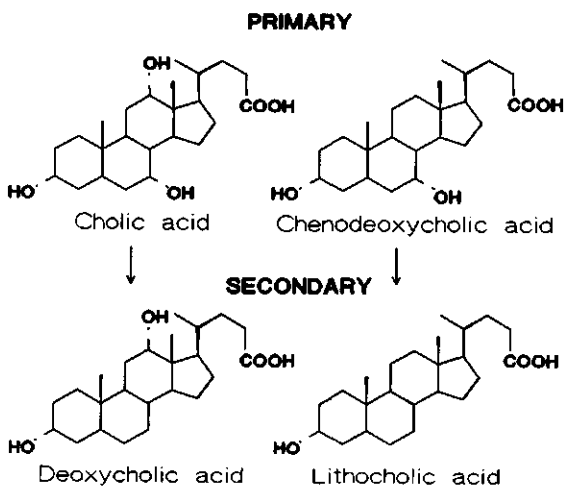


Figure 3 Metabolism of primary bile acids to secondary colonic bile acids.

The role of bile acids and fatty acids in colon carcinogenesis

The hypothesis that the possible causal relationship between dietary fat and colon carcinogenesis is mediated by the bile acid metabolism has led to numerous experimental studies in animal models.

Using tumor-inducers like dimethylhydrazine or N-methyl-N'-nitro-N-Nitrosoguanidine it has been shown in rats that intrarectal instillation of the secondary, colonic bile acids deoxycholate and lithocholate resulted in tumor-promotion (Narisawa *et al.*, 1974). These experiments have later been confirmed by a number of groups (e.g. Reddy *et al.*, 1977; McSherry *et al.*, 1989). Reddy *et al.* (1977) showed that the promoting effect of bile acids was much greater in conventional than in germ-free animals suggesting an additional role of the gut microflora, e.g. by converting primary bile acids to their secondary counterparts. The fecal stream seems to play an important role in the promotion of colonic carcinogenesis as demonstrated by Campbell *et al.* (1975) and Rainey *et al.* (1983).

Whether bile acids and fatty acids act specifically as tumor promoters or as cocarcinogens is not clear. Comutagenicity of secondary bile acids to known mutagens like 2-amino-anthracene in the Ames mutagenicity test (Wilpart & Roberfroid, 1986) and in a transformation assay (Kelsey & Pienta, 1979) has been reported. The relative importance of these observations is at present not clear. One important mechanism of tumor promotion and of cocarcinogenicity is an increased cell-proliferation. Measurements of colonic proliferation in rats and mice showed that bile acids and fatty acids instilled intrarectally or supplemented in diet increase proliferation rates (Deschner *et al.*, 1981; Bull *et al.*, 1983; Wargovich *et al.*, 1983, 1984; Bird *et al.*, 1986). In humans, diversion of the fecal stream caused regression of rectal polyps (Cole & Holden, 1959). Dietary fat given as a bolus to humans induces hyperproliferation of colonic epithelium (Stadler *et al.*, 1988). These studies support the hypothesis that bile acids and fatty acids may act as promoters of colon carcinogenesis or as cocarcinogens by inducing hyperproliferation of the colonic epithelium. The mechanism of this bile acid-induced hyperproliferation is not clear. Some studies indicate the involvement of reactive oxygen and protein kinase C in the induction of hyperproliferation (DeRubertis & Craven, 1984; Craven *et al.*, 1987) suggesting a direct tumor-promoting effect. Others stress the possible cell-damaging effects of the surface-active bile acids/fatty acids (Newmark *et al.*, 1984). Bile acids are toxic to different types of cells (Coleman *et al.*, 1979, Van der Meer *et al.*, 1991,

Velardi *et al.*, 1991). In this context it should be noted that as early as 1964, Fry and Staffeldt observed that a high dose of deoxycholate (2%) in diets of mice induced cell-necrosis in the jejenum and ileum. The cytotoxic effects followed by the compensatory cell-proliferation can be considered as a promotive as well as a cocarcinogenic effect. This is dependent on the time of action: An increased proliferation of normal cells resulting in a higher susceptibility to mutagens is cocarcinogenic. Proliferation of initiated cells is a tumor-promoting activity. Whether one or both mechanisms exist is not clear.

In conclusion, present knowledge shows that bile acids and, less extensively studied, fatty acids may act as promoters of colon carcinogenesis or as cocarcinogens by inducing hyperproliferation of the colonic epithelium. However, the mechanism of this process and consequently the possible chances for dietary intervention are not quite clear. In the next paragraphs two modulators of the promotive effect of fat on colon carcinogenesis are briefly discussed.

Fat, fibre and colon cancer

Dietary fibre is an important modulator of colonic carcinogenesis and has been studied since decades (e.g. Burkitt, 1971). The protective effect of fibre has been observed in most, but not all, observational and case-control studies as recently reported in a meta-analysis by Trock *et al.* (1990). The negative relationship between the intake of dietary fibre and colon cancer has invoked many experimental studies. Most of these studies do observe protective effects of fibre on colon carcinogenesis, but controversial results have been reported (see for review: Boutron *et al.*, 1991). One of the main methodological problems is that dietary fibre is not a homogeneous entity, but may contain different components with different properties. These components can be divided into several groups: fermentable and non-fermentable fibre or a subdivision into soluble and insoluble fibre (Topping, 1991). It can easily be understood that the mechanism of action of a fermentable fibre is different from that of a non-fermentable fibre (see also Figure 2 of this chapter). The differential modes of actions may also implicate that fibres may be effective in different phases of carcinogenesis. For instance, cellulose might be effective only in the initiating phase

whereas wheat bran is protective in the promotion phase (Wilpart, 1987). The overall effect of dietary fibre is commonly considered to be a protective one.

Possible mechanisms of the protective effects of fibre are: (1) increase of the bulk thereby diluting possible carcinogens and binding of bile acids (pectin and lignin); (2) acceleration of gut transit time thereby decreasing the contact time between the intestinal contents and the colonic epithelium, (3) fermentation of fibre components yielding short chain fatty acids which have specific effects on the colonic epithelium and which lower colonic pH. This lowering of colonic pH may inhibit the enzyme 7α-dehydroxylase which catalyzes the formation of cytotoxic secondary bile acids from the less toxic primary bile acids. (4) Special interest has been focussed on butyrate as a product of bacterial fermentation, because butyrate represents an important fuel for colonocytes and may act as an inducer of cell-differentiation.

Dietary fibre can thus be considered as an important protective factor in the etiology of colon cancer. The experimental studies reported suggest, but do not explain, the mechanism by which dietary fibre may lower the risk of colon cancer.

Fat, calcium and colon cancer

The relationship between calcium and colon cancer has been studied less extensively. In 1984 an attractive hypothesis by Newmark *et al.* was published which discussed the possible interactions between calcium and bile acids/fatty acids with regard to colon cancer. They suggested that ionic calcium could form insoluble salts with bile acids and fatty acids in the colonic lumen. In this way, calcium would inhibit the hyperproliferative effects of bile acids and fatty acids. This hypothesis has evoked many experimental studies using in vitro models, animal models and humans.

In vitro studies

Calcium may act as mediator in many cellular processes including cell-proliferation (Whitfield, 1991). Calcium may thus act directly on the colonic epithelium by influencing for instance protein-kinase C activity which is a known modulator of cell-proliferation (Hickie *et al.*, 1983). On the other hand, calcium can also act indirectly by binding bile acids and fatty acids and thus protecting the colonic epithelium from their hyperproliferative effects as hypothesized by Newmark *et al.* (1984).

Buset *et al.* (1986) found that in patients at risk for colon cancer calcium inhibited proliferation of human colonic epithelial cells in vitro. Similar results were obtained by Lipkin *et al.* (1989) using in vitro exposure of human colonic epithelial cells to increasing amounts of calcium. Growth inhibition was observed in human and mouse cancer cell lines at high extracellular calcium concentrations (Guo *et al.*, 1990). Van der Meer and De Vries (1985) demonstrated that glycine-conjugated bile acids bind to insoluble calcium phosphate. This is in contrast with the hypothesis of Newmark *et al.* (1984) that ionic calcium is responsible for the binding of bile acids and fatty acids in colon. Because in colon more than 95% of the bile acid pool is composed of the secondary bile acids deoxycholate and lithocholate, Van der Meer *et al.* (1991) studied binding of these bile acids to ionic calcium and insoluble calcium phosphate. Using lysis of erythrocytes as a model system they found that calcium phosphate but not ionic calcium precipitated these bile acids and inhibited cytotoxicity.

Animal studies

The protective effects of calcium on (pre)malignant changes in the colon have predominantly been studied in rats and mice. Several, but not all tumor-induction studies showed that in rats the promotive effect of a high-fat diet on the formation of colonic tumors could be abolished by dietary calcium supplementation (Appleton *et al.*, 1987; Pence & Buddingh, 1988; McSherry *et al.*, 1989; Behling *et al.*, 1990; Wargovich *et al.*, 1990).

Other studies used intrarectal instillation or perfusion with bile acids and fatty acids to induce hyperproliferation of colonic cells (Bull *et al.*, 1987; DeRubertis & Craven, 1984; Wargovich *et al.*, 1983,1984; Hu *et al.*, 1989). When different calcium sources were added simultaneously, the effects of bile acids and fatty acids on colonic proliferation were drastically decreased (Wargovich *et al.*, 1983, 1984; Hu *et al.*, 1989). Rafter *et al.* (1986) indicated that deoxycholate bound to calcium did not damage the colonic epithelium when perfused through the colon. Despite the protective effects of calcium found in these studies, it should be noted that their artificial design precludes the intestinal interaction between calcium, phosphate and bile acids/fatty acids and thus limits the physiological relevance of these studies. When calcium (as calcium phosphate) was provided in diet combined with bile acids/fatty acids or a fat bolus, colonic proliferation was drastically decreased compared to the groups without calcium

supplementation (Bird *et al.* 1986; Bird, 1986; Skraastad & Reichelt, 1988; Caderni *et al.*, 1988). In none of these studies fecal parameters have been studied thus providing no additional information on the mechanism by which dietary calcium inhibits colonic proliferation and tumor formation.

Human intervention studies

Most intervention trials focus on modulation of colonic epithelial proliferation as marker of the risk for colon cancer. This is based on the assumption that the effects of calcium on colon cancer are mediated by modulation of the cell-kinetic behavior of the colonic epithelium. Subjects at high risk for colon cancer are characterized by an increased colonic epithelial proliferation (Lipkin *et al.*, 1983; Terpstra *et al.*, 1987; Ponz de Leon *et al.*, 1988; Biasco *et al.*, 1990). Because proliferation is measured in biopsies, most intervention trials have been done in people at high risk for colon cancer such as patients previously treated for colon cancer. Several studies have found a protective effect of dietary calcium on proliferative indices in the colon (Lipkin & Newmark, 1985; Lipkin *et al.*, 1989; Rozen *et al.*, 1989). However, Bruce and coworkers could not demonstrate a decrease in proliferation by supplemental calcium in placebo-controlled studies using subjects with partial or subtotal colectomy (Gregoire *et al.*, 1989; Stern *et al.*, 1990). Considering the hypothesis with the suggested role of bile acids, it should be noted that these operations may alter bile acid metabolism (Cats *et al.*, 1991) and thus prevent the protective effect of calcium. The results of these studies by Gregoire *et al.* and by Stern *et al.* do not indicate a decreased proliferation by a direct effect of calcium on the colonic mucosa.

In conclusion, the in vitro, animal and human experiments suggest that dietary calcium decreases one of the main risk factors for colon cancer. However, few studies have been conducted regarding a mechanism by which calcium reduces proliferation. Investigation of this mechanism is essential to extrapolate the results of animal studies to the humans and to explain results of intervention studies. Figure 4 shows a hypothetical mechanism of the intestinal interactions between calcium, phosphate, bile acids and fatty acids with regard to the development of colon cancer which was chosen as a starting point for the present studies. First, a high intake of dietary fat

increases the concentrations of soluble bile acids and fatty acids in colon. Because of their surface-active properties, these acids damage the colonic epithelium. Subsequently, replication of epithelial cells becomes stimulated and may transform to premalignant lesions. Eventually these lesions may progress to carcinomas. Dietary calcium complexates with inorganic phosphate in the intestine (Fordtran & Locklear, 1966; Holdsworth, 1975; Spencer *et al.*, 1984; Van der Meer *et al.*, 1990). Soluble bile acids and fatty acids are bound to insoluble calcium phosphate and this binding inhibits their cytotoxicity. Thus, dietary calcium may inhibit proliferation of the colonic epithelium by binding luminal surfactants and inhibiting their lytic effects on the colonic epithelium.

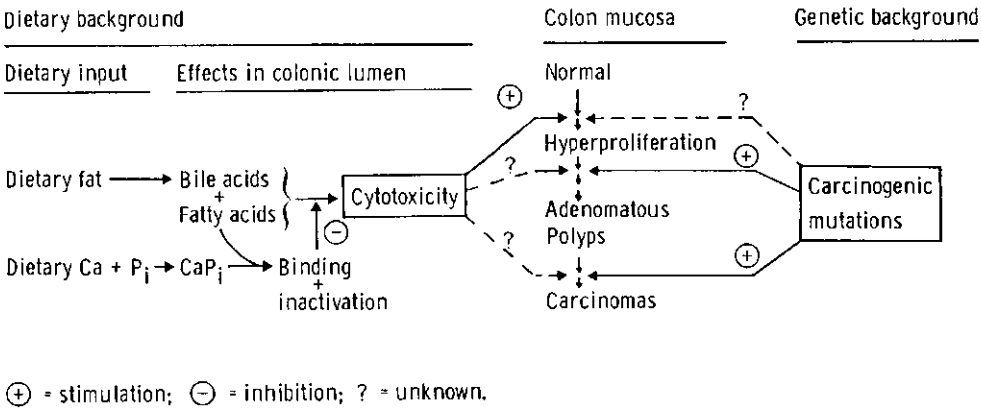


Figure 4 A hypothetical mechanism of the interactions between dietary fat and calcium with regard to the risk of colon cancer.

Scope of this thesis

As outlined above, in recent years evidence has been obtained about calcium as a promising protective factor in the etiology of colon cancer. However, present knowledge does not provide insight in the mechanism of this protective effect. At present, most of the studies with calcium focuss on the reduction of colonic proliferation in patients at high-risk for colon cancer. Therefore, the main emphasis in this thesis is laid upon the effects of calcium supplementation in the colonic lumen and the subsequent response of the colonic epithelium. To test the hypothetical

mechanism outlined in Figure 4, the following stepwise approach was used. First, in an in vitro study cytotoxicity of fatty acids and physiological mixtures of bile acids and fatty acids was investigated using a colonic epithelial cell-line (CaCo-2) and erythrocytes (chapter 3). Subsequently, it was tested whether a diet-dependent increase of soluble bile acids increased cytotoxicity of fecal water and colonic proliferation in rats (chapter 4). The next step consisted of dietary supplementation with $CaHPO_4$ to investigate whether the concentrations of soluble surfactants and cytotoxicity of fecal water were decreased by dietary calcium (chapter 5). Because bile acids and fatty acids are products of fat digestion, it was tested whether the type of dietary fat influences cytotoxicity of fecal water and the protective role of calcium phosphate (chapter 6). Subsequently, in chapter 7, the luminal effects of calcium supplementation are extended to effects on colonic epithelial proliferation using three different sources of human fat consumption. Finally, a pilot human intervention trial is described where the effects of dietary calcium on fecal water parameters and luminal cytotoxicity are compared with those observed in rats (chapter 8). Chapters 9 and 10 are the summary and concluding remarks in English and Dutch respectively.

REFERENCES

Cancer Incidence in Five Continents, vol. 4. *IARC Scientific Publications no. 42.* Lyon France 1982

Appleton GV, Davies PW, Bristol JB, Williamson RCN. Inhibition of intestinal carcinogenesis by dietary supplementation with calcium. *Br J Surgery* 1987;6:523-525

Behling AR, Kaup SM, Choquette LL, Gregor JL. Lipid absorption and intestinal tumour incidence in rats fed on varying levels of calcium and butterfat. *Br J Nutr* 1990;64:505-513

Biasco G, Paganelli GM, Miglioli M, Brillanti S, Di Febo G, Gizzi G, Ponz de Leon M, Campieri M, Barbara L. Rectal cell proliferation and colon cancer risk in ulcerative colitis. *Cancer Res* 1990;50:1156-1159

Bird RP. Effect of dietary components on the pathobiology of colon epithelium: possible relationship with colon tumorigenesis. *Lipids* 1986;21:289-291

Bird RP, Bruce WR. Effect of dietary fat levels on the susceptibility of colonic cells to nuclear-damaging agents. *Nutr Cancer* 1986;8:93-100

Bird RP, Schneider R, Stamp D, Bruce R. Effect of dietary calcium and cholic acid on the proliferative indices of murine colonic epithelium. *Carcinogenesis* 1986;7:1657-1661

Bjelke E. Epidemiological studies of cancer in stomach, colon and rectum; with special emphasis on the role of diet. *Scand J Gastroenterol* 1974; 9: Suppl 31:1-235.

Boutron MC, Wilpart M, Faivre J. Diet and colorectal cancer. *Eur J Cancer Prev* 1991;1(suppl.2):13-20

Boutwell RK. The function and mechanism of promoters of carcinogenesis. *CRC Critical Rev Tox*

1974;2:419-445

Bresalier RS, Itzkowitz SH, Kim YS. Colorectal cancer. In: Gitnick G. (Ed.): *Principles and practice of gastroenterology and hepatology.* 1988; Elsevier, New York: 497-523

Breuer N, Goebell H. The role of bile acids in colonic carcinogenesis. *Klin Wochenschr* 1985;63:97-105

Bull AW, Marnett LJ, Dawe EJ, Nigro ND. Stimulation of deoxythymidine incorporation in the colon of rats treated intrarectally with bile acids and fats. *Carcinogenesis* 1983;4:207-210

Burkitt DP. Epidemiology of cancer of the colon and rectum. *Cancer* 1971;28:3-13

Buset M. Lipkin M, Winawer S, Friedman E. Inhibition of human colonic epithelial cell proliferation in vivo and in vitro by calcium. *Cancer Res* 1986;46:5426-5430

Caderni G, Stuart EW, Bruce WR. Dietary factors affecting the proliferation of epithelial cells in the mouse colon. *Nutr Cancer* 1988;3:147-153

Campbell RL, Singh DV, Nigro ND. Importance of the faecal stream in the induction of colon tumours by azoxymethane in rats. *Cancer Res* 1975;35:1369-1371

Cats A, Kleibeuker JH, Kuipers F, Boersma W. Hardonk MJ, Verschueren RCJ, Grond J, Bouman JG, Vonk RJ, Mulder NH, De Vries EGE. Rectal epithelial proliferation and intestinal bile acids in familial adenomatous polyposis; effect of subtotal colectomy. *Gastroenterology* 1991;100:A353

Cole JW, Holden WD. Post-colectomy regression of adenomatous polyps in the human colon. *Arch Surg* 1959;79:385-392

Coleman R, Iqbal S, Godfrey PP, Billington D. Membranes and bile formation: Composition of several mammalian biles and their membrane-damaging properties. *Biochem J* 1979;178:201-208

Craven PA, Pfanstiel J, Saito R, DeRubertis FR. Role of activation of protein kinase C in the stimulation of colonic epithelial proliferation and reactive oxygen formation by bile acids. *J Clin Invest* 1987;79:532-541

Cummings JH, Wiggins HS, Jenkins DJA, Houston H, Jivray T, Drasar BS, Hill MJ. Influence of diets high and low in animal fat on bowel habit, gastrointestinal transit time, fecal microflora, bile acid, and fat excretion. *J Clin Invest* 1978;57:953-963

DeRubertis FR, Craven PA. Bile salt stimulation of colonic epithelial proliferation. *J Clin Invest* 1984;74:1614-1624

Deschner EE, Cohen BI, Raicht RF. Acute and chronic effect of dietary cholic acid on colonic epithelial cell proliferation. *Digestion* 1981;21:290-296

Doll R, Peto R. The causes of cancer: quantitative estimates of avoidable risks of cancer in the United States today. *J Natl Cancer Inst* 1981;66:1191-1308

Fearon ER, Hamilton SR, Vogelstein B. Clonal analysis of human colorectal tumors. *Science* 1987;238:193-197

Fordtran JS, Locklear TW. Ionic constituents and osmolality of gastric and small-intestinal fluids after eating. *Am J Dig Dis* 1966;11:503-521

Fry RJM, Staffeldt E. Effect of a diet containing sodium deoxycholate on the intestinal mucosa of the mouse. *Nature* 1964;203:1397-1398

Garland CF, Comstock GW, Garland FC, Helsing KJ, Shaw EK, Gorham ED. Serum 25-hydroxyvitamin D and colon cancer: eight-year prospective study. *Lancet* 1989;II:1176-1178

Gregoire RC, Stern HS, Yeung KS, Stadler J, Langley S, Furrer R, Bruce WR. Effect of calcium supplementation on mucosal proliferation in high risk patients for colon cancer. *Gut* 1989;30:376-382

Guo Y-S, Draviam E, Townsend CM Jr, Singh P. Differential effects of Ca^{2+} on proliferation of stomach, colonic, and pancreatic cancer cell lines in vitro. *Nutr Cancer* 1990;14:149-157

Haenszel W, Kurihara M. Mortality from cancer and other diseases among Japanes in the United States. *J Natl Cancer Inst* 1968;40:43-68

Hickie RA, Wei JW, Blyth LM, Wong D, Klaasen D. Cations and calmodulin in normal and neoplastic growth regulation. *Can J Biochem Cell Biol* 1983;61:934-941

Hill MJ, Lennard-Jones JE, Melville DM, Neale K, Ritchie JK. Faecal bile acids, dysplasia, and carcinoma in ulcerative colitis. *Lancet* 1987;ii:185-186

Holdsworth CD. In: *Intestinal absorption in man* (McGoll, I & Sladen, G.E., eds.). Academic Press New York 1975: 223-262

Hu PJ, Baer AR, Wargovich MJ. Calcium and phosphate: effect of two dietary confounders on colonic epithelial cellular proliferation. *Nutr Res* 1989;9:545-553

Jain M, Cook GM, Davis FG, Grace MG, Howe GR, Miller AB. A case-control study of diet and colorectal cancer. *Int J Cancer* 1980;26:757-768

Kelsey M, Pienta RJ. Transformation of hamster embryo cells by cholesterol epoxide and lithocholic acid. *Cancer Lett* 1979;19:322-327

Lipkin M. Phase 1 and phase 2 proliferative lesions of colonic epithelial cells in diseases leading to colon cancer. *Cancer Res* 1974;34:878-888

Lipkin M, Blattner WE, Fraumeni JF Jr, Lynch HT, Deschner E, Winawer S. Tritiated thymidine labelling distribution as a marker for hereditary predisposition to colon cancer. *Cancer Res* 1983;43:1899-1904

Lipkin M, Newmark H. Effect of added dietary calcium on colonic epithelial-cell proliferation in subjects at high risk for familial colonic cancer. *N Engl J Med* 1985; 313:1381-1384

Lipkin M, Friedman E, Winawer SJ, Newmark H. Colonic epithelial cell proliferation in responders and nonresponders to supplemental dietary calcium. *Cancer Res* 1989;49:248-254

McSherry CK, Cohen BI, Bokkenheuser VD, Mosbach EH, Winter J, Matoba N, Scholes J. Effects of calcium and bile acid feeding on colon tumors in the rat. *Cancer Res* 1989;49:6039-6043

Miller AB, Howe GR, Jain M, Craib KJP, Harrison L. Food items and food groups as risk factors in a case-control study of diet and colorectal cancer. *Int J Cancer* 1983;32:155-161

Narisawa T, Magadia NE, Weisburger JH, Wynder EL. Promoting effects of bile acids on colon carcinogenesis after intrarectal instillation of N-methyl-N'-nitro-N-Nitrosoguanidine in rats. *J Natl Cancer Inst* 1974;53:1093-1097

Newmark HL, Wargovich MJ, Bruce WR. Colon cancer and dietary fat, phosphate, and calcium: a hypothesis. *J Natl Cancer Inst* 1984;72:1323-1325

Nigro ND, Singh DV, Campbell RL, Pak MS. Effect of dietary beef fat on intestinal tumor formation by azoxymethane in rats. *J Natl Cancer Inst* 1975;54:439-442

Pence BC, Buddingh F. Inhibition of dietary fat promotion of colon carcinogenesis by supplemental

calcium and vitamin D. *Carcinogenesis* 1988;9:187-190

Phillips RL. Role of life style and dietary habits in risk of cancer among Seventh-Day Adventists. *Cancer Res* 1975;35:3513-3522

Ponz de Leon M, Roncucci L, Di Donato P, Tassi L, Smerieri O, Amorico MG, Malagoli G, De Maria D, Antonioli A, Chahin NJ, Perini M, Rigo G, Barberini G, Manenti A, Biasco G, Barbara L. Pattern of epithelial cell proliferation in colorectal mucosa of normal subjects and of patients with adenomatous polyps or cancer of the large bowel. *Cancer Res* 1988;48:4121-4126

Preston-Martin S, Pike MC, Ross RK, Jones PA, Henderson BE. Increased cell division as a cause of human cancer. *Cancer Res* 1990;50:7415-7421

Rafter JJ, Eng VWS, Furrer R, Medline A, Bruce WR. Effects of calcium and pH on the mucosal damage produced by deoxycholic acid in the rat colon. *Gut* 1986;27:1320-1329

Rainey JB, Davies PW, Bristol JB, Williamson RCN. Adaptation and carcinogenesis in defunctioned rat colon: divergent effect of faeces and bile acids. *Br J Cancer* 1983;48:477-484

Reddy BS. Role of bile metabolites in colon carcinogenesis. *Cancer* 1975;36:2401-2406

Reddy BS, Wynder EL. Metabolic epidemiology of colon cancer. Fecal bile acids in colon cancer patients and patients with adenomatous polyps. *Cancer* 1977;39:2533-2539

Reddy BS, Watanabe K, Weisburger JH, Wynder EL. Promoting effects of bile acids in colon carcinogenesis in germ free and conventional F344 rats. *Cancer Res* 1977;37:3238-3242

Reddy BS. Diet and excretion of bile acids. *Cancer Res* 1981;41:3766-3768

Rozen P, Fireman Z, Fine N, Wax Y, Ron E. Oral calcium suppresses increased rectal proliferation of persons at risk of colorectal cancer. *Gut* 1989;30:650-655

Skraastad O, Reichelt KL. An endogenous colon mitosis inhibitor and dietary calcium inhibit the increased colonic cell proliferation induced by cholic acid. *Scand J Gastroenterol* 1988;23:801-807

Sorenson AW, Slattery ML, Ford MH. Calcium and colon cancer: a review. *Nutr Cancer* 1988;11:135-145

Spencer H, Kramer L, Norris C, Osis D. Effect of calcium and phosphorous on zinc metabolism in man. *Am J Clin Nutr* 1984;40:1213-1218

Stern HS, Gregoire RC, Kashtan H, Stadler J, Bruce WR. Long-term effects of dietary calcium on risk markers for colon cancer in patients with familial polyposis. *Surgery* 1990;108:528-533

Stadler J, Stern HS, Yeung KS, Mcquire V, Furrer R, Marcon N, Bruce WR. Effect of high fat consumption on cell proliferation activity of colorectal mucosa and on soluble faecal bile acids. *Gut* 1988;29:1326-1331

Terpstra OT, Van Blankenstein M, Dees J, Eilers GA. Abnormal pattern of cell-proliferation in the entire colonic mucosa of patients with colon adenoma or cancer. *Gastroenterology* 1987;92:704-708

Topping DL. Soluble fiber polysaccharides: effects on plasma cholesterol and colonic fermentation. *Nutr Reviews* 1991;49:195-203

Trock B, Lanza E, Greenwald P. Dietary fiber, vegetables, and colon cancer: Critical review and meta-analyses of the epidemiologic evidence. *J Natl Cancer Inst* 1990;8:650-661

Tuyns AJ, Haelterman M, Kaaks R. Colorectal cancer and the intake of nutrients: oligosaccharides are a risk factor, fats are not. A case-control study in Belgium. *Nutr Cancer* 1987;10:185-196

Van der Meer R, De Vries HT. Differential binding of glycine- and taurine-conjugated bile acids to insoluble calcium phosphate. *Biochem J* 1985;229:265-268

Van der Meer R. Is the hypercholesterolemic effect of casein related to its phosphorylation state? *Atherosclerosis* 1983;49:339-341

Van der Meer R, Welberg JWM, Kuipers F, Kleibeuker JH, Mulder NH, Termont DSML, Vonk RJ, De Vries HT, De Vries EGE. Effects of supplemental dietary calcium on the intestinal association of calcium, phosphate and bile acids. *Gastroenterology* 1990;99:1653-1659

Van der Meer R, Termont DSML, De Vries HT. Differential effects of calcium ions and calcium phosphate on cytotoxicity of bile acids. *Am J Physiol* 1991;260:G142-G147

Velardi ALM, Groen AK, Oude Elferink RJP, Van der Meer R, Palasciano G, Tytgat GNJ. Cell-type dependent effect of phospholipid and cholesterol on bile salt cytotoxicity. *Gastroenterology* 1991;101:457-464

Verma AK, Boutwell RK. Effects of dose and duration of treatment with the tumor-promoting agent 12-O-tetradecanoyl-13-phorbol acetate on mouse-skin carcinogenesis. *Carcinogenesis* 1980;1:271-274

Vogelstein B, Fearon ER, Hamilton SR Genetic alterations during colorectal-tumor development. *N Eng J Med* 1988;319:525-532

Wargovich MJ, Eng VWS, Newmark HL, Bruce WR. Calcium ameliorates the toxic effect of deoxycholic acid on colonic epithelium. *Carcinogenesis* 1983;4:1205-1207

Wargovich MJ, Eng VWS, Newmark, Bruce WR. Calcium inhibits the damaging and compensatory proliferative effects of fatty acids on mouse colonic epithelium. *Cancer Lett* 1984;23:253-258

Wargovich MJ, Allnut D, Palmer C, Anaya P, Stephens LC. Inhibition of the promotional phase of azoxymethane-induced colon carcinogenesis in the F344 rat by calcium lactate: effect of simulating two human nutrient density levels. *Cancer Lett* 1990;53:17-25

Weisburger JH, Wynder EL. Etiology of colorectal cancer with emphasis on mechanism of action and prevention. In: DaVita VT, Hellman S, Rosenberg SA, eds. *Important advances in oncology 1987.* JB Lippincott, Philadelphia, 1987:197-220

Whitfield JF. Calcium switches, cell cycles, differentiation, and death. In: Lipkin M, Newmark HL, Kelloff G, eds. *Calcium, Vitamin D, and Prevention of colon cancer.* Boca Raton: CRC Press Inc. 1991:31-74

Willett WC. The search for the causes of breast and colon cancer. *Nature* 1989;338:389-394

Willett WC, Stampfer MJ, Colditz GA, Rosner BA, Speizer FE. Relation of meat, fat, and fiber intake to the risk of colon cancer in a prospective study among women. *N Eng J Med* 1990;323:1664-1672

Wilpart M. Dietary fat and fibre and experimental carcinogenesis: a critical review of published evidence. In: Faivre J, Hill MJ, eds. *Causation and Prevention of colorectal cancer.* Elsevier, Amsterdam, 1987:85-98

Wilpart M, Roberfroid M. Effects of secondary bile acids on the mutagenicity of MNNG, 2-AAF and 2-

nitrofluorene towards Salmonella typhimurium strains. *Carcinogenesis* 1986;7:703-706.

Ziegler RG, DeVesa SS, Fraumeni JF Jr. Epidemiologic patterns of colorectal cancer. In: DaVita VT, Hellman S, Rosenberg SA, eds. *Important advances in oncology 1987*. JB Lippincott, Philadelphia, 1987: 197-219

CHAPTER 2 DETERMINATION OF CYTOTOXICITY, EPITHELIOLYSIS AND COLONIC PROLIFERATION

Introduction

This chapter describes the main methods used in the studies reported in this thesis. First, preparation of fecal water is discussed, subsequently the methods to determine cytotoxicity of fecal water, intestinal epitheliolysis and colonic cell-proliferation are presented.

Preparation of fecal water of rats

Measurement of the cytotoxicity of the intestinal contents requires the use of fecal water. Fecal water is the fraction of feces which contains the water-soluble, not-bound components of the feces such as soluble bile acids and fatty acids which may determine the cytotoxicity of the intestinal contents.

In the animal experiments fecal water was prepared using freeze-dried feces reconstituted to 35% dry wt which mimics the dry weight of the colonic contents in rats. After mixing, the samples were incubated for one hour at 37°C in a shaking waterbath and subsequently centrifuged for 10 minutes at 15,000*g. No significant differences in pH, calcium, inorganic phosphate, bile acid and fatty acid concentrations were observed using longer incubation times (up to 120 min) or centrifugation times (up to 30 minutes at 15,000*g). Also the cytotoxicity of fecal water was not significantly influenced by varying these two parameters.

Fecal water was prepared from freeze-dried feces. Because freeze-drying may induce variable losses of volatile components like short-chain fatty acids and ammoniak, we checked if fecal water prepared from fresh rat feces differed significantly from fecal water prepared from freeze-dried rat feces. As shown in Table 1 significant differences were found only for pH. All other parameters were not significantly different between the two groups (two-sided Student's t-test for pairs).

Subsequently we tested the reproducibility of the preparation of fecal water. Fecal water was prepared five times from freeze-dried feces and analysed for pH, calcium, inorganic phosphate, bile acid and fatty acid concentration. The coefficients of variation were for pH 1%, Ca 3%, Pi 3%, BA 2%, FA 7%. These coefficients of variation were in an acceptable range and therefore in the experiments described in this thesis fecal water was prepared from freeze-dried feces using the procedure described above.

Table 1 Comparison between fecal water prepared from freeze-dried feces and from fresh feces of rats.

	Feces		
	fresh	freeze-dried	Difference
pH	7.6 ± 0.10	7.1 ± 0.04	0.5 ± 0.09*
Ca (mM)	3.9 ± 2.6	3.8 ± 1.5	0.1 ± 2.2
P_i (mM)	10.2 ± 4.5	9.3 ± 2.5	0.9 ± 2.2
BA (mM)	2.9 ± 0.8	2.7 ± 0.8	0.2 ± 0.1
FA (mM)	2.7 ± 0.4	2.4 ± 0.4	0.3 ± 0.2
Cytotoxicity (%)	61.0 ±13	59.0 ±15	2.3 ± 7.7

Values are means of 4 with their standard errors. * : $P<0.05$

Cytotoxicity of fecal water

Cytotoxicity of fecal water was determined as lysis of human erythrocytes according to the procedures described by Coleman *et al.* (1979) and by Rafter *et al.* (1987). Coleman *et al.* used pure bile acids to induce lysis of erythrocytes and quantitated hemolysis by measuring the absorbance of hemoglobin at 540 nm in the supernatants after centrifugation. Rafter *et al.* used fecal water to induce lysis of erythrocytes and quantitated hemolysis by counting the remainder of intact erythrocytes. This is probably done because fecal pigments interfere with the spectrophotometric measurement of hemoglobin. As an alternative method we considered measuring Fe-release by lysed cells using atomic absorption spectrophotometry (AAS). We compared both the cell-count method and the AAS method with the standard spectrophotometric measurement of hemolysis induced by glycodeoxycholate (Figure 1). It should be noted that lysis was calculated relative to samples which were hemolyzed in double-distilled water with 2.5 mM deoxycholate (100% lysis samples) to prevent formation of ghost cells.

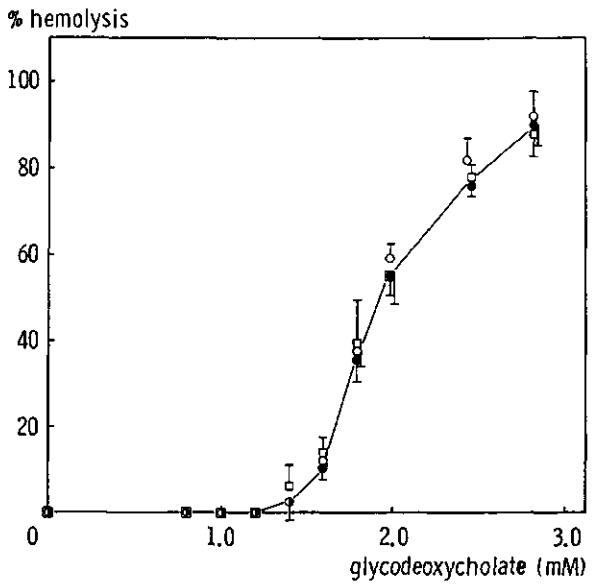


Figure 1 Concentration dependence of the hemolytic effect of glycodeoxycholate measured by spectrophotometry at 540 nm (O), by Fe-release determined by atomic absorption spectrophotometry (●) and by cell-count (□). The incubation mixture contained HEPES (final concentration 50 mM and pH 7.4), NaCl (to maintain a constant ionic strength of 150 mM), increasing concentrations of glycodeoxycholate, and human erythrocytes at a final hematocrit of 5%. Values shown are means of three experiments with their standard errors.

Both methods showed a pattern of hemolysis similar to that obtained with the standard spectrophotometric method. Subsequently, we compared the cell-count and Fe-release method using fecal water to induce lysis. As shown in Figure 2A, the cell-count method showed a hemolysis pattern which completely deviates from the normal concentration-dependent hemolysis by pure bile acids. In contrast, the Fe-release method showed a hemolysis pattern with a concentration-dependence comparable to hemolysis induced by pure bile acids (Figure 2B). This suggests that the cell-count method is liable to artifacts.

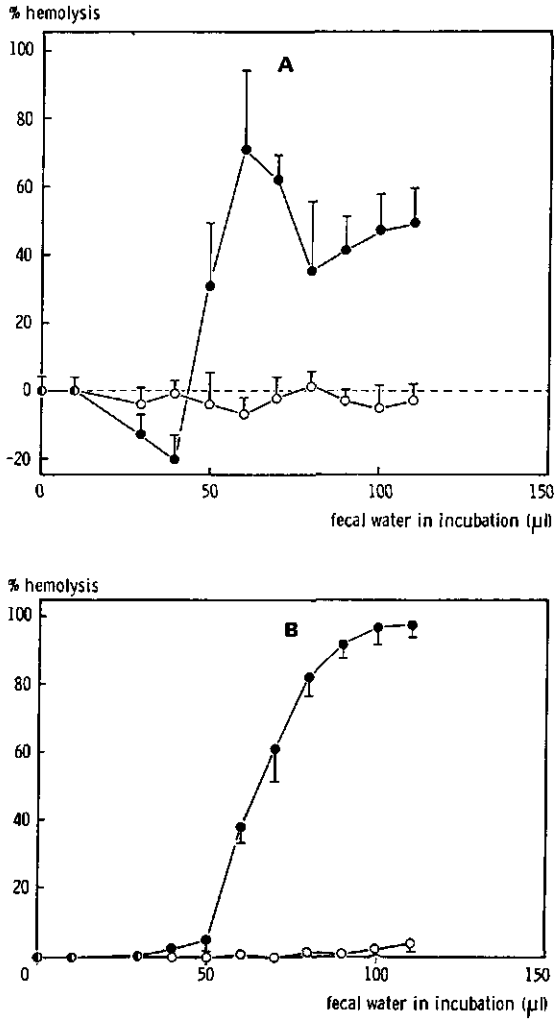


Figure 2 Concentration dependence of the hemolytic activity of rat fecal water measured by cell-count (Figure 2A) and by Fe-release determined by Atomic Absorption Spectrophotometry (Figure 2B). The incubation mixture contained increasing amounts of fecal water, 154 mM NaCl and erythrocytes at a final hematocrit of 5% in a total volume of 200 µl. Values shown are means of three experiments with their standard errors. O: fecal water from rats fed a control diet

●: fecal water from rats fed a steroid-supplemented diet

As shown in Figure 3A, counting of intact erythrocytes resulted in a linear relationship with the precentage hematocrit in the incubation. However, lysed erythrocytes in double-distilled water also produced significant counts. When lysed erythrocytes were added to fecal water less counts were detected, whereas addition to 2.5 mM deoxycholate produced the lowest counts. The particles counted are probably hemoglobin-free ghosts which are known to be formed under such conditions (Dodge *et al.*, 1963). In contrast to the cell-count method, measurement of Fe-release using the same samples showed a linear relation between Fe-release and percent hematocrit with lysed erythrocytes in double-distilled water, in fecal water and in 2.5 mM deoxycholate. No Fe-release was detected with intact erythrocytes (Figure 3B). These results showed that Fe-release is a reliable measure to determine cytotoxic effects of fecal water against erythrocytes. The variation coefficient (SD/Mean * 100%) for determining cytotoxicity by fecal water of rats varied from 4 to 7% with different samples.

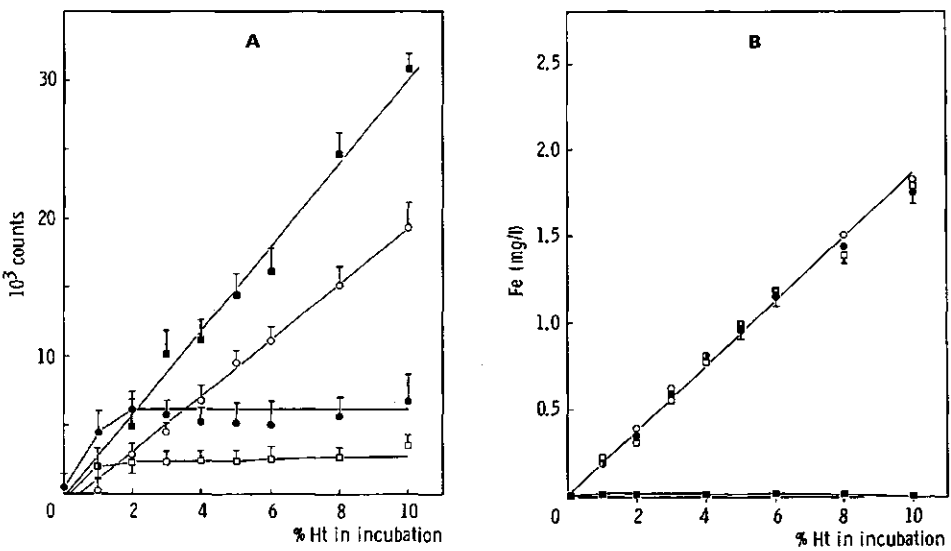


Figure 3 Intact erythrocytes in 154 mM NaCl (■), and lysed erythrocytes in double-distilled water (○), in 2.5 mM deoxycholate (□) and in fecal water (●) measured with cell-count (Figure 3A) and as Fe-release by Atomic Absorption Spectrophotometry (Figure 3B) as a function of the hematocrit percentage. Values shown are means of three experiments. Standard errors are either smaller than the size of the symbols or indicated by bars.

In conclusion, Fe-release determined by atomic absorption spectrophotometry is a reliable and reproducible method to measure the cytotoxicity of rat fecal water against human erythrocytes. In the first two animal studies (chapters 4 & 5) only one dilution of fecal water was used to determine cytotoxicity. In the last two animal studies, titration curves with fecal water were used and cytotoxicity was quantified as the area under the lytic curve (chapters 6 & 7). This was expressed as a percentage of the maximal area which implies 100% lysis at each dilution of fecal water. The same method was used in incubations with human fecal water (chapter 8).

Determination of intestinal epitheliolysis using alkaline phosphatase as marker

Intestinal epitheliolysis was determined by measuring the activity of intestinal alkaline phosphatase (intestinal ALP) in fecal water as membrane-marker (chapter 6 & 7). Intestinal ALP is one of the different ALP isozymes such as placental ALP and E. coli ALP. It is a dimeric Zn glycoprotein (120-140 kDa) which hydrolyzes organophosphates and is located as exoenzyme on the apical membrane of enterocytes (Ono, 1974). Consequently, damage of the apical membrane may bring ALP in the fecal stream which offers the possibility that this enzyme can be used as marker for intestinal epitheliolysis.

Total ALP activity was determined according to Bessey *et al.* (1946) using a glycine buffer (final: 100 mM, pH 9.8) in the presence of zinc (final: 2 mM) and magnesium (final: 5 mM). p-Nitrophenyl phosphate was used as substrate and the absorbance of the reaction product p-nitrophenol was determined spectrophotometrically at 405 nm. The concentration p-nitrophenol was calculated using a standard curve of known p-nitrophenol concentrations and ALP activity was expressed as µmol p-nitrophenol/min/ml fecal water (U/ml). Intestinal ALP activity was measured using 60 mM L-phenylalanine which acts as a specific uncompetitive inhibitor of the intestinal isozyme in humans and rats (Fishman *et al.*, 1962). No inhibition of intestinal ALP occurred with the enantiomer D-phenylalanine as inhibitor. Intestinal ALP was quantified as the difference between total (non-inhibited) ALP activity and the ALP activity after inhibition with L-phenylalanine. Because an antibody against rat intestinal ALP was not commercially available, we validated the enzyme-kinetic measurement of intestinal ALP with immunoprecipiatation of intestinal ALP in

human fecal water isolated from feces of healthy human volunteers consuming habitual diets. Human intestinal ALP activity was determined by immunoprecipitation using a commercially available rabbit anti-human-placenta polyclonal antibody (Dakopatts, Glostrup, Denmark) which is known for its cross-reactivity with the intestinal isozyme (Lehmann, 1975). Appropriate dilutions of human fecal water were incubated for 1 hour at 37°C with anti-serum or rabbit control-serum (Dakopatts, Glostrup, Denmark) at the same protein concentrations in 154 mM NaCl + 3% Polyethylene glycol (PEG) 6000. After 16 hours at 4°C, samples were centrifuged for twenty minutes at 15,000*g (Eppendorf 5415) and in the supernatants of samples incubated with anti-serum or control serum, ALP activity was determined. Control experiments showed that after immunoprecipitation no inhibitory effect of 60 mM L-phenylalanine could be measured which indicates that the intestinal isozyme has completely been precipitated by the antibody. Using immunoprecipitation by the antibody and enzyme-kinetic inhibition by L-phenylalanine to determine the activity of intestinal ALP in human fecal water a nearly perfect correlation between the two methods (r=0.98, n=29) was obtained as can be seen in Figure 4. Moreover, the regression equation (y=1.0x-0.01) indicates that both methods produce exactly the same values for intestinal ALP activities.

Experiments using feces derived from rats and humans consuming different diets showed that >95% of the activity of intestinal ALP was recovered in fecal water. Feces tested comprised feces of rats on control diets, on diets with extra steroids, on diets with supplemental calcium and feces of human volunteers with and without supplemental calcium. Thus determination of ALP activity in fecal water covers the total intestinal ALP activity in feces independent of the experimental diets.

Because of the well-known decreasing gradient in ALP specific activity along the gastrointestinal tract (Vanderhoof *et al.*, 1990), we investigated whether ALP in fecal water was derived from small intestine or colon. Inactivation experiments using ileum samples of rats showed that after 4 hours incubation at 37°C, 50% of ALP was inactivated probably by proteolytic activity. Correlation studies between ALP activity in ileum, colon and fecal samples showed that a weak correlation exists between ALP in distal ileum and feces (r=0.52, n=39, fecesALP = 0.42*ileumALP+50) and a very significant correlation between distal colon and feces (r=0.95, n=39, fecesALP= 1.0*colonALP-7.4). This indicates that the ALP activity in colon can adequately be estimated by the ALP activity in fecal water.

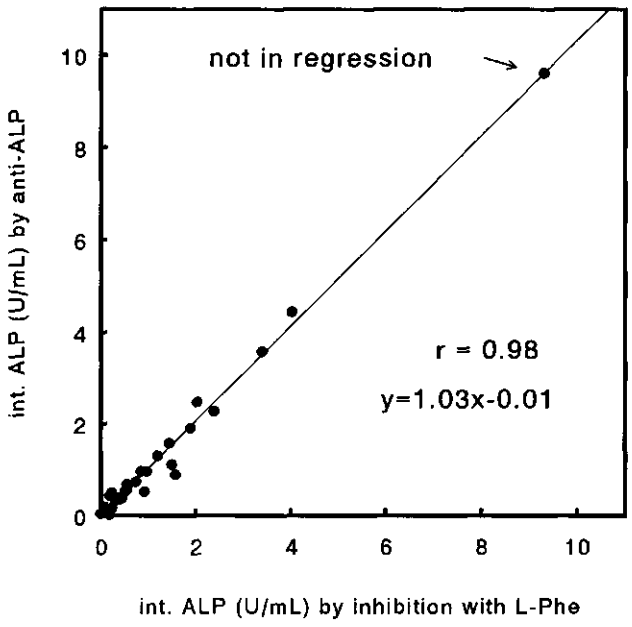


Figure 4 Correlation between measurement of intestinal alkaline phosphatase activity in human fecal water by specific uncompetitive inhibition (60 mM L-phenylalanine) and by immunoprecipitation using a polyclonal antibody against intestinal alkaline phosphatase.

Measurement of in vivo proliferation by ^{3}H-thymidine incorporation

Changes in the proliferative behavior of cells lining the large intestine have been considered as one of the sites of action where diet could affect the risk for colon cancer (Lipkin, 1974). Proliferation of colonic cells follows a steady-state kinetic pattern: cell loss is compensated for by cell-replacement (Deschner & Lipkin, 1976). The standard method for determining proliferation of colonic cells is by using incorporation of tritiated thymidine into DNA. This method is very attractive because it relies on a very simple principle. Radiolabelled thymidine will be incorporated into DNA during the S-phase of the cell-cycle. In vitro incubations of biopsies with tritiated thymidine has been extensively used to asses proliferation rates (eg. Lipkin & Newmark, 1985). However, these in vitro incubations do not necessarily reflect the kinetic behavior of the colonic cells in vivo. For that reason, we used incorporation of

tritiated thymidine in vivo in rats. As the rate of DNA synthesis expresses the mitotic activity, the radioactivity of the trichloroacetic acid precipitable material should reflect cell-proliferation. The crypt cell production rate per hour using a metaphase arrest technique as a measure for cell-proliferation is highly correlated with dpm ^3H/mg wet tissue (Sharp & Wright, 1984). It should be noted that there is an important difference between the metaphase arrest technique and measurement of tritiated thymidine incorporation into DNA. The scoring of labelled cells in the metaphase arrest technique is a semiquantitative measurement whereas determination of the incorporated radioactivity by scintillation counting gives direct quantitative information. Therefore, in this thesis in vivo colonic proliferation is measured as dpm ^3H/µg DNA.

Rats (non-fasted) were injected intraperitoneally at 9.00 a.m. with [methyl-^3H]-thymidine in saline (Amersham int., Buckinghamshire, England; s.a. 25 Ci/mmol) at a dose of 100 µCi/kg (Figure 5).

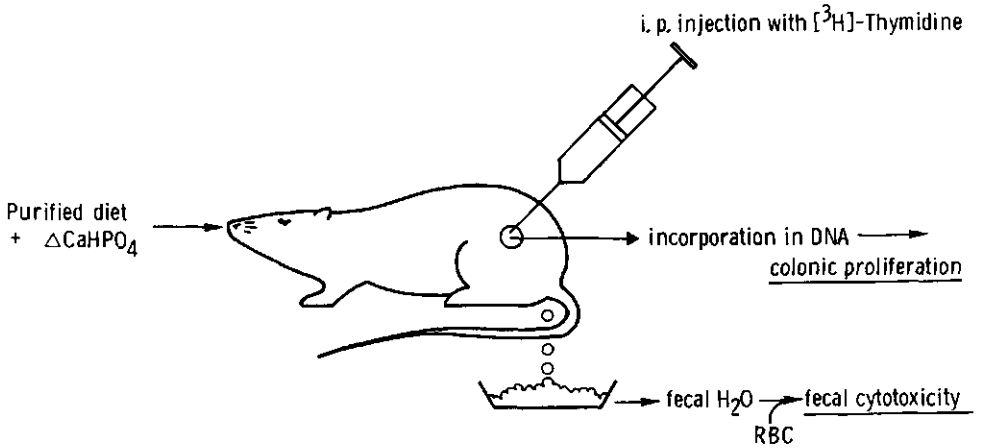


Figure 5 *Experimental design to study [methyl-^3H] thymidine incorporation in rats.*

After two hours, rats were killed by decapitation after anesthetization with CO_2. The colon was prepared free and excised. The length of the colon was opened and the intestinal contents were removed by rinsing with ice-cold 154 mM KCl. The opened colon was spread on a plastic table with the luminal side up and carefully scraped using a metal spatula. The scraping was solubilized in 1 ml ice-cold KCl and kept on ice. After homogenization, the macromolecular fraction was precipitated with 5%

trichloroacetic acid (wt/vol) during 15 minutes at room temperature. The samples were centrifuged for 15 minutes at 10,000*g and the supernatant was discarded. The pellet was resuspended in 1 M $HClO_4$ and hydrolyzed for 15 minutes at 70°C. After centrifugation (15 min./15,000*g) the hydrolyzate was removed and the hydrolyzation repeated. The hydrolyzates were combined and 1 ml hydrolyzate was dissolved in 20.0 ml Aqua Luma scintillation counting liquid (Lumac, 3M, Schaesberg, The Netherlands) and counted with corrections for quenching on a Beckman LS-7500 liquid scintillation cell-counter. DNA was determined using the diphenylamine reaction with calf thymus DNA (Sigma) as standard as described by Burton *et al.* (1956). Proliferation was expressed as dpm $^3H/\mu g$ DNA.

One of the main problems using mucosal scrapings is a contamination of the samples with non-epithelial cells. For instance, submucosal cells will probably less proliferate compared to the epithelial cells. Thus, the amount of radioactivity incorporated will be about equal when these cells are present in the scraped material, but the amount of DNA will be increased. Consequently, the ratio dpm/μg DNA will be decreased without any effect on proliferation. Therefore, an internal control which should be made is that the amount of DNA per scraping must be constant whereas the amount of radioactivity must be varying. Only then the ratio of dpm/μg DNA reflects DNA synthesis and proliferation. Unfortunately, this control analysis is neglected in most studies on the effect of bile acids on colonic proliferation measured in mucosal scrapings (e.g. DeRubertis & Craven, 1984).

REFERENCES

Bessey OA, Lowry OH, Brock MJ. A method for the rapid determination of alkaline phosphatase with five cubic millimeters of serum. *J Biol Chem* 1946;164:321-323

Burton K. A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acids. *Biochem J* 1956;62:315-323

Coleman R, Iqbal S, Godfrey PP, Billington D. Membranes and bile formation: Composition of several mammalian biles and their membrane-damaging properties. *Biochem J* 1979;178:201-208

DeRubertis FR, Craven PA. Bile salt stimulation of colonic epithelial proliferation. *J Clin Invest* 1984;74:1614-1624

Deschner EE, Lipkin M. Cell-proliferation in normal preneoplastic and neoplastic gastrointestinal cells. *Clin Gastroenterol* 1976;5:545-556

Dodge JT, Mitchell C, Hanahan DJ. The preparation and chemical characteristics of hemoglobin-free ghosts of human erythrocytes. *Arch Biochem Biophys* 1963;100;1190-130

Fishman WH, Green S, Inglis NI. Organ-specific behavior exhibited by rat intestine and liver alkaline phosphatase. *Biochim Biophys Acta* 1962;62:363-375

Lehmann FG. Immunological relationship between human placental and intestinal alkaline phosphatase. *Clin Chim Acta* 1975;65:257-264

Lipkin M. Phase 1 and phase 2 proliferative lesions of colonic epithelial cells in diseases leading to colon cancer. *Cancer Res* 1974;34:878-888

Lipkin M, Newmark H. Effect of added dietary calcium on colonic epithelial-cell proliferation in subjects at high risk for familial colonic cancer. *N Engl J Med* 1985; 313:1381-1384

Ono K. The localization of alkaline phosphatase activity in the small intestinal epithelium after fat and protein feeding. *Acta Histochem* 1974;51:124-137

Rafter JJ, Child P, Anderson AM, Alder R, Eng VWS, Bruce WR. Cellular toxicity of fecal water depends on diet. *Am J Clin Nutr* 1987;45:559-563

Sharp JG, Wright NA. Comparison of tritiated thymidine and metaphase arrest techniques of measuring cell production in rat intestine. *Dig Dis Sci* 1984;29:1153-1158

Vanderhoof JA, Park JHY, Mohammadpour H, Blackwood D. Effects of dietary lipids on recovery from mucosal injury. *Gastroenterology* 1990;99:1226-1231

CHAPTER 3 LYTIC EFFECTS OF MIXED MICELLES OF FATTY ACIDS AND BILE ACIDS

John A. Lapré, Denise S.M.L. Termont, Albert K. Groen and Roelof Van der Meer.
(Submitted for publication)

ABSTRACT

Bile acids and fatty acids are assumed to promote colon cancer. A proposed mechanism is a lytic effect of these surfactants resulting in a stimulation of colonic cell proliferation. Because in human fecal water the fatty acid concentration is about one order of magnitude higher than the bile acid concentration, we studied the lytic activity of fatty acids and physiological mixtures of fatty acids and bile acids using both erythrocytes and cultured CaCo-2 cells, a model system for intestinal epithelium. The hemolytic activity of fatty acids increased in the order $C_{8:0} < C_{10:0} < C_{18:0} < C_{16:0} < C_{12:0} < C_{14:0} << C_{18:1} = C_{18:2}$, *but was not dependent on their critical micellar concentration. Addition of a sublytic, submicellar concentration of cholate resulted in the formation of highly lytic mixed micelles. Lytic activity of these mixed micelles was closely associated with their micellar aggregation as determined in parallel incubations using a fluorescent micellar probe. Using identical concentrations of fatty acids and mixed micelles lysis of erythrocytes was highly correlated (r>0.95) with lysis of CaCo-2 cells measured by either release of the apical membrane-marker alkaline phosphatase or the cytosolic marker lactate dehydrogenase. This indicates that the cytolytic activity of these surfactants is not cell-type dependent. Addition of bile acids in concentrations as present in human fecal water resulted in an increased lytic activity of fatty acids. The increase in lytic activity was lowest for the primary bile acid cholate and highest for the more hydrophobic, colonic bile acids deoxycholate and lithocholate. These results may contribute to a better knowledge of the relevant determinants of lytic activity of human fecal water.*

INTRODUCTION

Epidemiological data show a correlation between a high fat intake and the incidence of colon cancer (Caroll, 1984; Willett *et al.*, 1990). It has been suggested that this effect is caused by a diet-dependent increase of bile acids and free fatty acids in colon (Newmark *et al.*, 1984; Weisburger & Wynder, 1987). These surfactants stimulate proliferation of colonic epithelium in rodents (Bull *et al.*, 1983; Deschner *et al.*, 1981; Wargovich *et al.*, 1983, 1984), probably because of their intrinsic lytic properties (Rafter *et al.*, 1986; Wargovich *et al.*, 1983, 1984). Also in humans dietary fat stimulates colonic proliferation (Stadler *et al.*, 1988). This hyperproliferation of

colonic epithelium is generally accepted as an important biomarker of the risk for colon cancer (Lipkin, 1988).

Recently, we have shown that lytic activity of bile acids (measured as lysis of erythrocytes) is strongly dependent on their structure and increases with increasing hydrophobicity (Van der Meer *et al.*, 1991). Lytic activity increases from the primary conjugated (duodenal) to the secondary (colonic) bile acids and is associated with their micellar aggregation (Van der Meer *et al.*, 1991). The physiological relevance of these studies has been ascertained in experiments with rats. A diet-induced increase of the bile acid concentration in fecal water results in a higher intestinal lytic activity (Lapré *et al.*, 1991) and in a higher colonic cell proliferation (Lapré & Van der Meer, 1992). In these animal studies the concentration of bile acids in fecal water was greater than that of fatty acids (Lapré *et al.*, 1991; Lapré & Van der Meer, 1992). However, in human fecal water the concentration of bile acids is low (about 250 μM) and approximately one order of magnitude lower than the concentration of fatty acids (Rafter *et al.*, 1987; Allinger *et al.*, 1989; Van der Meer *et al.*, 1990). Consequently, the fatty acid concentration in human fecal water might be a relevant determinant of luminal lytic activity in humans. For that reason, we studied the lytic properties of fatty acids in the absence and presence of bile acids using human erythrocytes as a model system for lytic activity. By measuring hemolysis as an endpoint of lytic activity, several studies (Coleman *et al.*, 1980, 1987; Van der Meer *et al.*, 1991) have shown that this model system can adequately be used for determining membrane damage by surfactants. To validate the physiological relevance of lysis of erythrocytes by surfactants, we studied the lytic effects of different kinds of surfactants on CaCo-2 cells, which are derived from a human colon adenomacarcinoma cell line. By culturing in a polarized monolayer these cells provide an excellent model system to study lytic effects on the apical membrane of intestinal epithelial cells. To quantify lysis of these cells we used both an apical membrane-marker (alkaline phosphatase) (Hidalgo *et al.*, 1989) as well as a cytosolic marker (lactate dehydrogenase).

MATERIALS AND METHODS

Materials. Human blood was obtained from healthy donors by venipuncture. Human erythrocytes were thoroughly washed and isolated exactly as described by Van der Meer *et al.* (1991). Bile acids, fatty acids and fat-free bovine serum albumin were

acquired from Sigma (St Louis, MO) and Fluka (Buchs, Switzerland) and were of the highest purity commercially available. The purity of bile acids and fatty acids used has been been verified by gaschromatographic analysis. N-Phenyl-1-Naphthylamine (NPN) was obtained from Eastman Kodak (Rochester, NY) and N-2-hydroxy-ethylpiperazine-N'-2-ethanesulfonic acid (HEPES) from Sigma. Other chemicals used were of analytical grade.

Methods. Fatty acids were dissolved in double-distilled water and saponified with potassium hydroxide. After saponification, the pH of the stock solutions was brought to pH 7.5-8.0. Concentrations of fatty acids were measured using a commercially available enzymatic kit (NEFA-C, Wako Chemicals, Germany). Concentrations of bile acids were measured using an enzymatic fluoresence method (Sterognost 3α Flu, Nycomed AS, Oslo, Norway) (Beher *et al.*, 1981).

Lytic properties of fatty acids and mixtures of fatty acids and bile acids were studied using hemolysis of erythrocytes at a final hematocrit 5%. To avoid peroxidation of the unsaturated fatty acids the experiments with oleate and linoleate were performed under nitrogen. However, control experiments showed that no difference in lytic activity occurred during our short-term incubations when the experiments were carried out aerobically. After a preincubation of 10 min, erythrocytes were added to tubes containing increasing concentrations of fatty acids and/or bile acids, HEPES (final concentration 50 mM and pH 7.4) and NaCl (in order to maintain a constant ionic strength of 150 mM). KCl was used to correct for the amount of potassium in the fatty acid stock solutions, thus maintaining a constant potassium concentration in the incubation of 60 mM. Control experiments showed that this potassium concentration did not affect hemolysis (data not shown). After 15 min of incubation at 37°C, the tubes were centrifuged for 1 min at 10,000*g (Eppendorf 5415). After dilution of the supernatants with double-distilled water, hemolysis was determined by measuring the absorbance at 540 nm. In control experiments it appeared that neither bile acids nor fatty acids affected the absorbance of hemoglobin at this wavelength. Percent hemolysis was calculated by comparing the absorbance of the samples with that of an identical amount of erythrocytes lysed in double-distilled water, which represents 100% hemolysis.

Monolayers of CaCo-2 cells were grown in microwell plates as described by Velardi *et al.* (1991). After washing of the postconfluent cultures with phosphate buffered saline (pH 7.4), cells were incubated for 45 minutes at 37°C with solutions

containing surfactants as described above. At the end of the incubation time the medium was collected and and the cells were treated with 0.1% Triton X-100. Lactate dehydrogenase (LDH) activity was measured according to Mitchell *et al.* (1980) and alkaline phosphatase (ALP) activity according to Bessey *et al.* (1946) in both medium as well as in Triton X-100 treated cells. Fat-free bovine serum albumin (final concentration 0.6%) was added to prevent interference of surfactants with the spectrophotometric assay of LDH activity. % Lysis was calculated as enzyme activity in medium divided by the total enzyme activity (medium + Triton X-100).

The apparent critical micellar concentration (CMC) was determined under the same conditions, but in the absence of cells, by using 2 μM of the fluorescent micellar probe NPN, as described by Brito and Vaz (1986). Results are given as means ± SD of at least three experiments.

RESULTS

Figure 1 shows the concentration dependence of the lytic effect of laurate ($C_{12:0}$). At low concentrations laurate was not lytic, but above a certain threshold concentration significant lysis was observed. The concentration at which 2% lysis occurred is defined as the critical lytic concentration (CLC). In addition, we used the lytic concentration required for 50% lysis (LC_{50}) as a second parameter to define the lytic curve. To investigate whether addition of bile acids enhances the lytic effect of fatty acids, we used 5 mM of the primary bile acid cholate. Addition of this submicellar concentration of cholate (Van der Meer *et al.*, 1991) to increasing concentrations of laurate caused a shift of both CLC and LC_{50} to lower values, which indicates an increased lytic activity (Figure 1). In the absence of laurate, no lytic activity was observed, which indicates that 5 mM cholate alone did not induce lysis.

Figure 2 shows the lytic potential, which is the reciprocal of LC_{50}, for a range of fatty acids. Lytic potential increased from $C_{8:0}$ to $C_{14:0}$ with increasing chain length. The long-chain saturated fatty acids ($C_{16:0}$ and $C_{18:0}$) were less lytic than the medium-chain fatty acids. Oleate and linoleate showed a higher lytic potential than stearate. Figure 2 also shows that the lytic potential of all fatty acids $\geq C_{12:0}$ was drastically increased by the addition of a sublytic concentration (5mM) of cholate. To determine whether this increase in lytic potential is due to the formation of mixed micelles, we determined the CMC of these mixtures using a fluorescent probe.

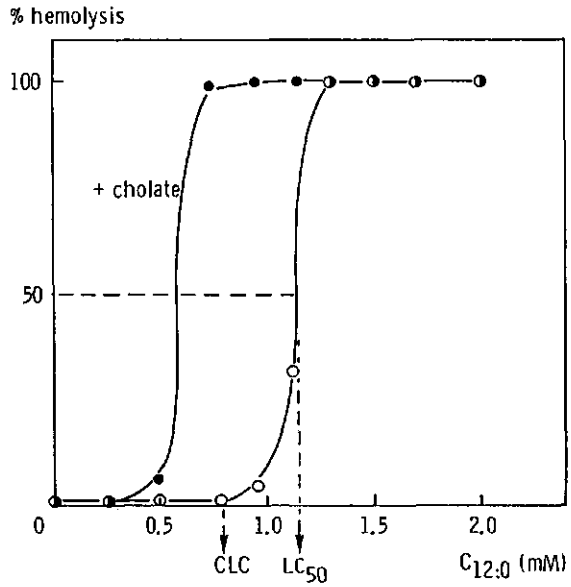


Figure 1 Concentration dependence of the lytic effect of laurate in the absence and presence of a sublytic concentration of cholate (5 mM). CLC is the critical lytic concentration required for 2% lysis and LC_{50} is the concentration required for 50% lysis.

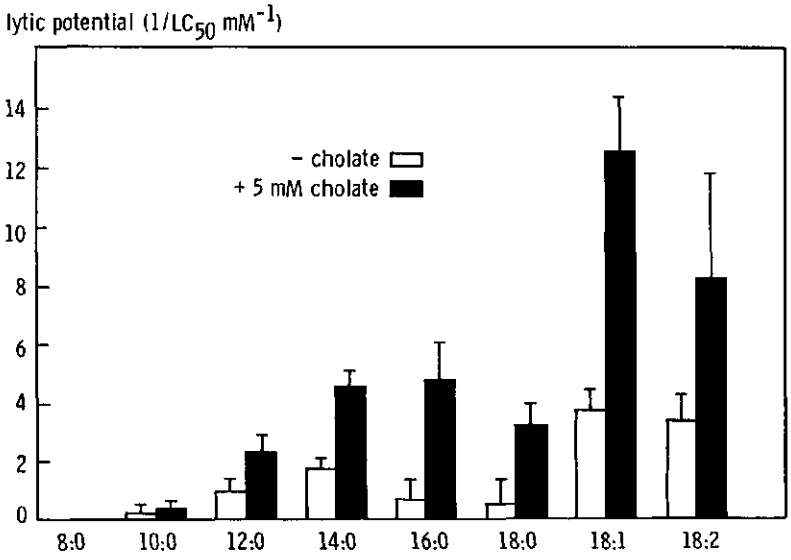


Figure 2 Lytic effects of fatty acids measured as hemolysis and expressed as lytic potential ($1/LC_{50}$) in the absence and presence of a sublytic concentration of cholate (5 mM). Bars represent SDs (n=3).

In contrast to bile acids (Van der Meer *et al.*, 1991), the CLC of fatty acids was not associated with their micellar aggregation (Figure 3). Addition of a submicellar concentration of cholate induced the formation of mixed micelles. Thus at concentrations below their individual CMC's the combination of bile acids and fatty acids induced the formation of mixed micelles. The CLC of these mixed micelles corresponded with their CMC (Figure 3), which is analogous to the relationship observed for bile acids (Van der Meer *et al.*, 1991). This indicates that the lytic activity of the bile acid-fatty acid mixtures is caused by mixed micelles.

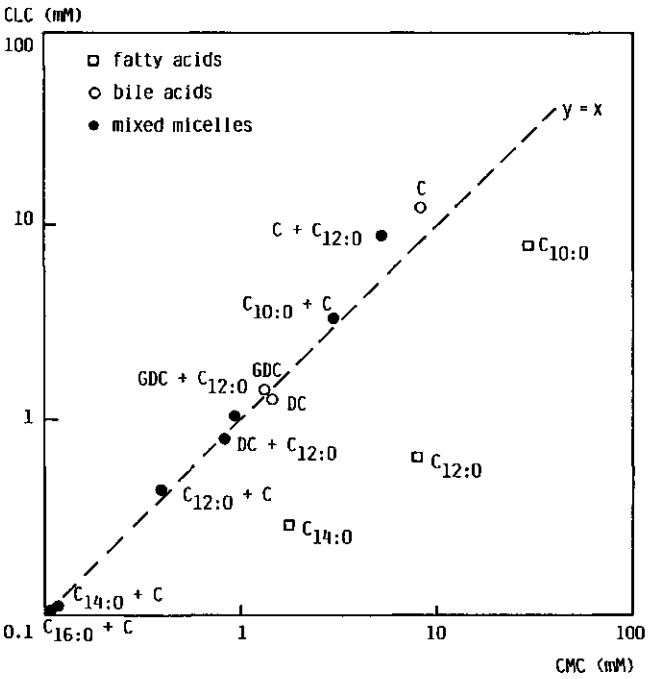


Figure 3 Correlation between critical lytic concentration (CLC) and critical micellar concentration (CMC) for bile acids, fatty acids, and mixtures of bile acids and fatty acids. Note that the scales are logarithmic. C is cholate, GDC is glycodeoxycholate and DC is deoxycholate. Mixed micelles consist of the different fatty acids combined with 5 mM cholate (CMC and CLC expressed as concentration of fatty acid). For the combinations $C+C_{12:0}$, $GDC+C_{12:0}$ and $DC+C_{12:0}$ the different bile acids were combined with 300 µM laurate (CMC and CLC expressed as concentration of bile acid).

Table 1 gives the results of the comparison between the effects of surfactants on lysis of CaCo-2 cells and on lysis of erythrocytes. It is clearly shown that the different surfactants (bile acids alone, fatty acids alone and combinations of fatty acids and bile acids) had comparable lytic effects on erythrocytes and CaCo-2 cells.

Table 1 Lytic effects of bile acids, fatty acids and mixtures of bile acids and fatty acids on CaCo-2 cells and on erythrocytes.

Surfactant	Lysis (%)		
	Erythrocytes	CaCo-2 cells	
		LDH-release	ALP-release
Control			
No surfactants	0 ± 0	6 ± 1	1 ± 1
Cholate			
16.5 mM	99 ± 5	96 ± 1	96 ± 1
$C_{10:0}$			
10.0 mM	1 ± 0	6 ± 1	2 ± 2
20.0 mM	102 ± 11	89 ± 3	98 ± 3
$C_{12:0}$			
0.6 mM	1 ± 0	6 ± 1	3 ± 2
1.4 mM	179 ± 19	59 ± 1	50 ± 4
$C_{10:0}$ + 5 mM cholate			
5.0 mM	30 ± 1	14 ± 2	10 ± 5
10.0 mM	94 ± 2	103 ± 3	106 ± 2
$C_{16:0}$ + 5 mM cholate			
0.3 mM	54 ± 10	57 ± 6	65 ± 16
0.6 mM	80 ± 5	80 ± 13	93 ± 5

Lysis of CaCo-2 cells was measured by lactate dehydrogenase (LDH) release and by alkaline phosphatase (ALP) release, and hemolysis by release of hemoglobin. Values are means ± SD (n=3). Correlation coefficients between hemolysis and LDH were 0.97 (Hemolysis = 1.03 LDH + 0.9), between hemolysis and ALP 0.96 (Hemolysis = 0.91 ALP - 7.2), and between LDH and ALP 0.99 (LDH = 0.89 ALP - 5.0).

Moreover, the high correlation coefficients ($r>0.95$) and the regression equations indicate that the lytic effects are similar on both cell-types under these conditions. However, it should be noted that the difference in incubation time (15 minutes for erythrocytes against 45 minutes for CaCo-2 cells) shows that erythrocytes are more sensitive to surfactant-induced lysis compared to CaCo-2 cells.

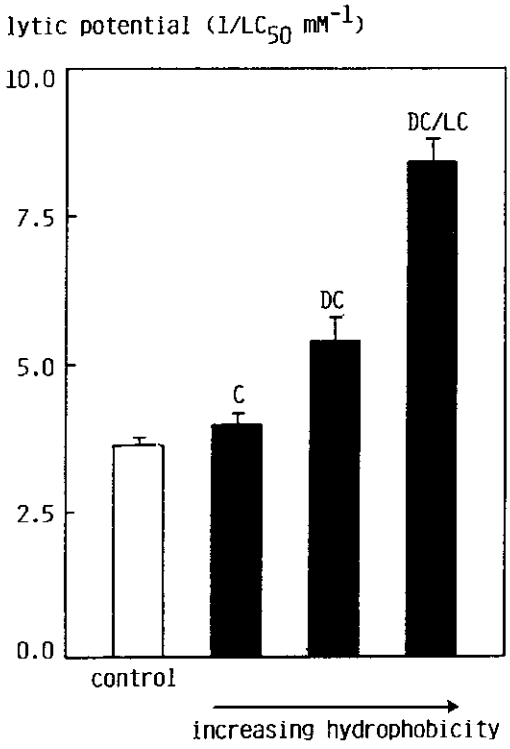


Figure 4 Effect of 250 μM cholate (C), deoxycholate (DC), and deoxycholate/lithocholate (DC/LC) (molar ratio 3:1) on lytic potential of oleate. Bars represent SDs (n=4).

Finally, to examine the effect of bile acid structure on the lytic activity of mixed micelles, we used an increasing concentration of oleate combined with 250 μM cholate, deoxycholate or a mixture of deoxycholate and lithocholate in a molar ratio of 3:1. Control experiments showed that 250 μM of these bile acids in the absence of

oleate is not lytic. This bile acid concentration corresponds with its concentration in human fecal water (Rafter *et al.*, 1987; Allinger *et al.*, 1989; Van der Meer *et al.*, 1990) and oleate is one of the most predominant fatty acids in human fecal water (unpublished data). As shown in Figure 4 a change in bile acid structure caused a signficant change in lytic potential. Addition of the primary bile acid cholate had little or no effect on oleate-induced lytic potential, whereas the secondary bile acid deoxycholate increased its lytic potential. The addition of the mixture of deoxycholate and lithocholate (predominantly present in the colon) induced the highest lytic potential. Comparable results were obtained using laurate as model for a medium-chain fatty acid and palmitate as long chain saturated fatty acid. For instance, addition of 250 μM cholate has little or no effect on the lytic potential of laurate or palmitate whereas 250 μM of deoxycholate + lithocholate stimulated lytic potential of laurate from 0.83 mM^{-1} to 3.0 mM^{-1} and of palmitate from 0.53 mM^{-1} to 1.1 mM^{-1}.

DISCUSSION

The results of the present study show that the lytic potential of fatty acids is at least of the same magnitude as that of bile acids (Coleman *et al.*, 1980, 1987; Van der Meer *et al.*, 1991). Buset *et al.* (1990) have shown that palmitate, oleate and linoleate decrease the viability of isolated colonocytes as quantified by ^{3}H-thymidine incorporation. This is probably due to the lytic effects of these fatty acids as observed in our present study. The high correlation between lysis of erythrocytes as a membrane-model and lysis of intact colonocytes strongly suggest that the cytotoxicity of bile acids and fatty acids is caused by membrane-damage and subsequent cytolysis.

In our experiments using an incubation temperature of 37°C, lytic activity of the saturated fatty acids increased with the increase in the number of carbon atoms, which reflects an increasing hydrophobicity. A similar relationship between bile acid hydrophobicity and lytic activity has been reported previously (Van der Meer *et al.*, 1991). However, palmitic and stearic acid were less lytic than would have been expected from their chain length. This anamolous behavior is probably due to the fact that our incubation temperature of 37°C is below the critical temperature of these long-chain fatty acids (Larsson, 1986), so that their lytic activity is decreased because of

gel formation (Chapman, 1969; Larsson, 1986). As shown by Csordas and Rybczynska (1988) this may drastically decrease the fatty-acid-induced lysis of erythrocytes. The observed increase in lytic activity with unsaturation of fatty acids (Figure 2) is in accordance with this explanation, because unsaturation of fatty acids decreases their critical temperature (Larsson, 1986).

The similarity of CMC and CLC values for bile acids and mixed micelles (Figure 3) shows that hydrophobicity and micellar aggregation are important determinants of their cytolytic effect. This indicates that lysis is due to disruption of the cellular membrane integrity by comicellization of membrane lipids (Coleman, 1980; Van der Meer *et al.*, 1991). Whether the same mechanism holds for the lytic effects of fatty acids per se is at present not clear. For instance, comparison of our CMC value of 7.1 mM for laurate, which is similar to the value of 7.5 mM published earlier (Corrin & Harkins, 1947), with the CLC value of 0.73 mM indicates that there is no association between micellar aggregation of fatty acids and fatty acid induced lysis of erythrocytes. It should be noted that this lack of association cannot be taken as evidence that lysis is mediated by fatty acid monomers. For instance, the sigmoidal concentration dependence of fatty acid-induced lysis (Figure 1) may reflect that aggregation of fatty acids is required for its lytic effects. This may indicate that this lytic activity is due to dimers or oligomers with a low aggregation number. Apparently our fluorescent probe does not intercalate with these small structures, but this requires further investigation.

Benzonana (1969) showed that high concentrations of oleate and deoxycholate at pH 9.0 form mixed micelles. Shilnikov *et al.* (1987) found that deoxycholate only interacted with fatty acids above a chain length of 9 C-atoms. These studies showed that fatty acids and bile acids at concentrations above their individual CMC form mixed micelles. The present study showed that mixed micelles are also formed at concentrations below their individual CMC and that this association increased their lytic activity. At present no information on the nature of this association and structure of these mixed micelles is available. A bile acid concentration of 250 μM of secondary bile acids like deoxycholate or a mixture of deoxycholate and lithocholate increased the lytic activity of fatty acids (laurate, palmitate and oleate). However, addition of 250 μM of a primary bile acid like cholate had almost no effect on lytic activity. These results indicate that changes in the hydrophobic/hydrophilic balance of bile acids in fecal water may have a drastic effect on intestinal lytic activity.

The significant effects of hydrophobicity of fatty acids and bile acids on micellar

aggregation and cytotoxicity may be of relevance for the integrity of the intestinal mucosa. The present results show that the lytic effects of these surfactants on CaCo-2 cells and erythrocytes are highly correlated (Table 1), and thus support and extend our recent findings (Velardi *et al.*, 1991). Our results indicate that CaCo-2 cells, compared to erythrocytes, are slightly more resistant to surfactant-induced lysis. This can be explained by the high content of glycolipids in the apical membrane of enterocytes (Simons & Van Meer, 1988), because it has been shown that differences in membrane glycolipids could affect the resistance to surfactant-induced lysis (Coleman *et al.*, 1980). With regard to this, it should be mentioned that lysis of CaCo-2 cells was measured using a cytosolic marker (LDH) and a marker almost exclusively located in the apical membranes of CaCo-2 cells and enterocytes (Hidalgo *et al.*, 1989). This indicates that lysis of enterocytes can be determined with the same accuracy by measuring the release of either an apical (ALP) or a cytosolic marker (LDH). Finally, we have recently demonstrated that a diet-induced increase in lytic activity of rat fecal water correlated highly with in vivo colonic proliferation (Lapré & Van der Meer, 1992). This also indicates that the in vitro experiments reported in this paper are of relevance to a proper understanding of the molecular determinants of luminal lytic activity.

Lipkin and Newmark (1985) found that dietary calcium supplementation reduces the proliferation in colonic biopsies of humans at high risk of colon cancer. Using the same study design Van der Meer *et al.* (1990) found that luminal lytic activity was decreased by oral calcium. In that study, the total bile acid concentration in fecal water (app. 250 μM) was not changed by calcium supplementation, but the fatty acid concentration in fecal water was decreased. The most predominant fatty acids in fecal water were $C_{6:0}$, $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$ (unpublished data). Our data showed that the physiologically relevant combination of palmitate or oleate with physiological concentrations of the colonic bile acids deoxycholate and lithocholate drastically increased lytic activity. Therefore, the present in vitro experiments may offer a molecular explanation for the calcium-dependent inhibition of lytic activity of fecal water because of the concomitant decrease in fatty acid concentration. An additional effect of calcium supplementation on the hydrophobic/hydrophilic balance of bile acids in fecal water is now under investigation.

In conclusion, fatty acids, bile acids and mixtures of fatty acids and bile acids have similar lytic effects in CaCo-2 cells and erythrocytes. Mixtures of fatty acids and bile acids synergistically increase lytic activity because of mixed micelle formation. The

lytic activity of these mixed micelles is drastically increased by the amount of fatty acids as well as by increasing hydrophobicity of bile acids. These mixed micelles may be important determinants of lytic activity of human fecal water. Therefore the results of the present study may contribute to a proper understanding of the mechanism of dietary effects on luminal lytic activity.

Acknowledgements: This study was supported by Grant 663/88-1.7 from the European Community and by the Netherlands Organization for Scientific Research (NWO), Medical Sciences (NWO-900-562-078). The authors wish to thank H. Van den Berg and E. Strootman for technical assistance.

REFERENCES

Allinger UG, Johansson GK, Gustafsson JA, Rafter JJ. Shift from a mixed to a lactovegetarian diet: influence on acidic lipids in fecal water - a potential risk factor for colon cancer. *Am J Clin Nutr* 1989; 50:992-996

Beher WT, Stradnieks S, Lin GJ, Sanfield J. Rapid analysis of human fecal bile acids. *Steroids* 1981;38:281-295

Benzonana G. Study of bile salts micelles: properties of mixed oleate-deoxycholate solutions at pH 9.0. *Biochim Biophys Acta* 1969;176:836-848

Bessey OA, Lowry OH, Brock MJ. A method for the rapid determination of alkaline phosphatase with five cubic millimeters of serum. *J Biol Chem* 1946;164:321-323

Brito RMM, Vaz WLC. Determination of the critical micellar concentration of surfactants using the fluorescent probe N-phenyl-1-naphthylamine. *Anal Biochem* 1986;152:250-255

Bull AW, Marnett LJ, Dawe EJ, Nigro ND. Stimulation of deoxythymidine incorporation in the colon of rats treated intrarectally with bile acids and fats. *Carcinogenesis* 1983;4:207-210

Buset M, Galand R, Lipkin M, Winawer S, Friedman E. Injury induced by fatty acids or bile acids in isolated human colonocytes prevented by calcium. *Cancer Lett* 1990;5:221-226

Caroll KK. Role of lipids in tumorigenesis. *JAOCS* 1984;61:1888-1891

Chapman D. *Introduction to lipids.* McGraw-Hill, London, England, 1969.

Coleman R, Iqbal S, Godfrey PP, Billington D. Membranes and bile formation: Composition of several mammalian biles and their membrane-damaging properties. *Biochem J* 1978;178:201-208

Coleman R, Lowe PJ, Billington D. Membrane lipid composition and susceptibility to bile salt damage. *Biochim Biophys Acta* 1980;599:294-300

Corrin ML, Harkins WD. The effect of salts on the critical concentration for the formation of micelles in colloidal electrolytes. *J Am Chem Soc* 1947;69:683-688

Csordas A, Rybczynska M. Critical temperatures for the interaction of free fatty acids with the

erythrocyte membrane. *Biochim Biophys Acta* 1988;944:155-163

Deschner EE, Cohen BI, Raicht RF. Acute and chronic effect of dietary cholic acid on colonic epithelial cell proliferation. *Digestion* 1981;21:290-296

Hidalgo IJ, Raub TJ, Borchardt RT. Characterization of the human colon carcinoma cell line (CaCo-2) as a model system for intestinal permeability. *Gastroenterology* 1989;96:736-749

Lapré JA, Van der Meer R. Diet-induced increase in colonic bile acids stimulates lytic activity of fecal water and proliferation of colonic cells. *Carcinogenesis* 1992;13:41-44

Lapré JA, De Vries HT, Van der Meer R. Dietary calcium phosphate inhibits cytotoxicity of fecal water. *Am J Physiol* 1991;261:G907-G912

Larsson K. Physical properties- structural and physical characteristics. In: *The Lipid Handbook*, Gunstone FD, Harwood JL, Padley FB eds. Chapman and Hall, New York, 1986

Lipkin M. Biomarkers of increased susceptibility to gastrointestinal cancer: new application to studies of cancer prevention in human subjects. *Cancer Res* 1988;48:235-245

Lipkin M, Newmark HL. Effect of added dietary calcium on colonic epithelial-cell proliferation in subjects at high risk for familial colonic cancer. *N Engl J Med* 1985;313:1381-1384

Mitchell DB, Santone KS, Acosta D. Evaluation of cytotoxicity in cultured cells by enzyme leakage. J *Tissue Culture Methods* 1980;6:113-116

Newmark HL, Wargovich MJ, Bruce WR. Colon cancer and dietary fat, phosphate, and calcium: a hypothesis. *J Natl Cancer Inst* 1984;72:1323-1325

Rafter JJ, Child P, Anderson AM, Alder R, Eng VWS, Bruce WR. Cellular toxicity of fecal water depends on diet. *Am J Clin Nutr* 1987;45:559-563

Rafter JJ, Eng VWS, Furrer R, Medline A, Bruce WR. Effects of calcium and pH on the mucosal damage produced by deoxycholic acid in the rat colon. *Gut* 1986;27:1320-1329

Shilnikov GV, Sarvazyan AP, Okon M, Zakrzewska J, Hranisavljevic J, Vucelic D. Interaction of fatty acid sodium salts with sodium deoxycholate. *J Lipid Res* 1987;28:1259-1262

Simons K, Van Meer G. Lipid sorting in epithelial cells. *Biochemistry* 1988;27:6197-6202

Stadler J, Stern HS, Yeung KS, McGuire V, Furrer R, Marcon N, Bruce WR. Effect of high fat consumption on cell proliferation activity of colorectal mucosa and on soluble faecal bile acids. *Gut* 1988;29:1326-1331

Van der Meer R, Termont DSML, De Vries HT. Differential effects of calcium ions and calcium phosphate on cytotoxicity of bile acids. *Am J Physiol* 1991;260:G142-G147

Van der Meer R, Lapré JA, Kleibeuker JH, De Vries EGE, De Vries HT. Effects of supplemental dietary calcium on composition and cytotoxicity of fecal water. *Gastroenterology* 1990;98:A317

Velardi ALM, Groen AK, Oude-Elferink RJP, Van der Meer R, Palasciano G, Tytgat GNJ. Cell-type dependent effect of phospholipid and cholesterol on bile salt cytotoxicity. *Gastroenterology* 1991; 101:457-464

Wargovich MJ, Eng VWS, Newmark HL. Calcium inhibits the damaging and compensatory proliferative effects of fatty acids on mouse colon epithelium. *Cancer Letters* 1984;23:253-258

Wargovich MJ, Eng VWS, Newmark HL, Bruce WR. Calcium ameliorates the toxic effect of deoxycholic acid on colonic epithelium. *Carcinogenesis* 1983;4:1205-1207

Weisburger JH, Wynder EL. Etiology of colorectal cancer with emphasis on mechanism of action and prevention. In: DaVita VT, Hellman S, Rosenberg SA eds. *Important advances in oncology* 1987. Philadelphia, Lippincott, 1987:197-220

Willett WC, Stampfer MJ, Colditz GA, Rosner BA, Speizner FE. Relation of meat, fat and fiber intake to the risk of colon cancer in a prospective study among women. *N Eng J Med* 1990;323:1664-1742

CHAPTER 4 DIET-INDUCED INCREASE OF COLONIC BILE ACIDS STIMULATES LYTIC ACTIVITY OF FECAL WATER AND PROLIFERATION OF COLONIC CELLS.

John A. Lapré and Roelof Van der Meer.
Carcinogenesis 1992;13:41-44

ABSTRACT

The proposed intermediate steps in the relationship between a diet-dependent increase in colonic bile acids and proliferation of colonic cells were studied in rats. Male Wistar rats were fed diets supplemented with increasing amounts of steroids to increase the bile acid concentration of the colon. After two weeks, in vivo colonic proliferation was measured using tritiated thymidine incorporation into DNA. Luminal lytic activity was measured as lysis of erythrocytes by fecal water. To quantify hemolysis in the presence of fecal water, a method was developed which measures Fe-release using atomic absorption spectrophotometry. This method proved to be superior to the cell-counter method published earlier. Our results showed that steroid supplementation increased, in a dose-dependent manner, the total fecal and the soluble bile acid concentration as well as lytic activity of fecal water and colonic proliferation. A highly significant correlation between lytic activity of fecal water and colonic proliferation ($r=0.85$, $n=24$, $P<0.001$) was observed. These results indicate that the increase in colonic proliferation is mediated by diet-dependent increases in soluble colonic bile acid concentration and luminal lytic activity. This sequence of effects illustrates how diet could influence the risk for colon cancer.

INTRODUCTION

Epidemiological data indicate that colon cancer is associated with a high fat-intake (Willett, 1989; Weisburger & Wynder, 1987; Willett *et al.*, 1990). It has been suggested that this promotive effect of dietary fat is caused by a diet-dependent increase of bile acids and/or fatty acids in colon (Weisburger & Wynder, 1987; Newmark *et al.*, 1984). Measurements of colonic cell-proliferation using tritiated thymidine incorporation showed that in rodents both bile acids and fatty acids, either instilled intrarectally (Bull *et al.*, 1983; DeRubertis & Craven, 1984; Rafter *et al.*, 1986; Wargovich *et al.*, 1984) or supplemented with diet (Deschner *et al.*, 1981; Bird *et al.*, 1986), can induce hyperproliferation. Also in humans, dietary fat given as a bolus induces hyperproliferation of colonic epithelium (Stadler *et al.*, 1988). This increased colonic epithelial proliferation is an important biomarker of the increased risk for colon cancer (Lipkin, 1988).

It has been hypothesized that the diet-dependent induction of hyperproliferation is caused by an increase in luminal lytic activity which results in damage of the

epithelial cells (Newmark *et al.*, 1984). The first step in the induction of hyperproliferation should then consist of a membrane-damaging effect of luminal surfactants like bile acids on colonic epithelial cells. Consequently, this epitheliolysis should then result in a compensatory increase in proliferation of crypt cells. Several studies using erythrocytes as a membrane-model have shown that bile acids are lytic to cells (Coleman *et al.*, 1979; Coleman *et al.*, 1980; Gaetti *et al.*, 1988; Salvioli *et al.*, 1985; Van der Meer *et al.*, 1991). These in-vitro experiments stress the lytic potential of these surfactants. However, the proposed intermediate steps between an increase in colonic bile acids and colonic proliferation have never been quantified in vivo. Therefore, the present study was designed to investigate the diet-dependent increases in colonic bile acids, soluble colonic surfactant concentrations, luminal lytic activity and colonic proliferation in rats fed diets differing in steroid content.

MATERIALS AND METHODS

Animals and diets. Male outbred Wistar rats (Small Animal Research Center of the Wageningen Agricultural University), eight weeks old, were housed individually. For two weeks, all rats were fed purified diets differing only in steroid content. To increase the colonic bile acid concentration six steroid levels were used: Control (no steroids added); 0.5% cholesterol + 0.05% or 0.15% deoxycholate; 1% cholesterol + 0.05% or 0.10% or 0.15% deoxycholate. The composition of the basic diet was (g/kg): casein (acid casein, DMV Veghel, The Netherlands) 200, dextrose (monohydrate) 519, sun flower oil 200, cellulose 20, mineral mix 35, vitamin mix 10, $CaHPO_4 \cdot 2H_2O$ 3.4, washed sand 12.6. Steroids were added in exchange for sand. The compositions of the vitamin and mineral mixtures have been described elsewhere (Schaafsma & Visser, 1980; AIN, 1977). Food and water were supplied ad libitum. Animal weights were recorded weekly and food intake was measured every two days. Feces were collected quantitatively during days 11-14 of the experiment.

Total fecal analyses. Fecal bile acid excretion was measured as described earlier (Van der Meer *et al.*, 1985). Briefly, freeze-dried feces were extracted with a t-butanol-water (1:1 vol/vol) mixture and subsequently bile acids were assayed using a fluorimetric enzymatic assay (Sterognost 3α-Flu, Nycomed AS, Oslo, Norway) (Beher *et al.*, 1981). Results obtained with this method correlate highly with those obtained

by the standard GLC-procedure (Van der Meer *et al.*, 1985).

[^{3}H]Thymidine incorporation. After the experimental feeding period, rats (non-fasted) were injected i.p. with [methyl-^{3}H]thymidine in saline (Amersham Int., Buckinghamshire, England; s.a. 25 Ci/mmol; dose 100 μCi/kg). Two hours later they were killed by decapitation after anesthetization with CO_2 and the lower third part of the large bowel was excised and opened; intestinal contents were removed by rinsing with ice-cold 154 mM KCl. After homogenization of the distal colon in 154 mM KCl, the macromolecular fraction was precipitated with 5% TCA (wt/vol), followed by 10 min centrifugation at 15,000*g as described by Bull *et al.* (1983). The supernatant was discarded and the pellet resuspended in 1 M $HClO_4$ and hydrolyzed twice at 70°C for 15 min according to Burton (1956). An aliquot (1.0 ml) of the hydrolysate was dissolved in 20.0 ml Aqua Luma (Lumac, 3M, Schaesberg, The Netherlands) and counted with corrections for quenching on a Beckman LS-7500 liquid scintillation counter. DNA was determined using the diphenylamine reaction with calf thymus DNA (Sigma) as standard as described by Burton (1956).

Preparation of fecal water. Fecal water was prepared by reconstituting freeze-dried feces with double-distilled water to 35% dry weight, which mimics the conditions in the distal colon (unpublished data). Control experiments showed that freeze-drying did not affect lytic activity of fecal water. Lytic activity was 61% ±13 for fecal water prepared from fresh feces and 59% ±15 for fecal water prepared from freeze-dried feces with a paired difference of 2% ±8 for four samples. After mixing, samples were incubated for 1 h at 37°C in a shaking waterbath followed by centrifugation for 10 min at 15,000*g (Eppendorf 5415). Centrifugation for 20 or 30 min showed no significant differences in bile acid content of fecal water nor did it affect the lytic activity. The supernatant was carefully aspirated and stored at -20°C until further use.

Fecal water analyses. Bile acids in fecal water were determined using the fluorimetric enzymatic assay mentioned above. Control experiments in which pure bile acids were added to fecal water showed that the fecal pigments in the fecal water did not influence this fluorimetric assay. Recovery of added pure bile acids (deoxycholate or deoxycholate+lithocholate) to fecal water always exceeded 95%. Free fatty acids were determined using a commercially available enzymatic assay (NEFA-C, Wako chemicals, Germany). Control experiments using pure fatty acids showed that the fecal pigments did not affect this determination of free fatty acids in fecal water. Recoveries of fatty acid standards to fecal water was >91%.

Lytic activity assay. The assay described in this paper is a modification of the methods described by Coleman *et al.* (1979) and Rafter *et al.* (1987). Human blood with EDTA as anti-coagulant and thimerosal as bacteriostatic agent was centrifuged for 15 min at 1,500*g. Plasma and buffy coat were removed and 1 volume of cells was washed three times with 5 volumes of buffer (1.5 mM HEPES, 154 mM NaCl; pH 7.4) before use. The incubation mixture contained fecal water or bile acids, buffer and/or NaCl, and erythrocytes (final hematocrit 5%). In each experiment blanks without erythrocytes, erythrocytes in double-distilled water (=100 % lysis) and erythrocytes in 154 mM NaCl (=0% lysis) were incubated simultaneously. Samples were incubated at 37°C for 30 min in a shaking waterbath. For the cell counter method, samples were diluted in buffer and counted with a cell counter (Sysmex microcell counter CC130) coupled to a multichannel analyzer (Tracer Northern Inc. TN-7200). Lysis was calculated as the relative decrease in counts compared with intact erythrocytes. Direct release of hemoglobin was determined in samples centrifuged for 1 min at 10,000*g (Eppendorf 5415). Supernatants were carefully aspirated and diluted with double-distilled water. Hemoglobin in the supernatant was measured as absorbance at 540 nm. As an alternative method for the determination of hemolysis, Fe-content was analyzed using a flame atomic absorption spectrophotometer (Perkin-Elmer 1100). Lysis was calculated relative to 100% lysed cells.

Statistics. Values are given as means with their standard errors. Every data point for one animal is the mean of triplicate measurements. Analysis of variance was performed for treatment effects of steroid supplementation on fecal bile acid concentration, bile acids and fatty acids in fecal water, lytic activity and [^{3}H]-thymidine incorporation into DNA. Differences between means of the groups for the different parameters were tested by Fisher's protected least significant difference (LSD) test (Steel & Torrie, 1980) using SPSS/PC+ v2.0 (SPSS Inc., Chicago, USA). Differences were regarded as significant if $P<0.05$. Linear regression analysis was performed on data comparing hemolysis and thymidine incorporation using SPSS/PC+ v2.0.

RESULTS

Feed intake (16.9±0.2 g) and growth characteristics (61.6±1.7 g/14 days) of the rats were not significantly affected by the diets. Fecal output (0.71±0.02 g dry/day) and percentage dry weight (72±2 % dry wt) were not significantly different between the

groups. Fecal bile acid concentration was drastically increased in a dose-dependent manner by supplementation with steroids (Table 1). Dietary supplementation with steroids increased colonic proliferation in vivo comparable with the increase in fecal bile acid concentration (Table 1). It should be noted that the basic cell-proliferation (control diet) was approx. 40 dpm/μg DNA, which is in accordance with other studies using male rats fed ad libitum (Bull *et al.*, 1983; DeRubertis & Craven, 1980). These increases in colonic proliferation after dietary supplementation with steroids have also been found by others (Deschner *et al.*, 1981; Bird *et al.*, 1986).

Because Newmark *et al.* (1984) hypothesized that the bile acid-induced colonic hyperproliferation is mediated by increases in soluble colonic surfactant concentrations, we quantified the bile acid and fatty acid concentrations in fecal water. Dietary supplementation with steroids increased the soluble bile acid concentration in a dose-dependent manner (Table 1). Whereas the fatty acid concentration was significantly lower on the control diet no further dose-dependent increase with dietary steroids was observed.

Table 1 Effects of dietary steroid supplementation on total and soluble bile acid concentrations, soluble fatty acid concentrations, lytic activity of fecal water and in vivo colonic proliferation measured as [^{3}H]-thymidine incorporation in DNA.

Diet	Fecal bile acids (μmol/g dry wt)	Soluble bile acids (mM)	Soluble fatty acids (mM)	Lytic activity (% hemolysis)	Colonic proliferation (dpm/μg DNA)
Control	20.3±0.8	4.4±0.5	2.8±0.6^{a}	0.3± 0.2	43.4±6.2^{a}
0.5% C+0.05% DC	66.9±2.7^{a}	21.7±1.5^{a}	6.4±2.2	30.5±11.4^{a}	62.8±7.8^{b,c}
0.5% C+0.15% DC	84.5±9.3^{b}	26.0±3.7^{a,b}	6.7±0.8	44.4± 7.0^{a}	59.5±3.9^{b}
1.0% C+0.05% DC	79.0±3.0^{b}	22.9±1.5^{a}	6.2±0.7	32.7± 3.2^{a}	62.7±6.5^{b}
1.0% C+0.10% DC	98.4±2.8^{c}	27.1±1.4^{a,b}	6.9±1.3	65.9± 4.0^{b}	70.4±1.9^{c}
1.0% C+0.15% DC	105.6±5.1^{c}	31.9±3.3^{b}	6.7±1.2	91.0± 2.7^{c}	81.4±4.6^{d}
ANOVA	P<0.001	P<0.001	P>0.3	P<0.001	P<0.004

% C and % DC indicate % cholesterol and % deoxycholate in diet, respectively. Values are Means ± SEs for 4 rats per group. Values in the same column not sharing the same superscript are signficantly different: P<0.05 (LSD-test). ANOVA denotes the F-probability for treatment effects.

These diet-dependent increases in soluble surfactant concentrations should, according to the hypothesis (Newmark *et al.*, 1984), result in an increased luminal lytic activity. Therefore, we ascertained whether luminal lytic activity can adequately be determined. Rafter *et al.* (1987) proposed that this can be achieved by measuring lysis of erythrocytes incubated with fecal water. Probably because fecal pigments interfere with the photometric measurement of hemoglobin as measure for hemolysis, they quantified lysis by means of cell-counting. First, we validated their method and compared it with an alternative method in which hemolysis is determined as Fe-release, measured with atomic absorption spectrophotometry.

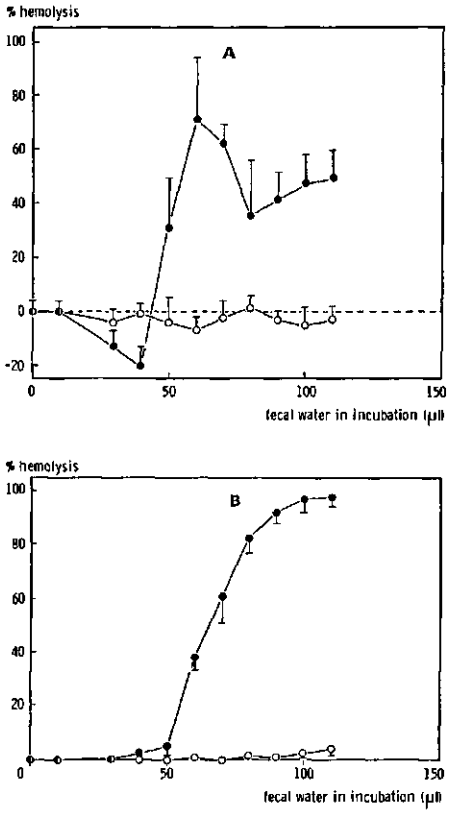


Figure 1 *Concentration dependence* of the hemolytic activity of rat fecal water measured by cell-count (Figure 1A) and by Fe-release determined by Atomic Absorption Spectrophotometry (Figure 1B). The incubation mixture contained increasing amounts of fecal water, 154 mM NaCl and erythrocytes at a final hematocrit of 5% in a total volume of 200 µl. Values shown *are means of three experiments* with their standards errors. ○: fecal water from rats fed a control diet

●: fecal water from rats fed a steroid-supplemented diet

Control experiments, using increasing concentrations of bile acids to induce hemolysis, showed that both methods gave identical lytic curves which were not different from the sigmoidal curves obtained with the standard photometric determination of hemoglobin at 540 nm (unpublished data). However, after incubation of erythrocytes with increasing amounts of fecal water, the cell-counting method resulted in an anamolous lytic curve (Figure 1A). In control experiments it appeared that lysed erythrocytes, added to fecal water, were still partly counted by this method. These results indicate that cell-counting in the presence of fecal water is liable to artifacts. In contrast, measurement of Fe-release gave a normal sigmoidal curve (Figure 1B) which is comparable with the lytic curves observed earlier (Van der Meer *et al.*, 1991) for the concentration dependence of bile-acid-induced hemolysis. Moreover, measurement of Fe was perfect linear (r=0.99, n=9) with the amount of lysed erythrocytes added to fecal water (not shown). Because of these results we concluded that measurement of Fe-release can adequately be used for the determination of the lytic activity of fecal water.

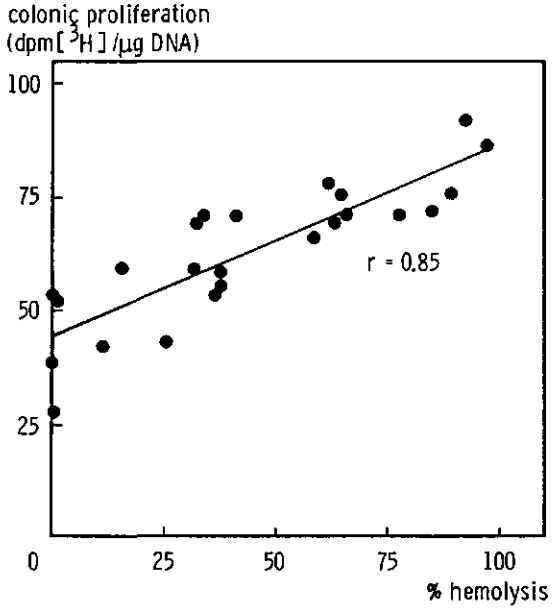


Figure 2 Relationship between colonic proliferation measured as [^{3}H]-thymidine incorporation in rats and lytic activity of fecal water measured as % hemolysis. The incubation mixture contained 10 µl fecal water, 150 µl 154 mM NaCl and 40 µl erythrocytes (final hematocrit: 5%). $y = 0.417x + 44.6$

As shown in Table 1, lytic activity of fecal water was drastically increased by dietary steroids analogous to the dose-dependent increases in total bile acid and soluble bile acid concentrations. To determine whether this increase in lytic activity is related to the observed colonic hyperproliferation, we correlated these data for the individual rats. As shown in Figure 2, a highly significant positive correlation ($r=0.85$, $n=24$, $p<0.001$) between lytic activity and colonic proliferation in vivo was observed, which is consistent with the cause-and-effect relationship proposed by Newmark *et al.* (1984).

DISCUSSION

Hyperproliferation of colonic epithelium is an important biomarker of the increased risk for colon cancer (Lipkin, 1988). As hypothesized by Newmark *et al.* (1984), diet may increase this risk because diet-dependent increases in colonic bile acids may damage colonic epithelial cells, resulting in a compensatory increase in proliferation of crypt cells. With regard to this, it has been shown that bile acids and fatty acids, either instilled intrarectally (Bull *et al.*, 1983; DeRubertis & Craven, 1984; Rafter *et al.*, 1986; Wargovich *et al.*, 1984) or added to diet (Deschner *et al.*, 1981; Bird *et al.*, 1986), induce proliferation of colonic epithelium. But in none of these studies the proposed intermediate steps between total bile acids and hyperproliferation have been quantified. As shown in Table 1, dietary steroid supplementation significantly increased the total bile acid concentration in a dose-dependent manner. This increased bile acid concentration then results in an increased concentration of soluble bile acids. In vitro experiments have shown that not the total but only the soluble bile acid concentration determine the lytic activity of bile acids (Van der Meer *et al.*, 1991). The fatty acid concentration is increased by steroid supplementation compared to the control diet probably due to a fatty acid-solubilizing effect of bile acids (Graham & Sackman, 1983), but no dose-dependent response was observed. Therefore our results indicate that the diet-dependent increase in luminal lytic activity is mainly due to the diet-dependent increase in soluble bile acids. The results of the present study show, to our knowledge for the first time, that diet-dependent lytic activity is highly correlated with colonic proliferation measured as tritiated thymidine incorporation into DNA ($r=0.85$, $P<0.001$). With regard to this validation it should be mentioned that this lytic activity should not be interpreted as lysis of epithelial cells.

Luminal lytic activity measures the lytic potency of the luminal surfactants. Lysis of epithelial cells, however, is determined by this lytic potency as well as by the susceptibility of the apical membrane exposed to these surfactants. Therefore, our data should not be interpreted as a quantitative measure of intestinal epitheliolysis caused by these surfactants, because determinants of the epithelial susceptibility are at present largely unknown.

The sequence of effects observed in the present study suggest that the relationship between total bile acid concentrations and colonic proliferation is mediated by soluble colonic bile acids and an increased luminal lytic activity. It should be stressed that this strong correlation does not prove a cause-and-effect relationships, because our study was done under steady-state conditions. However, these effects observed in vivo are consistent with cause-and-effect relationships observed in vitro (Van der Meer *et al.*, 1991; Velardi *et al.*, 1991). Firstly, it has been shown that only soluble bile acids are lytic (Van der Meer *et al.*, 1991). Secondly, the sigmoidal concentration dependence of the lytic activity of fecal water (Figure 1B) is similar to those observed for soluble bile acids (Van der Meer *et al.*, 1991). Thirdly, bile acid-induced lysis of erythrocytes is quantitatively comparable to bile acid-induced lysis of polarized colonic epithelial cells in vitro (Velardi *et al.*, 1991). Eventually, lysis of these epithelial cells is then compensated for by an increased colonic proliferation (Wargovich *et al.*, 1983). In addition, Craven *et al.* (1986) showed that intrarectal instillation of deoxycholate results in cell-sloughing and stimulation of tritiated thymidine incorporation into DNA. Whether activation of protein kinase C (Craven *et al.*, 1987) is involved in this compensatory hyperpoliferation is at present not known and requires further investigation.

For obvious reasons the relationship between colonic proliferation in vivo and lytic activity of fecal water cannot be quantified in humans. Therefore, we studied this relationship in the rat, which is generally considered to be a suitable animal model for studying effects on colonic proliferation. For instance, effects of dietary fat and calcium are qualitatively similar in rodents and humans (Deschner *et al.*, 1981; Bird *et al.*, 1986; Stadler *et al.*, 1988, Bird *et al.*, 1985; Fry & Staffeldt, 1964; Lipkin & Newmark, 1985; Lipkin *et al.*, 1989). Because of this similarity, it is reasonable to assume that our assay for screening luminal lytic activity might also be of relevance for studies with humans. Further support for this assumption can be obtained by comparison with proliferation in colonic biopsies and by studying the effects of known dietary

modulators of colonic proliferation like calcium (Lipkin *et al.*, 1989). Preliminary results from our diet-controlled study with healthy volunteers indeed indicate that supplemental dietary calcium inhibits lytic activity of fecal water (Van der Meer *et al.*, 1990).

In conclusion, dietary supplementation with steroids induces in rats an increase of the soluble bile acid concentration in fecal water. This results in an increased luminal lytic activity which probably results in intestinal cell-damage. Finally, this is followed by a compensatory increase in colonic proliferation. These intermediate steps between a diet-dependent increase in total bile acid concentration and colonic proliferation may explain how diet could affect proliferation of the colonic cells and consequently influence the risk for colon cancer.

Acknowledgements: This study was supported by Grant 663/88-1.7 from the European Community. The authors wish to thank G. Van Tintelen for taking care of the animals, H.T. De Vries for expert technical assistance and the Department of Toxicology of the Agricultural University Wageningen for the use of the cell counter.

REFERENCES

American Institute of Nutrition. Report of the American institute of nutrition ad hoc committee on standards for nutritional studies. *J Nutr* 1977;107:1340-1348

Beher WT, Stradnieks S, Lin GJ, Sanfield J. Rapid analysis of human fecal bile acids. *Steroids* 1981;38:281-295

Bird RP, Medline A, Furrer RJ, Bruce WR. Toxicity of orally administered fat to the colonic epithelium of mice. *Carcinogenesis* 1985;6:1063-1066

Bird RP, Schneider R, Stamp D, Bruce WR. Effect of dietary calcium and cholic acid on the proliferative indices of murine colonic epithelium. *Carcinogenesis* 1986;7:1657-1661

Bull AW, Marnett LJ, Dawe EJ, Nigro ND. Stimulation of deoxythymidine incorporation in the colon of rats treated intrarectally with bile acids and fats. *Carcinogenesis* 1983;4:207-210

Burton K. A study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem J* 1956;62:315-323

Coleman R, Iqbal S, Godfrey PP, Billington D. Membranes and bile formation: Composition of several

biles and their membrane-damaging properties. *Biochem J* 1979;178:201-208

Coleman R, Lowe PJ, Billington D. Membrane lipid composition and susceptibility to bile salt damage. *Biochim Biophys Acta* 1980;599:294-300

Craven PA, Pfanstiel J, Saito R, DeRubertis FR. Relationship between loss of rat colonic surface epithelium induced by deoxycholate and initiation of the subsequent proliferative response. *Cancer Res* 1986;46:5754-5759

Craven PA, Pfanstiel J, DeRubertis FR. Role of activation of protein kinase C in the stimulation of colonic epithelial proliferation and reactive oxygen formation by bile acids. *J Clin Invest* 1987;79:532-541

DeRubertis FR, Craven PA. Bile salt stimulation of colonic epithelial cell-proliferation. *J Clin Invest* 1984; 74:1614-1624

DeRubertis FR, Craven PA. Early alterations in rat colonic mucosal cyclic metabolism and protein kinase activity induced by 1,2-dimethylhydrazine. *Cancer Res* 1980;40:4589-4598

Deschner EE, Cohen BI, Raicht RF. Acute and chronic effect of dietary cholic acid on colonic epithelial cell-proliferation. *Digestion* 1981;21:290-296

Fry RJM, Staffeldt E. Effect of a diet containing sodium deoxycholate on the intestinal mucosa of the mouse. *Nature* 1964;203:1397-1398

Gaetti E, Salvioli GF, Gualdi GL, Lugli R. Effect of bile salts (BS) on red blood cell (RBC) membrane: a method to evaluate their membrane-damaging effect. Abstract *Int. Meeting on Pathochemistry, Pathophysiology and Pathomechanics of the biliary system.* Bologna, Italy 1988

Graham DY, Sackman JW. Solubility of calcium soaps of long-chain fatty acids in a simulated intestinal environment. *Dig Dis Sci* 1983;28:733-736

Lipkin M, Newmark HL. Effect of added dietary calcium on colonic epithelial cell-proliferation in subjects with a high risk for familial colonic cancer. *N Eng J Med* 1985;313:1381-1384

Lipkin M. Biomarkers of increased susceptibility to gastrointestinal cancer: a new application to studies of cancer prevention in human subjects. *Cancer Res* 1988;48:235-245

Lipkin M, Friedman E, Winawer SJ, Newmark HL. Colonic epithelial cell-proliferation in responders and nonresponders to supplemental dietary calcium. *Cancer Res* 1989;49:248-254

Newmark HL, Wargovich MJ, Bruce WR Colon cancer and dietary fat, phosphate and calcium: a hypothesis. *J Natl Cancer Inst* 1984;72:1323-1325

Rafter JJ, Eng VWS, Furrer R, Medline A, Bruce WR. Effects of calcium and pH on the mucosal damage produced by deoxycholic acid in the rat colon. *Gut* 1986;27:1320-1329

Rafter JJ, Child P, Anderson AM, Alder R, Eng VWS, Bruce WR. Cellular toxicity of fecal water depends on diet. *Am J Clin Nutr* 1987;45:559-563

Salvioli G, Mambrini A, Lugli R, Berlotani R, Zirilli E. Effects of different bile salts on erythrocyte membranes. In: Barbara L, Dowling RH, Hofmann AF, Roda E. (eds). *Recent advances in bile acid research.* Raven Press, New York, 1985:17-27

Schaafsma G, Visser R. Nutritional interrelationships between calcium, phosphorus and lactose in rats. *J Nutr* 1980;110:1101-1111

Stadler J, Stern HS, Yeung KS, McGuire V, Furrer R, Marcon N, Bruce WR. Effect of high fat consumption on cell proliferation activity of colorectal mucosa and on soluble faecal bile acids. *Gut* 1988;29:1326-1331

Steel RGD, Torrie JH. *Principles and Procedures of Statistics; A Biometrical Approach.* McGraw-Hill, Tokyo, 1980.

Van der Meer R, De Vries HT, Glatz JFC. t-Butanol extraction of feces: a rapid procedure for enzymic determination of fecal bile acids. In: Beynen AC, Geelen MJH, Katan MB, Schouten JA (eds). *Cholesterol Metabolism in Health and Disease: Studies in The Netherlands.* Ponsen & Looijen, Wageningen 1985:113-119

Van der Meer R, Termont DSML, De Vries HT. Differential effects of calcium ions and calcium phosphate on cytotoxicity of bile acids. *Am J Physiol* 1991;260:G142-G147

Van der Meer R, Lapré JA, Kleibeuker JH, De Vries EGE, De Vries HT. Effects of supplemental dietary calcium on composition and cytotoxicity of fecal water. *Gastroenterology* 1990;98:A317

Velardi ALM, Groen AK, Oude Elferink RJP, Van der Meer R, Palasciano G, Tytgat GNJ. Cell-type dependent effect of phospholipid and cholesterol on bile salt cytotoxicity. *Gastroenterology* 1991;101:457-464

Wargovich MJ, Eng VWS, Newmark HL, Bruce WR. Calcium ameliorates the toxic effect of deoxycholic acid on colonic epithelium. *Carcinogenesis* 1983;4:1205-1207

Wargovich MJ, Eng VWS, Newmark HL. Calcium inhibits the damaging and compensatory proliferative effects of fatty acids on mouse colon epithelium. *Cancer Letters* 1984;23:253-258

Weisburger JH, Wynder EL. Etiology of colorectal cancer with emphasis on mechanism of action and prevention. In: DaVita VT, Hellman S, and Rosenberg SA (eds) *Important advances in oncology 1987*, JB Lippincot, Philadelphia, 1987:197-220

Willett WC. The search for the causes of breast and colon cancer. *Nature* 1989;338:389-394

Willett WC, Stampfer MJ, Colditz GA, Rosner BA, Speizer FE. Relation of meat, fat, and fiber intake to the risk of colon cancer in a prospective study among women. *N Eng J Med* 1990;323:1664-1672

CHAPTER 5 DIETARY CALCIUM PHOSPHATE INHIBITS CYTOTOXICITY OF FECAL WATER.

John A. Lapré, Hielke T. De Vries and Roelof Van der Meer.
Am J Physiol 1991;261:G907-G912

ABSTRACT

The effects of dietary steroid and $CaHPO_4$ supplementation on the solubility of bile acids and cytotoxicity of fecal water were studied in rats. Dietary steroid supplementation increased the bile acid concentration of both feces and fecal water. $CaHPO_4$ supplementation produced a slight increase in total fecal bile acid concentration, but resulted in a drastic decrease in soluble bile acid concentration. Cytotoxicity of fecal water on control and steroid supplemented diets decreased with $CaHPO_4$ supplementation analogous to the decrease in soluble bile acid concentration. The concentrations of precipitated Ca and P_i were highly correlated ($r>0.90$) with the concentration of precipitated bile acids and with inhibition of cytotoxicity. But there was no significant correlation between the logarithms of soluble calcium and soluble bile acids indicating that solubility of bile acids is not determined by soluble calcium. Concentrations of calcium and phosphate in fecal water indicated the formation of insoluble calcium phosphate in the intestine. Thus, dietary $CaHPO_4$ causes a decrease in soluble bile acid concentration, which is probably due to the formation of an insoluble bile acid calcium phosphate complex. Consequently, cytotoxicity of fecal water is inhibited, which might have implications for the protective effect of dietary calcium with regard to colonic cancer in humans.

INTRODUCTION

Epidemiological data suggest that dietary fat may promote the risk of colorectal cancer (Weisburger & Wynder, 1987; Zaridze, 1983). With regard to the mechanism of this promotive effect, it has been proposed that dietary fat increases the colonic concentration of surfactants, like bile acids and fatty acids (Bruce, 1987). In animal studies these acids have been shown to stimulate proliferation of colonic epithelial cells, probably because of their intrinsic cytotoxic properties (Bull *et al.*, 1983; Wargovich *et al.*, 1983; DeRubertis *et al.*, 1984; Rafter *et al.*, 1986). Also in humans dietary fat stimulated proliferation of colonic epithelium (Stadler *et al.*, 1988). This hyperproliferation of colonic epithelium is an important biomarker of increased susceptibility to colon cancer (see Lipkin, 1988 for review).

It was shown that only **soluble** colonic bile acids (Rafter *et al.*, 1986) and fatty acids (Wargovich, 1988) are toxic to cells and consequently increase proliferation of crypt cells. This indicates that the soluble bile acids and fatty acids in fecal water are

physiologically relevant determinants of intestinal cytotoxicity. Dietary components may modulate intestinal cytotoxicity and hyperproliferation by changing the solubility of these surfactants in the intestinal lumen. For instance, dietary calcium supplementation inhibits colonic cell proliferation in rodents (Wargovich *et al.*, 1983; Bird, 1986; Bird *et al.*, 1986; Rafter *et al.*, 1986; Skraastad & Reichelt, 1988) and in humans (Lipkin & Newmark, 1985; Buset *et al.*, 1986; Wargovich, 1988; Lipkin *et al.*, 1989). One study failed to find a protective effect of dietary calcium on proliferation of colonic cells in patients at high risk for colon cancer (Gregoire *et al.*, 1989). With regard to the mechanism of this protective effect of calcium, Newmark *et al* (1984) proposed that soluble calcium (Ca^{2+}) precipitates luminal bile acids and fatty acids and thus inhibits cytotoxicity and hyperproliferation. They also hypothesized that a calcium ligand like phosphate should inhibit this protective effect of Ca^{2+} because of formation of insoluble calcium phosphate (CaP_i) in the intestine. This proposed molecular mechanism is in contrast to an alternative hypothesis (Van der Meer & De Vries, 1985) implying that bile acids bind to insoluble CaP_i, which is supported by binding studies (Van der Meer & De Vries, 1985; Van der Meer *et al.*, 1991) and by resolubilization studies (Van der Meer *et al.*, 1990a). Recently we (Van der Meer *et al.*, 1991) investigated the molecular mechanisms of these opposing hypotheses and found that cytotoxicity of bile acids is stimulated by Ca^{2+} (see also Child & Rafter, 1986), which is in contrast to the hypothesis of Newmark *et al.* (1984). On the contrary, cytotoxicity was inhibited by CaP_i, which was due to binding of carboxylic, primary and secondary, bile acids. The present study addresses the physiological relevance of this protective effect of CaP_i observed in vitro, in a nutritional experiment with rats. Instead of colonic instillation used in other studies (Wargovich *et al.*, 1983; Bull *et al.*, 1984; DeRubertis *et al.*, 1984; Wargovich *et al.*, 1984), steroids were supplemented in the diet to increase the total concentration of bile acids in the colon. Diets were also supplemented with increasing amounts of $CaHPO_4$ since in both animal and human diets, calcium is always associated with at least equimolar amounts of phosphate. It should be noted that the lowest level of $CaHPO_4$ in the diets is almost comparable to the human intake of dietary calcium. Cytotoxicity of fecal water was measured using hemolysis of human erythrocytes quantified by measuring Fe-release by atomic absorption spectrophotometry. This determination of cytotoxicity was highly correlated with the in vivo determination of colonic cell proliferation (Lapré & Van der Meer, 1990).

MATERIALS and METHODS

Animals and diets. Female outbred Wistar rats (Small Animal Research Center of the Wageningen Agricultural University) six weeks old were housed individually at a constant temperature of 21°C and fed a laboratory chow (RHMB-Hope Farms, Woerden, The Netherlands) for two weeks. During the experimental period of four weeks, groups of rats (six rats per group) were fed a purified diet which differed only in $CaHPO_4$ content and in supplementation with 1% (wt/wt) cholesterol and 0.15% (wt/wt) sodium deoxycholate. Cholesterol supplementation is known to stimulate bile acid synthesis and excretion in rats. Thus, the extent of the colonic interactions of bile acids, calcium and phosphate could be increased. The composition of the control, low $CaHPO_4$ diet was (g/kg diet): casein (acid casein, DMV Veghel, The Netherlands) 200, dextrose 489, sun-flower oil 200, cellulose 20, mineral mix 35, vitamin mix 10, $CaHPO_4{\cdot}2H_20$ 3.4 and acid-washed sand 42.6. In the control, high $CaHPO_4$ diet sand was 12 g/kg diet. In the steroid supplemented diets sand is 31, 25.9 and 0.4 g/kg diet for the low, middle and high $CaHPO_4$ diets, respectively. The levels of $CaHPO_4$ supplementation are shown in Table 1. The compositions of the vitamin and mineral mixtures have been described (Schaafsma & Visser, 1980; AIN, 1977). Feed and water were supplied ad libitum. Animal weights were recorded weekly and feed intake was measured every 2 days. Feces were collected quantitatively during days 25-28 of the experiment.

Fecal water preparation. Fecal water was prepared by reconstituting freeze-dried feces with double-distilled water to 35% dry weight, which reflects the wet weight to dry weight condition in the distal rat colon (unpublished data). Control experiments showed that fecal water from freeze-dried feces using our procedure did not differ significantly from fecal water prepared from fresh feces in calcium (99% relative to fecal water prepared from fresh feces), phosphate (93%), bile acid (93%) and fatty acid (88%) concentrations. Only a slight, but significant decrease in pH of 0.4 units was observed after freeze-drying, probably caused by evaporation of NH_3. After homogenizing, the samples were incubated for one hour at 37°C in a shaking waterbath followed by centrifugation for 10 min at 15,000*g (Eppendorf 5415). Centrifugation for 20 or 30 min produced no significant differences in bile acid, calcium and phosphate content of fecal water nor did it affect its cytotoxicity (data not shown). The supernatant was carefully aspirated and pH was measured at 37°C. Samples were stored at -20°C until further use.

Hemolysis assay. The hemolysis assay described in this paper is a modification of the method described by Rafter *et al.* (1987). Lysis could not be determined properly by spectrophotometric measurement of hemoglobin (540 nm), because of the fecal pigments present. Since we found in control experiments that counting of the remainder of intact cells using a cell-counter is liable to artifacts (Lapré & Van der Meer, 1990), we used Fe-release quantified by atomic absorption spectrophotometry as a measure of hemolysis. Human blood drawn by venipuncture, with EDTA as anti-coagulant (final concentration 4 mM) and thimerosal as bacteriostatic agent, was centrifuged for 15 min at 1,500*g. Plasma and buffy coat were removed and 1 volume of cells was washed three times with 5 volumes of buffer (1.5 mM HEPES, 154 mM NaCl pH 7.4). The incubation mixture contained 50 µl fecal water, 110 µl 154 mM NaCl and 40 µl cells (final hematocrit: 5%). No differences in cytotoxicity of fecal water were observed when the incubation was carried out at a constant pH of 7.4 using HEPES (final concentration 30 mM). In each experiment, blanks without erythrocytes, erythrocytes in double-distilled water (= 100% lysis) and erythrocytes in 154 mM NaCl (= 0% lysis) were incubated simultaneously. Samples were incubated at 37°C for 30 min (control and steroid supplemented diets) or 120 min (control diets only) in a shaking waterbath, then centrifuged for 1 min at 10,000*g (Eppendorf 5415). Supernatants were carefully aspirated and diluted with double-distilled water. Fe-content as a measure of erythrocyte lysis was analyzed using an atomic absorption spectrophotometer (Perkin-Elmer 1100). Lysis was calculated relative to the 100% lysis samples.

Total feces analyses. Fecal bile acid excretion was measured as described elsewhere (Van der Meer *et al.*, 1985). Briefly, freeze-dried feces were extracted with a t-butanol-water (1:1, vol/vol) mixture and subsequently bile acids were assayed enzymatically (Turley & Dietschy, 1978). Calcium was measured after extraction with trichloroacetic acid (TCA) (final concentration: 5% wt/vol) in an atomic absorption spectrophotometer and inorganic phosphate was determined in the TCA-extract using the method described by Fiske and Subbarow (1925).

Fecal water analyses. Bile acids in fecal water were determined using a fluorimetric enzymatic assay (Sterognost 3α-Flu, Nyegaard, Norway) (Turley & Dietschy, 1978). Free fatty acids were assayed using an enzymatic method (NEFA-C kit, Wako Chemicals, West Germany). Calcium and inorganic phosphate were measured after extraction with TCA as described above. All analyses were performed in triplicate and

appropriate reference samples and standards were measured simultaneously.

Solubility product analyses. Na_2HPO_4 (final concentration: 20 mM) was added to solutions containing $CaCl_2$ (final concentration: 20 mM), buffer (final concentration: 100 mM) and NaCl to maintain a constant ionic strength of 150 mM. MES (2[N-Morpholino]-ethanesulfonic acid) was used as buffer in the pH range 5.5-6.5 and MOPS (3-[N-Morpholino]-propanesulfonic acid) in the range 6.6-7.4. After 15 min incubation at 37°C, samples were centrifuged for 2 min at 10,000*g. The supernatants were collected and the pH was measured. After acidification with TCA (final concentration: 5% wt/vol), calcium and phosphate were measured as described above.

Statistics. Values are given as means of six rats per group with standard errors. After analysis of variance the differences between the means of the calcium groups were tested with a two-sided Student's t-test for comparing group means. Differences were regarded as significant if $P<0.05$.

RESULTS

Feed intake and body weight gain were not significantly affected by steroid and $CaHPO_4$ supplementation, nor was total fecal output (mean 0.84, SE 0.03 g dry wt/day) affected. The experimental diets did not induce any changes in dry weight to wet weight ratio (mean 0.78, SE 0.02). As shown in Table 1, the bile acid concentration in feces was drastically increased by dietary supplementation with steroids and slightly increased by dietary $CaHPO_4$. Fecal calcium and fecal inorganic phosphate were significantly increased by dietary $CaHPO_4$.

Fecal water pH increased significantly with increasing concentrations of dietary $CaHPO_4$ (Table 1). The concentrations of precipitated calcium and phosphate were drastically increased by dietary calcium phosphate supplementation. Calcium supplementation from 20 µmol $CaHPO_4$/g diet to 50 µmol $CaHPO_4$/g diet increased the soluble calcium concentration. However, additional supplementation to 200 µmol $CaHPO_4$/g diet did not further increase soluble calcium concentrations, but drastically increased precipitated calcium concentrations.

Table 1 Effects of dietary steroid and $CaHPO_4$ supplementation on fecal parameters and on fecal excretion of minerals and bile acids expressed in µmol/g dry wt.

	CONTROL (no added steroids)		DIET WITH STEROIDS		
$CaHPO_4$ in diet (µmol/g diet)	20	200	20	50	200
Bile Acids					
Total	4.9 ± 0.5	6.5 ± 0.7[a]	39.3 ± 2.7	40.1 ± 2.7	51.7 ± 2.4[a]
Soluble	1.5 ± 0.1	1.0 ± 0.1[a]	21.4 ± 2.4	16.0 ± 1.1[a]	7.5 ± 1.3[b]
Precipitated	3.4 ± 0.5	5.5 ± 0.7[a]	17.9 ± 1.4	24.1 ± 1.8[a]	44.2 ± 2.4[b]
Calcium					
Total	31.3 ± 5.7	1690 ±20[a]	56.6 ±10.3	255 ±15[a]	1830±44[b]
Soluble	4.9 ± 1.3	18.5 ± 2.5[a]	9.5 ± 2.1	26.2± 2.8[a]	21.6± 2.2[a]
Precipitated	26.4 ± 4.4	1670 ±24[a]	47.1 ± 8.4	229. ±14[a]	1807±45[b]
Inorganic Phosphate					
Total	55.3 ±11	1157 ±40[a]	85.8 ± 7.6	200 ±12[a]	1200±90[b]
Soluble	23.1 ± 5.2	23.7± 8.6	41.2 ± 3.8	33 ± 2.4[a]	14.6± 2.0[b]
Precipitated	32.2 ± 6.0	1133 ±35[a]	44.6 ± 7.3	167 ±10[a]	1184±39[b]
pH of fecal water					
	5.97 ± 0.03	6.29 ± 0.06[a]	5.89 ± 0.05	5.97 ± 0.04	6.41 ±0.06[a]

Values are means of six rats ± SE. Values in the same row for control diets or for diets without steroids not sharing the same superscript are significantly different: $P< 0.05$.

Subsequently, we ascertained whether fecal soluble calcium and phosphate were in equilibrium with precipitated calcium phosphate. Therefore we quantified this equilibrium in vitro and compared the results with those obtained in feces. As shown in Figure 1, this equilibrium is in vitro reflected by a linear relationship between pH and the logarithm of the concentration product of calcium and phosphate in the equilibrated supernatants. This linear relationship for the solubility product versus pH is in accordance with the theory of calcium phosphate precipitation. For fecal calcium and phosphate also a linear relationship ($r=-0.94$) was observed, which paralleled the curve for the solubility product of insoluble calcium phosphate in vitro (Figure 1). It should be noted that in vitro the precipitate had an apparent stoichiometry of tricalcium phosphate ($Ca_3(PO_4)_2$) which is similar to the stoichiometry of precipitated calcium and phosphate in feces of the $CaHPO_4$ supplemented groups (Table 1).

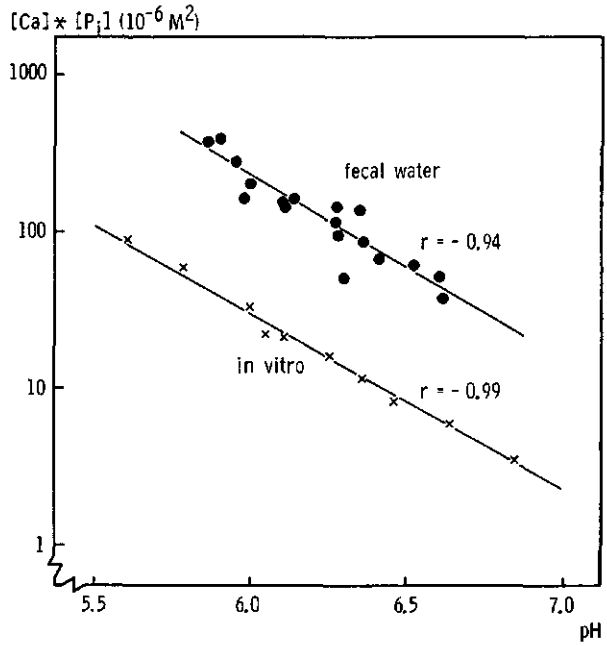


Figure 1 Logarithm of solubility product of calcium and phosphate in vitro at a ionic strength of 150 mM and in vivo in fecal water of rats fed diets supplemented with $CaHPO_4$ vs. pH

Free fatty acid concentration in fecal water showed a slight, but not significant decrease with dietary $CaHPO_4$ supplementation in the steroid-supplemented groups (Figure 2A). In the control groups there was a slight, but not significant increase in free fatty acid concentration. Dietary steroid supplementation drastically increased the bile acid concentration in fecal water (Figure 2B). This increase was counteracted by dietary $CaHPO_4$ supplementation. Instead of the observed slight increase in total bile acid concentration in feces with increasing $CaHPO_4$ levels, the soluble bile acid concentration is decreased by dietary $CaHPO_4$ (Table 1 & Fig. 2b). As can be seen in Table 1, the concentration of precipitated bile acid was drastically increased by dietary $CaHPO_4$ supplementation. The logarithms of the concentrations of precipitated bile acid (µmol/g feces) and precipitated calcium were highly correlated (r=0.91, n=18), as were the logarithms of the concentrations of precipitated bile acid and precipitated phosphate (r=0.91, n=18). No significant correlation was found between the logarithms of the soluble calcium and soluble bile acid concentrations (r=-0.23, n=18).

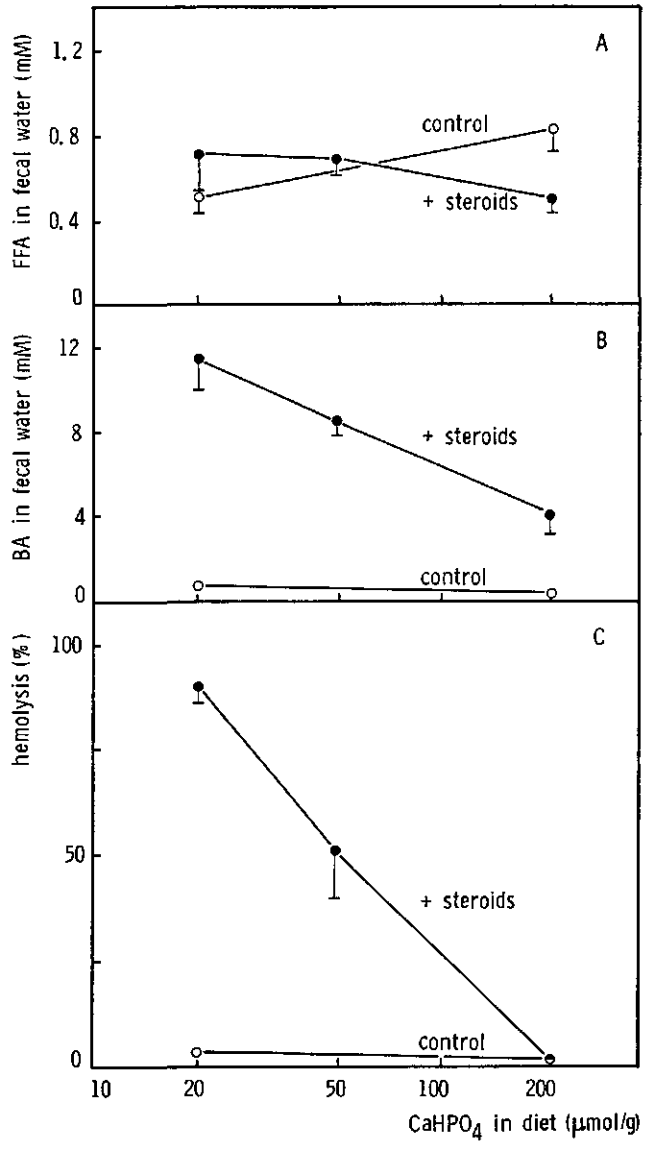


Figure 2 Free fatty acid concentration (A), bile acids concentration (B), and hemolysis (C) in fecal water of rats on control diets and diets supplemented with steroids vs. $CaHPO_4$ level in diet (log scale). Values are means of 6 rats. SEs are either smaller than size of symbols or indicated by bars.

Hemolysis by fecal water was dramatically increased by dietary steroid supplementation at low dietary $CaHPO_4$ levels. In contrast, dietary $CaHPO_4$ supplementation completely counteracted this increase to a level comparable with the

control groups (Figure 2C). The same protective effects of dietary $CaHPO_4$ supplementation were observed when the hemolysis assay was carried out at a constant pH of 7.4. This decrease in cytotoxic potential by dietary $CaHPO_4$ supplementation paralleled the decrease in bile acid concentration of the fecal water, as can be seen in Figure 2. Concentrations of precipitated calcium, phosphate and bile acid were significantly negatively correlated with cytotoxicity (resp. r=-0.91, r=-0.91, r=-0.90, n=18). The soluble calcium concentration was slightly negatively correlated with cytotoxicity (r=-0.49, n=18).

It should be noted that dietary $CaHPO_4$ also significantly decreased (from 4.0% ± 0.5 (SE) to 0.6% ± 0.0 (SE)) the cytotoxicity of fecal water. In Figure 2C this effect is difficult to see because the incubation time was dictated by the high cytotoxicity of the steroid supplemented samples. We have also incubated the control fecal waters for two hours instead of thirty minutes to increase the sensitivity of this assay. Under these conditions, $CaHPO_4$ very significantly decreased the cytotoxicity from 41.4% ± 4.5 (SE) to 8.7% ± 0.1 (SE). This illustrates that the protective effects of dietary calcium phosphate on cytotoxicity of fecal water exist on both steroid-supplemented and control diets.

DISCUSSION

This study shows the importance of the distribution of bile acids in feces in relation to intestinal cytotoxicity. Dietary steroid supplementation drastically increased the bile acid concentration of both feces and fecal water in rats. Dietary $CaHPO_4$ supplementation slightly increased total fecal bile acid concentration, which is in accordance with Van der Meer *et al.*, who found an increased fecal bile acid excretion in humans supplemented with calcium carbonate (Van der Meer *et al.*, 1990a). In contrast, in fecal water of the rats the soluble bile acid concentration was drastically decreased by dietary $CaHPO_4$ supplementation.

This study confirms in an in vivo model the results found in in vitro studies that soluble bile acids are bound by insoluble CaP_i (Van der Meer & De Vries, 1985; Van der Meer *et al.*, 1990a; Van der Meer *et al.*, 1991). These in vitro studies indicated that this binding is due to adsorption of bile acid micelles to insoluble calcium phosphate. As a consequence of this binding, cytotoxicity is inhibited (Van der Meer *et al.*, 1991). In addition, resolubilization studies in feces of human volunteers supplemented with calcium carbonate showed that calcium, inorganic phosphate and

bile acids were closely associated (Van der Meer *et al.*, 1990a). It should be noted that the ratio of precipitated calcium and precipitated phosphate is about 1.5 which is in accordance with the stoichiometry of insoluble calcium phosphate observed in our in vitro experiments (Van der Meer & De Vries, 1985; Van der Meer *et al.*, 1990a; Van der Meer *et al.*, 1991). The positive correlations between precipitated calcium and precipitated phosphate with respect to the concentration of precipitated bile acids support the proposed mechanism that bile acids bind to insoluble calcium phosphate. In contrast, the logarithm of the soluble calcium concentration was not significantly correlated with the logarithm of the soluble bile acid concentration ($r=-0.23$, $n=18$). When the precipitation of bile acids was due to formation of $Ca(BA)_2$ as proposed by Newmark *et al.* (1984), then a significant negative correlation should have existed. The negative correlations between precipitated concentrations of calcium, phosphate and bile acids in relation to cytotoxicity also indicate a protective effect of intestinal Ca-Pi-bile acid complexes.

For the $CaHPO_4$-supplemented groups, the concentration product of calcium and phosphate in fecal water plotted versus pH of fecal water results in a correlation coefficient of -0.94 ($P<0.001$) (Figure 1). This line parallels the pH dependency of the solubility product of calcium and phosphate in vitro. This clearly indicates that the soluble concentrations of calcium and phosphate are in equilibrium with insoluble calcium phosphate. It should be noted that the in vivo regression line shows a different intercept. This can be explained by the lower activity coefficients for calcium and phosphate in fecal water compared to the in vitro experiment. This is probably due to the higher viscosity of fecal water, but this requires further investigation.

No association between hemolysis and free fatty acid concentration in fecal water was found, whereas the decrease in hemolysis caused by dietary $CaHPO_4$ supplementation was analogous to the decrease in bile acid concentration of fecal water. Clearly, in this study using steroid-supplemented diets the role of fatty acids in cell damage was overwhelmed by the role of bile acids. However, in the control groups without added steroids, all parameters tested were significantly decreased by supplemental calcium except for the fatty acid concentration in fecal water. The possible role of fatty acids concerning the cytotoxicity of fecal water has to be evaluated in experiments using different amounts or different sources of dietary fat in combination with supplemental calcium.

This nutritional experiment with rats supports the hypothesis that bile acids in

the intestinal lumen are precipitated due to binding to insoluble calcium phosphate. The few other studies using rats or mice where both calcium and bile acids/fatty acids were supplemented in the diet used a Ca:P_i ratio of 1 in diet, which is comparable to our study (Bird, 1986; Bird *et al.*, 1986; Caderni *et al.*, 1988; Skraastad & Reichelt, 1988). These studies showed a decreased colonic cell-proliferation following dietary $CaHPO_4$ supplementation. This implies the absence of an inhibitory effect of dietary phosphate on the prevention of colonic cell-proliferation by calcium. The studies cited above did not present any data concerning the excretion of bile acids, calcium and phosphate in the feces and in fecal water, which would have provided additional information about the association of bile acids, calcium and phosphate in feces nor was intestinal cytotoxicity tested. The results of the studies cited above and of our study are in contrast with a recent study by Hu *et al.* (1989), who showed an inhibitory effect of phosphate on the antiproliferative effect of calcium. In this study, bile acids were given intrarectally and calcium and phosphate by a short oral intubation (about 24 h) instead of by dietary supplementation. In our opinion, this artificial design precludes the normal physiological interactions between calcium, phosphate and bile acids in the small and large intestine and thus limits the physiological relevance of this study.

In conclusion, our results showed that supplemental $CaHPO_4$ lowers the soluble bile acid concentration in fecal water probably by binding of bile acids to insoluble calcium phosphate. This results in a decreased cytotoxicity of fecal water analogous to our in-vitro studies (Van der Meer *et al.*, 1991). Our observations that the concentrations of precipitated bile acid, precipitated calcium and precipitated phosphate were closely associated, indicate that phosphate takes part in the complexation of calcium and bile acids. The present findings suggest that the results from our in vitro experiments can be extrapolated to animal studies and that this might have implications for the protective effect of dietary calcium with regard to colonic cancer in humans (Van der Meer *et al.*, 1990b).

Acknowledgements: This study was supported by Grant 663/88-1.7 from the European Community and by the Netherlands Organization for Scientific Research (NWO), Medical Sciences by Grant 900-562-078. The authors wish to thank Dr. G.C.Van der Plas for kindly providing human blood and G. Van Tintelen for taking care of the animals.

REFERENCES

American Institute of Nutrition. Report of the American institute of nutrition ad hoc committee on standards for nutritional studies. *J Nutr* 1977;107:1340-1348

Bird RP. Effect of dietary components on the pathobiology of colonic epithelium: Possible relationship with colon tumorigenesis. *Lipids* 1986;21:289-292

Bird RP, Schneider R, Stamp D, Bruce WR. Effect of dietary calcium and cholic acid on the proliferative indices of murine colonic epithelium. *Carcinogenesis* 1986;7:1657-1661

Bruce WR. Recent hypotheses for the origin of colon cancer. *Cancer Res* 1987;47:4237-4242

Bull AW, Marnett LJ, Dawe EJ, Nigro ND. Stimulation of deoxythymidine incorporation in the colon of rats treated intrarectally with bile acids and fats. *Carcinogenesis* 1983;4:207-210

Buset M, Lipkin M, Winawer S, Swaroop S, Friedman E. Inhibition of human colonic epithelial cell proliferation in vivo and in vitro by calcium. *Cancer Res* 1986;46:5426-5430

Caderni G, Stuart EW, Bruce WR. Dietary factors affecting the proliferation of epithelial cells in the mouse colon. *Nutr Cancer* 1988;11:147-153

Child P, Rafter J. Calcium enhances the hemolytic action of bile salts. *Biochim Biophys Acta* 1986;8 55:357-364

DeRubertis FR, Craven PA, Saito R. Bile salt stimulation of colonic epithelial proliferation. *J Clin Invest* 1984;74:1614-1624

Fiske CH, Subbarow Y. The colorimetric determination of phosphorus. *J Biol Chem* 1925;66:375-400

Gregoire RC, Stern HS, Yeung KS, Stadler J, Langley S, Furrer R, Bruce WR. Effect of calcium supplementation on mucosal cell proliferation in high risk patients for colon cancer. *Gut* 1989;30:376-382

Holdsworth CD. *Intestinal Absorption in Man.* New York, Academic Press 1975:223-262

Hu PJ, Baer AR, Wargovich MJ. Calcium and phosphate: effect of two dietary confounders on colonic epithelial cellular proliferation. *Nutr Res* 1989;9:545-553

Lapré JA, Van der Meer R. Relationship between diet-induced cytotoxicity of fecal water and in-vivo proliferation of colonic cells. *Gastroenterology* 1990;98:A292

Lipkin M. Biomarkers of increased susceptibility to gastrointestinal cancer: a new application to studies of cancer prevention in human subjects. *Cancer Res* 1988;48:235-245

Lipkin M, Friedman E, Winawer SJ, Newmark H. Colonic epithelial cell-proliferation in responders and nonresponders to supplemental dietary calcium. *Cancer Res* 1989;49:248-254

Lipkin M, Newmark HL. Effect of added dietary calcium on colonic epithelial-cell proliferation in subjects with a high risk for familial colonic cancer. *N Eng J Med* 1985;313:1381-1384

Newmark HL, Wargovich MJ, Bruce WR. Colon cancer and dietary fat, phosphate and calcium: a hypothesis. *J Natl Cancer Inst* 1984;72:1323-1325

Rafter JJ, Child P, Anderson AM, Alder R, Eng VWS, Bruce WR. Cellular toxicity of fecal water depends on diet. *Am J Clin Nutr* 1987;45:559-563

Rafter JJ, Eng VWS, Furrer R, Medline A, Bruce WR. Effects of calcium and pH on the mucosal damage produced by deoxycholic acid in the rat colon. *Gut* 1986;27:1320-1329

Schaafsma G, Visser R. Nutritional interrelationships between calcium, phosphorus and lactose in rats.

J Nutr 1980;110:1101-1111

Skraastad D, Reichelt KL. An endogenous colon mitosis inhibitor and dietary calcium inhibit the increased colonic cell-proliferation induced by cholic acid. *Scand J Gastroenterology* 1988;23:801-807

Stadler J, Stern HS, Yeung KS, McGuire V, Furrer R, Marcon N, Bruce WR. Effect of high fat consumption on cell proliferative activity of colorectal mucosa and on soluble faecal bile acids. *Gut* 1988;29:1326-1331

Turley SD, Dietschy JM. Re-evaluation of the 3α-hydroxysteroid dehydrogenase assay for total bile acids in bile. *J Lipid Res* 1978;19:924-928

Van der Meer R, De Vries HT. Differential binding of glycine- and taurine conjugated bile acids to insoluble calcium phosphate. *Biochem J* 1985;229:265-268

Van der Meer R, De Vries HT, Glatz JFC. *Cholesterol Metabolism in Health and Disease: Studies in the Netherlands.* Wageningen, Ponsen & Looijen; 1985:113- 119

Van der Meer R, Termont D, De Vries HT. Differential effects of calcium ions and calcium phosphate on cytotoxicity of bile acids. *Am J Phys* 1991;260:G142-G147

Van der Meer R, Welberg JWM, Kuipers F, Kleibeuker JH, Mulder NH, Termont DSML, Vonk RJ, De Vries HT, De Vries EGE. Effects of supplemental calcium on the intestinal association of calcium, phosphate and bile acids. *Gastroenterology* 1990a;99:1653-1659

Van der Meer R, Lapré JA, Kleibeuker JH, De Vries EGE, De Vries HT. Effects of supplemental dietary calcium on composition and cytotoxicity of fecal water. *Gastroenterology* 1990b;98:A317

Wargovich MJ, Eng VWS, Newmark HL. Calcium inhibits the damaging and compensatory proliferative effects of fatty acids on mouse colon epithelium. *Cancer Letters* 1984;23:253-258

Wargovich MJ, Eng VWS, Newmark HL, Bruce WR. Calcium ameliorates the toxic effect of deoxycholic acid on colonic epithelium. *Carcinogenesis* 1983;4:1205-1207

Wargovich MJ. Calcium and colon cancer. *J Am Coll Nutr* 1988;7:295-300

Weisburger JH, Wynder EL. *Important advances in oncology 1987.* Philadelphia, Lippincott 1987:197-220

Zaridze DG. Environmental etiology of large-bowel cancer. *J Natl Cancer Inst* 1983;70:389-400

CHAPTER 6 CYTOTOXICITY OF FECAL WATER IS DEPENDENT ON THE TYPE OF DIETARY FAT AND IS DECREASED BY SUPPLEMENTAL CALCIUM PHOSPHATE.

John A. Lapré, Hielke T. De Vries, and Roelof Van der Meer

(Submitted for publication)

ABSTRACT

The effects of the type of dietary fat (18% wt/wt) and of $CaHPO_4$ supplementation (25 μmol/g vs 225 μmol/g diet) on luminal solubility of fatty acids and bile acids, cytotoxicity of fecal water and intestinal epitheliolysis were studied in rats. The fecal excretion of fatty acids was drastically stimulated by supplemental $CaHPO_4$ and was dependent on the type of dietary fat. Both on the low and high $CaHPO_4$ diets fecal excretion of fatty acids decreased in the order palm oil > milk fat > corn oil. On the low $CaHPO_4$ diet palm oil also induced the highest soluble fatty acid concentration followed by milk fat and corn oil. The fat-type dependent differences in concentrations of luminal surfactants resulted in a fat-type dependent cytotoxicity of fecal water. The concentrations of soluble fatty acids as well as soluble bile acids were, however, drastically decreased by $CaHPO_4$ supplementation dependent on the type of dietary fat. This decrease in concentration of soluble surfactants resulted in a decreased cytotoxicity of fecal water. Cytotoxicity of fecal water and concentrations of soluble surfactants were highly correlated by multiple regression analysis ($R=0.89$). Intestinal epitheliolysis measured as alkaline phosphatase in fecal water was decreased comparable to the decrease in cytotoxicity by supplemental $CaHPO_4$. Intestinal epitheliolysis and cytotoxicity of fecal water were highly correlated ($r=0.92$, $P<0.001$). It is concluded that the type of dietary fat influences the soluble surfactant concentrations and consequently affects cytotoxicity of fecal water and intestinal epitheliolysis. Dietary calcium phosphate inhibits cytotoxicity of fecal water and intestinal epitheliolysis by lowering the concentrations of soluble surfactants. These interactions between dietary fat and calcium may explain how diet could affect the risk of colonic cancer.

INTRODUCTION

Epidemiologic studies suggest that a high incidence of colon cancer is positively associated with a high fat intake (Willett, 1989; Willett *et al.*, 1990; Weisburger, 1991) and negatively with the intake of dietary calcium (see Sorenson *et al.*, 1988 for review). Higher levels of saturated fat (20% compared to 5% wt/wt) strongly promote experimental carcinogenesis in rodents (Nigro *et al.*, 1975; Reddy *et al.*, 1977; Reddy, 1981; Bull *et al.*, 1979). Polyunsaturated fats may induce higher tumor incidences compared to saturated fats (Broitman *et al.*, 1977; Lockniskar *et al.*, 1985; Reddy *et*

al., 1985; Sakaguchi *et al.*, 1984). However, Nicholson *et al.* (1990) found that fats high in linoleic acid produced lower tumor yields compared to saturated fats. High levels of dietary calcium counteract the promotive effects of several types of dietary fat (20% wt/wt) (Appleton *et al.*, 1987; Pence & Buddingh, 1988; McSherry *et al.*, 1989; Behling *et al.*, 1990; Wargovich *et al.*, 1990).

With regard to the mechanism of the promotive effect of fat, Newmark *et al.* (1984) hypothesized that cytotoxic bile acids and fatty acids damage the colonic epithelial cells. This may result in an increased proliferation of the colonic epithelium which is an important biomarker of an increased susceptibility for colon cancer (Lipkin, 1988). Dietary calcium may complexate with bile acids and fatty acids in the intestinal lumen and thus reduce their promotive effects. It has repeatedly been shown that bile acids and fatty acids stimulate hyperproliferation of the colonic epithelium and that calcium reduces their hyperproliferative effects (Wargovich *et al.*, 1983; Bird *et al.*, 1986; Skraastad & Reichelt, 1988; Wargovich *et al.*, 1984; Caderni *et al.*, 1988).

Previous studies from our laboratory have shown that calcium phosphate is capable of binding bile acids (Van der Meer & De Vries, 1985; Van der Meer *et al.*, 1991) in vitro and that this binding decreases their cytotoxicity (Van der Meer *et al.*, 1991). In rats, dietary supplementation with calcium phosphate decreases cytotoxicity of fecal water by lowering the concentration of soluble bile acids (Lapré *et al.*, 1991a). Cytotoxicity of fecal water is highly correlated ($r=0.85$, $n=24$, $P<0.001$) with proliferation of the colonic epithelium (Lapré & Van der Meer, 1992) and can be considered as an intermediate step in the induction of hyperproliferation of the colonic epithelium.

In vitro studies showed that fatty acids are also important cytotoxic surfactants (Lapré *et al.*, 1990; Buset *et al.*, 1990) whose cytotoxicity may be blocked with supplemental calcium. With regard to this, it should be noted that cytotoxicity of human fecal water is strongly dependent on the concentrations of soluble fatty acids. Supplemental calcium decreases fatty acid concentration as well as cytotoxicity of fecal water (Van der Meer *et al.*, 1990). Because bile acids and fatty acids are products of fat digestion, cytotoxicity of fecal water and the protective effect of calcium phosphate may be dependent on the type of dietary fat. Therefore, we studied the effects of different types of dietary fats on luminal surfactants and cytotoxicity of fecal water, and their interaction with calcium phosphate in rats. To investigate whether an increased cytotoxicity of fecal water results in an increased damage of the colonic epithelium, we determined intestinal alkaline phosphatase (ALP) in fecal water as a

marker for intestinal epitheliolysis. Intestinal ALP is located as exoenzyme on the apical membrane of the enterocyte (Ono, 1974). Damage of the apical membrane may bring this enzyme in the fecal stream. This marker measured in fecal water correlates highly with cytotoxicity of fecal water and with proliferation of colonic cells in rats fed diets supplemented with steroids (Lapré *et al.*, 1991b).

MATERIALS AND METHODS

Animals and diets. Male outbred Wistar rats (Small Animal Research Center of the Wageningen Agricultural University) eight weeks old were housed individually at a constant temperature of 21°C. During the experimental period of two weeks, groups of rats (seven rats per group) were fed a purified diet which differed in $CaHPO_4$ content (25 and 225 µmol/g) and in type of dietary fat. Three types of dietary fat were used: milk fat, palm oil and corn oil. The fatty acid composition of the dietary fats is given in Table 1. Correction was made for the cholesterol content of milk fat by supplementing the other diets with 0.04% (wt/wt) cholesterol. The composition of low $CaHPO_4$ diet was (g/kg diet): casein (acid casein, DMV Veghel, The Netherlands) 200, dextrose 495, corn oil 20, milk fat, palm oil or corn oil 180, cellulose 20, mineral mix 35, vitamin mix 10, $CaHPO_4{\cdot}2H_20$ 4.3 and acid-washed sand 35.7. Cholesterol and $CaHPO_4$ were added in exchange of sand. The compositions of the vitamin and mineral mixtures have been described (Schaafsma & Visser, 1980; AIN, 1977). The control diets mimic Western high-risk diets containing 40 en-% fat, low fibre and low calcium. Feed and water were supplied ad libitum. Animal weights were recorded weekly and feed intake was measured every 2 days. Feces were collected quantitatively during days 11-14 of the experiment.

Total feces analyses. Fecal bile acid excretion was measured as described elsewhere (Van der Meer *et al.*, 1985). Briefly, freeze-dried feces were extracted with a t-butanol-water (1:1, vol/vol) mixture and subsequently bile acids were assayed enzymatically using a fluorimetric enzymatic kit (Sterognost 3α-FLU, Nycomed AS, Norway). Total free fatty acids in feces were extracted three times with diethyl ether after acidification with HCl (final concentration: 4 M). After evaporation of the diethyl ether under nitrogen and subsequent resolubilization in ethanol, free fatty acids were assayed enzymatically (NEFA-C kit, Wako Chemicals, Germany). Appropriate standards and reference samples were assayed simultaneously. The recovery of

added standards in these procedures to measure bile acids and fatty acids in feces always exceeded 95%. Calcium was measured after extraction with trichloroacetic acid (TCA) (final concentration: 5% wt/vol) in an atomic absorption spectrophotometer and inorganic phosphate was determined in the TCA-extract using the method described by Fiske and Subbarow (1925).

Table 1 Average fatty acid composition of the different dietary fats as % of weight (NPR 6305).

	Milk Fat	Palm oil	Corn oil
Saturated			
$C_{4:0}$ - $C_{10:0}$	11	--	--
$C_{12:0}$	4	--	--
$C_{14:0}$	11	1	--
$C_{16:0}$	27	43	11
$C_{18:0}$	11	5	2
Mono-unsaturated			
$C_{16:1}$	3	--	--
$C_{18:1}$	25	39	27
Poly-unsaturated			
$C_{18:2}$	2	11	57
Minor fatty acids	6	1	3

Fecal water preparation. Fecal water was prepared by reconstituting freeze-dried feces with double-distilled water to 35% dry weight, which reflects the wet weight to dry weight condition in the distal rat colon (unpublished data). After homogenizing, the samples were incubated for one hour at 37°C in a shaking waterbath followed by centrifugation for 10 min at 15,000*g (Eppendorf 5415). Centrifugation for 20 or 30 min produced no significant differences in bile acid, calcium and phosphate content of fecal water nor did it affect its cytotoxicity (data not shown). The supernatant was carefully aspirated and pH was measured at 37°C. Samples were stored at -20°C until further use. Control experiments showed that fecal water from freeze-dried feces using this procedure did not differ significantly from fecal water prepared from fresh feces for the parameters studied (Lapré *et al.*, 1991a; Lapré & Van der Meer, 1992).

Cytotoxicity assay. Cytotoxicity of fecal water was tested as described previously (Lapré *et al.*, 1991a) with the following minor modifications. The incubation mixture

contained 40, 80, 120 or 160 µl of fecal water, 154 mmol/L NaCl to a total volume of 160 µl and 40 µl of washed human erythrocytes (final hematocrit: 5%). The incubation time was two hours at 37°C. Cytotoxicity of each fecal water was quantified as the area under the lytic curve. This cytotoxicity is expressed as a percentage of the maximal area, which implies 100% lysis at each dilution of fecal water.

Fecal water analyses. Free fatty acids were assayed using an enzymatic method (NEFA-C kit, Wako Chemicals, Germany). Bile acids in fecal water were determined using a fluorimetric enzymatic assay (Sterognost 3α-Flu, Nycomed AS, Oslo, Norway). Appropriate reference samples and standards were measured simultaneously. Standards added to samples were always recovered for more than 92%.

Total ALP activity was determined according to Bessey *et al.* (1946) using a glycine buffer (final: 100 mM, pH 9.8) in the presence of zinc (final: 2 mM) and magnesium (final: 5 mM). p-Nitrophenyl phosphate was used as substrate and the absorbance of the reaction product p-nitrophenol was determined spectrophotometrically at 405 nm. The concentration p-nitrophenol was calculated using a standard curve of known p-nitrophenol concentrations and ALP activity was expressed as µmol p-nitrophenol/min/ml fecal water (U/ml). Intestinal ALP activity was inhibited using 60 mM L-phenylalanine which acts as a specific uncompetitive inhibitor of the intestinal isozyme in humans and rats (Fishman *et al.*, 1962). The difference in activity between total (non inhibited) and the activity after inhibition with L-phenylalanine is the activity of the intestinal isoenzyme. No inhibition of intestinal ALP occurred with the enantiomer D-phenylalanine as inhibitor. Because an antibody against rat intestinal ALP was not commercially available, we validated the enzyme-kinetic measurement of intestinal ALP with immunoprecipitation of intestinal ALP in human fecal water isolated from feces of healthy human volunteers consuming habitual diets. Immunoprecipitation was done using a commercially available rabbit anti-human-placenta polyclonal antibody (Dakopatts, Glostrup, Denmark) which is known for its cross-reactivity with the intestinal isozyme (Lehmann, 1975). Appropriate dilutions of human fecal water were incubated for 1 hour at 37°C with anti-serum or rabbit control-serum (Dakopatts, Glostrup, Denmark) at the same protein concentrations in 154 mM NaCl + 3% polyethylene glycol (PEG) 6000. After 16 hours at 4°C, samples were centrifuged for twenty minutes at 15,000*g (Eppendorf 5415) and in the supernatants of samples incubated with anti-serum or control serum, ALP activity was determined. Control experiments showed that after immunoprecipitation

no inhibitory effect of 60 mM L-phenylalanine could be measured which indicates that the intestinal isoenzyme has been completely precipitated by the antibody. Using immunoprecipitation by the antibody and enzyme-kinetic inhibition by L-phenylalanine to determine the activity of intestinal ALP in human fecal water a nearly perfect correlation between the two methods ($r=0.98$, $n=29$, $P<0.001$) was obtained (Figure 1). Moreover, the regression equation ($y=1.0x-0.01$) indicated that both methods produced exactly the same values for intestinal ALP activities. Experiments using feces of different dietary origin showed that >95% of the activity of intestinal ALP was recovered in fecal water. Feces tested comprised feces of rats on control diets, on diets with extra steroids and on diets with supplemental calcium.

Statistics. Values are the means of seven rats with their standard errors. After analysis of variance the differences between the means of the groups were tested using Fisher's protected least significant difference test (two-sided). Differences were regarded significant if $P<0.05$. Data comparing the enzyme-kinetic inhibition of intestinal alkaline phosphatase with immunoprecipitation and the comparison of cytotoxicity of fecal water with intestinal epitheliolysis were analyzed with single linear regression analysis. Multiple regression analysis of luminal surfactants on cytotoxicity was done using a commercially available statistical package (SPSS/PC+ v2.0).

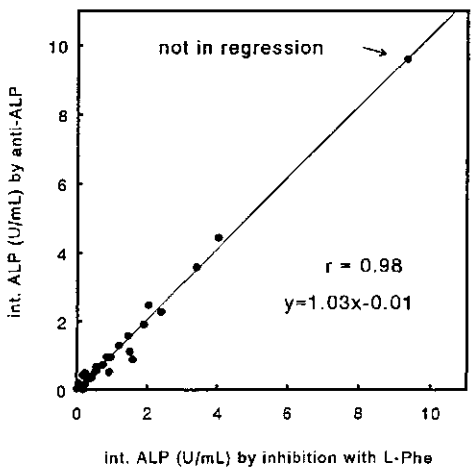


Figure 1 Relationship between measurement of intestinal alkaline phosphatase activity in human fecal water by specific uncompetitive inhibition (60 mM L-phenylalanine) and by immunoprecipitation using a rabbit-polyclonal antibody against human intestinal alkaline phosphatase ($r = 0.98$, $n=29$, $y=1.0x-0.01$).

RESULTS

Feed intake (mean: 20.7 g/day) and body weight gain (mean: 5.8 g/day) were not significantly affected by the type of dietary fat and by $CaHPO_4$ supplementation. Supplemental $CaHPO_4$ signficantly increased fecal output on the milk fat and palm oil diets (Table 2). On the low $CaHPO_4$ diet palm oil significantly increased fecal calcium compared to the milk fat or corn oil diets. Calcium and inorganic phosphate excretion were drastically stimulated by $CaHPO_4$ supplementation on all three fats as would be expected (Table 2).

Fatty acid excretion in feces was drastically stimulated by $CaHPO_4$ supplementation. It should be noted that the total fecal excretion of fatty acids is directly related to the type of dietary fat. Palm oil produced the highest excretion of fatty acids on the low and on the high $CaHPO_4$ levels followed by milk fat. The corn oil diet resulted in the lowest fecal fatty acid excretion. Fecal bile acid excretion was significantly lower on the corn oil diet and was slightly stimulated by $CaHPO_4$ supplementation on the corn oil diet only.

The effects of dietary calcium phosphate supplementation on total and soluble fatty acids clearly showed that despite an increase in total fecal fatty acid concentration, the concentration of soluble fatty acids was drastically decreased (Figure 2). The palm oil diet resulted in the highest concentrations of both total and soluble fatty acids compared to the other two dietary fats on the low and high $CaHPO_4$ level respectively.

Figure 3 shows that supplemental calcium phosphate does not affect the total fecal bile acid concentration on the milk fat and corn oil diets and decreased the bile acid concentration on the palm oil diet. This effect is due to the increased fecal mass (g dry/day) on the palm oil diet after $CaHPO_4$ supplementation which is caused by the huge increase in fatty acid excretion (Table 2). In contrast to their total concentration, the concentration of soluble bile acids is drastically decreased by supplemental calcium phosphate on all three types of dietary fat. Consistent with the effects on the total fecal bile acid concentration, the corn oil diet produced the lowest concentration of soluble bile acids.

Table 2 Effects of dietary fat and supplemental calcium phosphate on fecal mass and on fecal excretion of minerals, bile acids and fatty acids.

	MILK FAT		PALM OIL		CORN OIL	
$CaHPO_4$ in diet (μmol/g diet)	25	225	25	225	25	225
Feces (g dry/day)	1.10±0.07[a]	1.35 ±0.05[b]	1.14±0.04[a]	1.74 ±0.10[c]	1.14±0.07[a]	1.24 ±0.04[a]
Calcium (μmol/day)	25.5±1.5[a]	2543±171[b]	53.1±7.0[c]	2703±178[b]	28.5±6.1[a]	2546±319[b]
Inorganic phosphate (μmol/day)	46.6±6.3[a]	1911±167[b]	70.8±18.0[a]	1663±121[b]	48.9±8.6[a]	1731± 95[b]
Fatty acids (μmol/day)	25.1±3.3[a]	937± 66[b]	60.0± 4.9[c]	2416±134[d]	10.8±1.6[e]	193±22[f]
Bile acids (μmol/day)	23.0±2.5[b,d]	25.3.± 2.2[b]	25.3± 2.6[b]	24.6 ± 4.0[b,c]	15.3±1.2[a]	18.7 ± 1.2[c,d]

Values are means of seven rats ± SEs. Values in the same row not sharing the same superscript are significantly different: $P<0.05$.

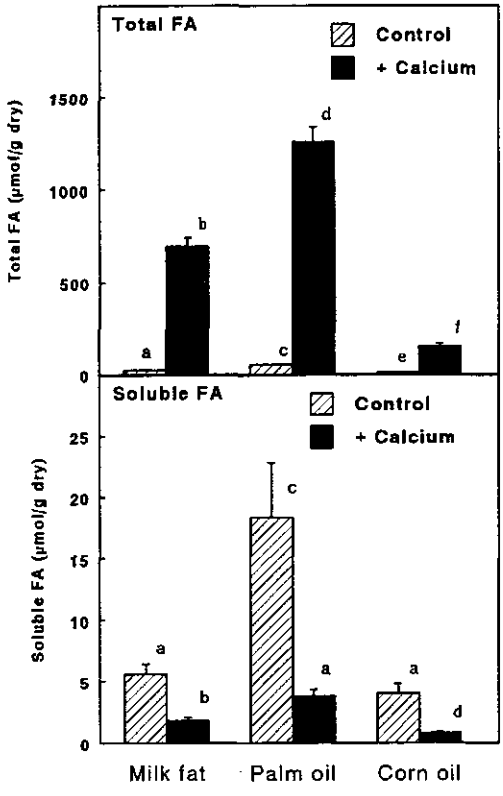


Figure 2

Total and soluble fecal free fatty acid concentrations (µmol/ g dry) of rats fed diets containing different types of dietary fat at 25 µmol $CaHPO_4$/g diet (Control) and 225 µmol $CaHPO_4$/g diet (+ Calcium). Values are means of seven rats with their SEs. Bars not sharing the same character are significantly different: $P<0.05$.

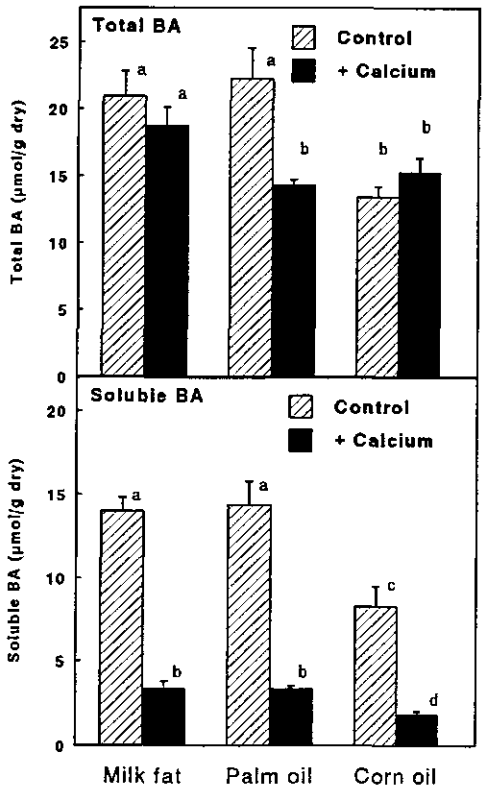


Figure 3

Total and soluble fecal bile acid concentrations (µmol/g dry) of rats fed diets containing different types of dietary fat at 25 µmol $CaHPO_4$/g diet (Control) and 225 µmol $CaHPO_4$/g diet (+ Calcium). Values are means of seven rats with their SEs. Bars not sharing the same character are significantly different: $P<0.05$.

Because cytotoxicity of the intestinal contents seems to be dependent on the concentrations of soluble surfactants (Lapré *et al.*, 1991a; Lapré & Van der Meer, 1992), we measured cytotoxicity of fecal water using lysis of erythrocytes. On the low calcium phosphate diets palm oil induced the highest cytotoxicity, followed by milk fat and corn oil (Figure 4).

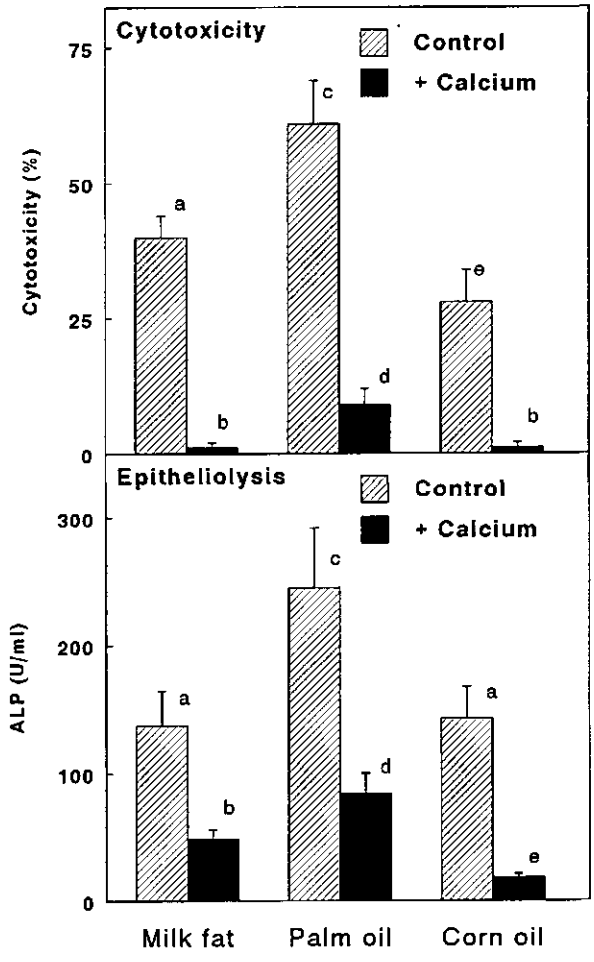


Figure 4 Cytotoxicity of fecal water and intestinal epitheliolysis of rats fed diets containing different types of dietary fat at 25 µmol $CaHPO_4$/g diet (Control) and 225 µmol $CaHPO_4$/g diet (+ Calcium). Values are means of seven rats with their SEs. Bars not sharing the same character are significantly different: $P<0.05$.

Calcium phosphate supplementation drastically decreased cytotoxicity of fecal water on all three dietary fats. However, the palm oil diet still resulted in the highest cytotoxicity compared to the milk fat and corn oil diets. In order to ascertain whether cytotoxicity of fecal water is determined by the concentrations of soluble fatty acids and bile acids, we compared these parameters by multiple regression analysis. This resulted in highly significant associations with a multiple correlation coefficient of 0.89. The regression equation (% cytotoxicity=1.97*FA_{sol}+1.91*BA_{sol}, SEs 0.27 and 0.37 respectively) showed that bile acids and fatty acids in fecal water are the main determinants of cytotoxicity with almost equal relative importance. No significant associations were found for between cytotoxicity and total fecal concentrations of fatty acids and bile acids.

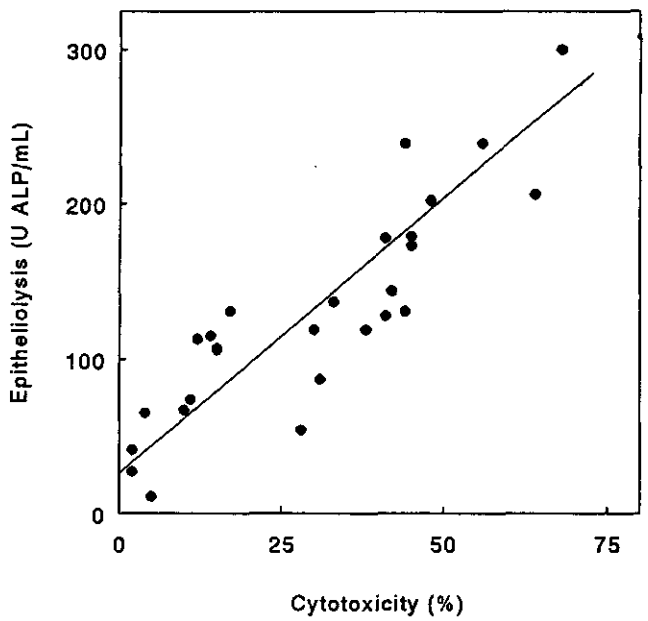


Figure 5 Relationship between cytotoxicity of fecal water and intestinal epitheliolysis in rats fed diets differing in type of fat and amount of $CaHPO_4$ ($r = 0.92$, $n=26$).

Using alkaline phosphatase activity in fecal water as marker for intestinal epitheliolysis (Lapré *et al.*, 1991b), effects similar to those for cytotoxicity were observed (Figure 4). Comparison of the individual data for cytotoxicity and

epitheliolysis of the same rats resulted in a highly significant correlation ($r=0.92$, $n=26$, $P<0.001$) (Figure 5). It should be noted that the samples which showed no cytotoxicity ($n=16$) are left out of this comparison to prevent formation of a cluster around cytotoxicity 0%. Inclusion of these samples resulted in a correlation coefficient of 0.94. Thus the higher concentrations of soluble surfactants on the palm oil diet induced a higher cytotoxicity of fecal water and resulted in a higher lysis of epithelial cells. Dietary calcium phosphate supplementation drastically decreased cytotoxicity of fecal water and intestinal epitheliolysis on all three types of dietary fat.

DISCUSSION

Our present study shows, to our knowledge for the first time in quantitative terms, that not the total bile acid and fatty acid concentrations, but their soluble concentrations are the mediators of intestinal cytotoxicity. In this study, both fatty acids and bile acids in fecal water explained 80% (R-squared) of the cytotoxicity of fecal water. Appleton *et al.* (1991) concluded that free fatty acids are bound intraluminally by calcium because supplemental calcium stimulates fecal excretion of fatty acids. Our study showed that the increase in fecal fatty acid excretion by dietary calcium is dependent on the type of dietary fat. This is consistent with the different affinities of calcium for the different fatty acids. Calcium soap formation is enhanced with long-chain saturated fatty acids and impaired with polyunsaturated fatty acids (Cheng *et al.*, 1949). This indicates that the drastic increases in fecal fatty acid excretion found on the high calcium phosphate diets is mainly due to the formation of insoluble calcium soaps. Whether phosphate is involved in this complexation analogous to the binding of bile acids by calcium phosphate (Van der Meer & De Vries, 1985; Van der Meer *et al.*, 1991; Van der Meer *et al.*, 1990; Qiu *et al.*, 1991) is at present not known and requires further investigation. With regard to this it should be mentioned that several studies suggest the occurrence of fatty acid-calcium-phosphate complexes in feces (Swell *et al.*, 1956; Richards & Carroll, 1959).

Analogous to the effects observed in our earlier studies (Lapré *et al.*, 1991a; Van der Meer & Govers, 1991), dietary calcium did not increase total fecal bile acid excretion (μmol/day), but decreased the concentration of soluble bile acids. Appleton *et al.* (1991) found a significant decrease in fecal bile acid concentration (mg/g dry feces) by supplemental calcium which we did not observe in our study. However, our

study showed that the bile acid concentration in fecal water is more important than the total fecal bile acid concentration with regard to cytotoxicity of fecal water. The bile acid concentration in fecal water is drastically decreased by dietary calcium analogous to our previous studies.

Not only the total fatty acid concentration, but also the concentration of soluble fatty acids is directly influenced by the type of dietary fat and by supplemental $CaHPO_4$. The differences in the concentrations of soluble fatty acids might be responsible for the higher cytotoxicity observed on the palm oil diets compared to the milk fat diets because no differences in the concentrations of soluble bile acids were observed. On the corn oil diets the concentrations of soluble bile acids were signficantly lower compared to the other diets, but the cytotoxicity of fecal water was comparable to that of the milk fat diets. This effect can be explained by our in vitro studies which have shown that the combination of linoleic acid with bile acids is highly cytotoxic (Lapré *et al.*, 1990). Supplemental $CaHPO_4$ decreased the concentrations of soluble fatty acids in our present study and this decrease combined with the decrease in soluble bile acids is responsible for the $CaHPO_4$-induced fall of cytotoxicity and epitheliolysis. The cytotoxic, fatty acid dependent effects observed in the present study are consistent with those found by others. For instance, fatty acids alone (Lapré *et al.*, 1990; Buset *et al.*, 1990) and in combination with bile acids (Lapré *et al.*, 1990) are cytotoxic for different cell-types including human colonocytes. Addition of calcium to the culture medium blocks the cytotoxicity of fatty acids completely (Buset *et al.*, 1990). Triglycerides or free fatty acids instilled intrarectally results in damage of the colonic epithelium (Wargovich *et al.*, 1984) and in induction of hyperproliferation (Wargovich *et al.*, 1983; Bull *et al.*, 1983; Jacobs & Amorde, 1986). This effect can be abolished by simultaneous administration of calcium (Wargovich *et al.*, 1983, 1984; Bird *et al.*, 1986; Skraastad & Reichelt, 1988; Caderni *et al.*, 1988).

Awad *et al.* (1989) studied the effects of beef fat (saturated), butter fat (saturated) and safflower oil (polyunsaturated) on fecal lipids at a calcium phosphate level of 125 μmol $CaHPO_4$/g. They found no significant effect on total fecal bile acid concentration and an increase in fecal fatty acid concentration with the more saturated fats which is in line with our study. They also found that the beef fat diet resulted in greater loss of protein bands from plasma membranes of colonocytes compared to butter fat and safflower oil. In another study they showed that a high level of dietary calcium (375 μmol/g) reduced the concentrations of bile acids and fatty acid in highly

diluted water extracts of feces and decreased the loss of protein bands of colon mucosal cells compared to the control diet (125 µmol Ca/g diet) (Awad *et al.*, 1990). The effects observed on loss of protein bands might be attributable to detergent-like effects of bile acids and free fatty acids as clearly observed in our erythrocyte assay with fecal water. It can be speculated that one of the protein bands represents alkaline phosphatase but this requires further investigation.

Using alkaline phosphatase in fecal water as marker for intestinal epitheliolysis, the present study shows that the higher concentrations of soluble fatty acids on the palm oil diet resulted in a higher cytotoxicity of fecal water and induced more lysis of epithelial cells compared with the milk fat and corn oil diets. These effects are largely reduced at the high calcium phosphate level consistent with the decrease in soluble surfactants and in cytotoxicity. Thus the effects of diet on soluble surfactant concentrations and on intestinal cytotoxicity are reflected by effects on the damage of the colonic epithelium suggesting possible cause-and-effects relationships.

In conclusion, our nutritional study with rats fed Western-type high risk diets shows that the type of dietary fat affects the concentrations of soluble bile acids and fatty acids and consequently cytotoxicity of fecal water and intestinal epitheliolysis. Supplemental calcium phosphate decreases the solubility of bile acids and fatty acids, luminal cytotoxicity and intestinal epitheliolysis independent of the type of dietary fat. These results offer a molecular explanation for the lower cytotoxicity and intestinal epitheliolysis.

Acknowledgements: The authors wish to thank M. Peters for taking care of the animals. This study was supported by Grant 663/88-1.7 from the European Community and by the Netherlands Organization for Scientific Research (NWO), Medical Sciences by Grant 900-562-078.

REFERENCES

American Institute of Nutrition. Report of the American institute of nutrition ad hoc committee on standards for nutritional studies. *J Nutr* 1977;107:1340-1348

Appleton GV, Davies PW, Bristol JB, Williamson RCN. Inhibition of intestinal carcinogenesis by dietary supplementation with calcium. *Br J Surgery* 1987;6:523-525

Appleton GVN, Owen RW, Wheeler EE, Challacombe DN, Williamson RCN. Effect of dietary calcium on the colonic luminal environment. *Gut* 1991;32:1374-1377

Awad AB, Short ME, Fink CS. Effect of excess dietary calcium on colon mucosal membranes and fecal lipids. *Lipids* 1990;25:379-382

Awad AB, Chattopadhyay JP, Danahy ME. Effect of dietary fat composition on rat colon plasma membranes and fecal lipids. *J Nutr* 1989;119:1376-1382

Behling AR, Kaup SM, Choquette LL, Gregor JL. Lipid absorption and intestinal tumour incidence in rats fed varying levels of calcium and butterfat. *Br J Nutr* 1990;64:505-513

Bessey OA, Lowry OH, Brock MJ. A method for the rapid determination of alkaline phosphatase with five cubic millimeters of serum. *J Biol Chem* 1946;164:321-323

Bird RP, Schneider RH, Stamp D, Bruce WR. Effect of dietary calcium and cholic acid on the proliferative indices of murine colonic epithelium. *Carcinogenesis* 1986;7:1657-1661

Broitman SA, Vitale JJ, Vavrousek-Jakuba E, Gottlieb LS. Polyunsaturated fat, cholesterol and large bowel tumorigenesis. *Cancer* 1977;40:2455-2563

Bull AW, Soullier BK, Wilson PS, Hayden MT, Nigro ND. Promotion of azoxymethane-induced intestinal cancer by high-fat diets in rats. *Cancer Res* 1979;39:4956-4959

Bull AW, Marnett LJ, Dawe EJ, Nigro ND. Stimulation of deoxythymidine incorporation in the colon of rats treated intrarectally with bile acids and fats. *Carcinogenesis* 1983;4:207-210

Buset M, Galand P, Lipkin M, Winawer S, Friedman E. Injury induced by fatty acids or bile acid in isolated human colonocytes prevented by calcium. *Cancer Lett* 1990;50:221-226

Caderni GE, Stuart EW, Bruce WR. Dietary factors affecting the proliferation of epithelial cells in the mouse colon. *Nutr Cancer* 1988;11:147-153

Cheng ALS, Morehouse MG, Deuel HJ. The effect of the level of dietary calcium and magnesium on the digestibility of fatty acids, simple triglycerides, and some natural and hydrogenated fats. *J Nutr* 1949;37: 237-250.

Fishman WH, Green S, Inglis NI. Organ specific behavior exhibited by rat intestine and liver alkaline phosphatase. *Biochim Biophys Acta* 1962;62:363-375

Fiske CH, Subbarow Y. The colorimetric determination of phosphorus. *J Biol Chem* 1925;66:375-400

Jacobs LR, Amorde K. Source and saturation of dietary fats determine regional alterations in rat colonic cell proliferation. *Gastroenterology* 1986;90:A1473

Lapré JA, Strootman EG, Van der Meer R. Cytotoxic effects of mixed micelles of bile acids and fatty acids. In: *Abstract Book Second Int. Meeting on Pathochemistry, Pathophysiology and Pathomechanics of the Biliary System.* Bologna March 19-21, 1990:177

Lapré JA, De Vries HT, Van der Meer R. Dietary calcium phosphate inhibits cytotoxicity of fecal water. *Am J Physiol* 1991a;261:G907-G912

Lapré JA, Kleibeuker JH, Van der Meer R. Intestinal alkaline phosphatase in fecal water reflects epitheliolysis and is decreased by dietary calcium. *Gastroenterology* 1991b;100:A378

Lapré JA, Van der Meer R. Diet-induced increase in colonic bile acids stimulate lytic activity of fecal water and proliferation of colonic cells. *Carcinogenesis* 1992;13:41-44

Lehmann FG. Immunological relationship between human placental and intestinal alkaline phosphatase.

Clin Chim Acta 1975:65:257-264

Lipkin M. Biomarkers of increased susceptibility to gastrointestinal cancer: a new application to studies of cancer prevention in human subjects. *Cancer Res* 1988;48:235-245

Lockniskar M, Nauss KM, Kauffman P, Newbeane PM. Interaction of dietary fat and route of carcinogen administration on 1,2-dimethylhydrazine-induced colon tumorigenesis in rats. *Carcinogenesis* 1985;6:349-354

McSherry CK, Cohen BI, Bokkenheuser VD, Mosbach EH, Winter J, Matoba N, Scholes J. Effects of calcium and bile acid feeding on colon tumors in the rat. *Cancer Res* 1989;49:6039-6043

NPR 6305. Vegetable and animal oils and fats - Interpretation of results obtained by chromatographic examination. Netherlands Normalisation Institute (NNI).

Newmark HL, Wargovich MJ, Bruce WR. Colon cancer and dietary fat, phosphate, and calcium: a hypothesis. *J Natl Cancer Inst* 1984;72:1323-1325

Nigro ND, Singh DV, Campbell RL, Pak MS. Effect of dietary beef fat on intestinal tumor formation by azoxymethane in rats. *J Natl Cancer Inst* 1975;54:439-442

Nicholson ML, Neoptolemos JP, Clayton HA, Talbot IC, Bell PRF. Inhibition of experimental colorectal carcinogenesis by dietary N-6 polyunsaturated fats. *Carcinogenesis* 1990;11:2191-2197

Ono K. The localization of alkaline phosphatase activity in the small intestinal epithelium after fat and protein feeding. *Acta Histochem* 1974;51:124-137

Pence BC, Buddingh F. Inhibition of dietary fat promotion of colon carcinogenesis by supplemental calcium and vitamin D. *Carcinogenesis* 1988;9:187-190

Qiu S, Wen G, Hirakawa N, Soloway RD, Hong N, Crowther RS. Glycochenodeoxycholic acid inhibits calcium phosphate precipitation in vitro by preventing the transformation of amorphous calcium phosphate to calcium hydroxyapatite. *J Clin Invest* 1991;88:1265-1271

Reddy BS, Watanabe K, Weisburger JH. Effect of a high-fat diet on colon carcinogenesis in F344 rats treated with 1,2-dimethylhydrazine, methylazoxymethanol acetate or methylnitrosurea. *Cancer Res* 1977;37: 4156-4259

Reddy BS. Dietary fat and its relationship to large bowel cancer. *Cancer Res* 1981;41:3700-3705

Reddy BS, Tanka T, Simi B. Effect of different levels of dietary trans fat or corn oil on azoxymethane-induced colon carcinogenesis in F344 rats. *J Natl Cancer Inst* 1985;75:791-798

Richards JF, Carroll KK. Studies on calcium phosphate fatty acid salts occurring in fecal lipids. *Can J Biochem Phys* 1959;37:725-730

Sakaguchi M, Hiramatsu Y, Takade H, Yamamura M, Hioki K, Saito K, Yamamoto M Effect of dietary unsaturated and saturated fats on azoxymethane-induced colon carcinogenesis in rats. *Cancer Res* 1984;44:1472-1477

Schaafsma G, Visser R. Nutritional interrelationships between calcium, phosphorus and lactose in rats. *J Nutr* 1980;110:1101-1111

Skraastad D, Reichelt KL. An endogenous colon mitosis inhibitor and dietary calcium inhibit the increased colonic cell-proliferation induced by cholic acid. *Scand J Gastroenterol 1988;23:801-807*

Sorenson AW, Slattery ML, Ford MH. Calcium and colon cancer: a review. *Nutr Cancer* 1988;11:135-

145

Swell L, Trout EC, Field H, Treadwell CR. Effect of dietary fat and fatty acid on fecal excretion of a calcium oleate phosphate complex. *J Biol Chem* 1956;22:613-615

Van der Meer R, De Vries HT, Glatz JFC. *Cholesterol metabolism in health and disease: Studies in the Netherlands.* Ponsen and Looijen, Wageningen, The Netherlands; 1985:113-119

Van der Meer R, De Vries HT. Differential binding of glycine- and taurine-conjugated bile acids to insoluble calcium phosphate. *Biochem J* 1985;229:265-268

Van der Meer R, Welberg JWM, Kuipers F, Kleibeuker JH, Mulder NH, Termont DSML, Vonk RJ, De Vries HT, De Vries EGE. Effects of supplemental dietary calcium on the intestinal association of calcium, phosphate and bile acids. *Gastroenterology* 1990;99:1653-1659

Van der Meer R, Termont DSML, De Vries HT. Differential effects of calcium ions and calcium phosphate on cytotoxicity of bile acids. *Am J Physiol* 1991;260:G142-G147

Van der Meer R, Govers MJAP. Dietary phosphate does not inhibit the protective effects of calcium on luminal solubility and cytotoxicity of bile acids and fatty acids. *Gastroenterology* 1991;100:A407

Wargovich MJ, Allnut D, Palmer C, Anaya P, Stephens LC. Inhibition of the promotional phase of azoxymethane-induced colon carcinogenesis in the F344 rat by calcium lactate: effect of simulating two human nutrient density levels. *Cancer Lett* 1990;53:17-25

Wargovich MJ, Eng VWS, Newmark HL, Bruce WR. Calcium ameliorates the toxic effect of deoxycholic acid on colonic epithelium. *Carcinogenesis* 1983;4:1205-1207

Wargovich MJ, Eng VWS, Newmark HL. Calcium inhibits the damaging and compensatory proliferative effects of fatty acids on mouse colon epithelium. *Cancer Lett* 1984;23:253-258

Weisburger JH. Causes, relevant mechanisms, and prevention of large bowel cancer. *Seminars in oncology* 1991;18:316-336

Willett WC. The search for the causes of breast and colon cancer. *Nature* 1989;338:389-394

Willett WC, Stampfer MJ, Colditz GA, Rosner BA, Speizer FE. Relation of meat, fat, and fiber intake to the risk of colon cancer in a prospective study among women. *N Eng J Med* 1990;323:1664-1672

CHAPTER 7 THE ANTI-PROLIFERATIVE EFFECT OF DIETARY CALCIUM ON COLONIC EPITHELIUM IS MEDIATED BY LUMINAL SURFACTANTS AND DEPENDENT ON THE TYPE OF DIETARY FAT.

John A. Lapré, Hielke T. De Vries, Jan H. Koeman and Roelof Van der Meer

(Submitted for publication)

ABSTRACT

Bile acids and fatty acids may promote colon cancer by inducing colonic hyperproliferation. Dietary calcium inhibits the promoting effects of bile acids and fatty acids possibly by precipitating these surfactants and lowering their cytotoxicity. Because bile acids and fatty acids are products of fat digestion, their effects may be dependent on the type of dietary fat. The effects of the type of dietary fat (40 en-%) and of $CaHPO_4$ supplementation (25 µmol/g diet vs 225 µmol/g diet) on luminal solubility of surfactants, cytotoxicity, epitheliolysis and in vivo colonic proliferation were studied in rats fed Western high risk diets. The different types of commercially available fats were: butter, saturated margarine and polyunsaturated margarine. Supplemental calcium drastically increased fecal fatty acid excretion dependent on the type of fat and slightly stimulated fecal bile acid excretion. Soluble surfactant concentrations were drastically decreased with all types of dietary fat. Consequently, cytotoxicity of fecal water was decreased by supplemental calcium. These luminal effects of calcium resulted in a lower intestinal epitheliolysis. The compensatory proliferation of the colonic epithelium was decreased by supplemental $CaHPO_4$ for the butter and saturated margarine diets. Despite $CaHPO_4$-dependent decreases in luminal effects and epitheliolysis, no significant decrease in proliferation on the polyunsaturated margarine diet was observed. Multiple regression analysis of soluble surfactants with cytotoxicity (R=0.76), epitheliolysis (R=0.74) and colonic proliferation (R=0.84) showed highly significant associations. Cytotoxicity and epitheliolysis as well as epitheliolysis and proliferation were highly correlated (resp. r=0.97 and r=0.88, n=36) for control and Ca-supplemented diets suggesting cause-and-effect relationships. We conclude that the anti-proliferative effect of dietary calcium is mediated by luminal effects on soluble surfactants and is dependent on the type of dietary fat.

INTRODUCTION

Several types of epidemiological studies have reported positive associations between the incidence of colon cancer and the dietary intake of fat (Jain *et al.*, 1980; Weisburger, 1991; Willett *et al.*, 1990). Negative correlations have been found for a high calcium intake and colon cancer (Sorenson *et al.*, 1988; Garland *et al.*, 1989).

Tumor-induction studies with rodents have shown that bile acids and fatty acids

may act as promoters/cocarcinogens (Narisawa *et al.*, 1974; Reddy *et al.*, 1977; McSherry *et al.*, 1989). One important mechanism for this promotive effect is the induction of colonic hyperproliferation, which can be considered as a biomarker of an increased susceptibility of colon cancer (Lipkin, 1988). Several studies using a semi-physiological design with intrarectal instillation of bile acids or fatty acids in rodents have shown that these surfactants may indeed induce higher proliferation rates in colon (Bull *et al.*, 1983; Wargovich *et al.*, 1983, 1984). Simultaneous administration of calcium reduces these hyperproliferative effects (Wargovich *et al.*, 1983, 1984).

With regard to the mechanism of the promotive effect of dietary fat and the protective effect of dietary calcium, Newmark *et al.* (1984) have proposed that a high-fat diet raises the concentrations of potentially cytotoxic bile acids and fatty acids in colon. Damage of the colonic epithelium by these surfactants is then compensated for by an increased epithelial proliferation. Calcium may complexate in the intestinal lumen with the cytotoxic surfactants thus reducing their cytotoxic and hyperproliferative effects (Newmark *et al.*, 1984). Several lines of experimental evidence now support this proposed sequence of effects. In vitro studies have shown that bile acids and fatty acids are toxic to different types of cells (Coleman *et al.*, 1980; Buset *et al.*, 1986, 1990; Lapré *et al.*, 1990; Van der Meer *et al.*, 1991; Velardi *et al.*, 1991). Bile acids are bound to calcium phosphate (Van der Meer & De Vries, 1985; Van der Meer *et al.*, 1991; Qiu *et al.*, 1991) which reduces their cytotoxicity (Van der Meer *et al.*, 1991). Cytotoxicity of fatty acids is blocked by calcium (Buset *et al.*, 1990). Nutritional studies with rodents have demonstrated that a diet-induced increase in colonic surfactant concentrations stimulates cytotoxicity of fecal water as well as colonic proliferation (Lapré & Van der Meer, 1992). Dietary $CaHPO_4$ decreases concentrations of soluble surfactants and cytotoxicity of fecal water (Lapré *et al.*, 1991a). Proliferation induced by feeding of bile acids is also decreased by supplemental dietary $CaHPO_4$ (Bird *et al.*, 1986; Skraastad & Reichelt, 1988). In recent studies Newmark *et al.* (1991) showed that a diet high in fat and phosphate, and low in calcium and vitamin D induced higher proliferation rates compared to a control diet. In humans, cytotoxicity of fecal water is lowered by supplemental calcium (Van der Meer *et al.*, 1990b) as is proliferation of colonic epithelium (Lipkin & Newmark, 1985; Rozen *et al.*, 1989). However, the different steps in the mechanism i.e. effects on solubility of surfactants, luminal cytotoxicity, intestinal epitheliolysis and the compensatory proliferation of the colonic epithelium have, as far as we know, never been quantified in one study. In the

present study we investigated this proposed sequence of effects in rats fed Western high-risk diets (control) or diets supplemented with $CaHPO_4$. We used three different types of human fat consumption mimicking a diet with a high saturated medium-chain triglycerides content (butter), a diet with a high long-chain saturated fat content (saturated margarine) and a diet with a high polyunsaturated fat content (polyunsaturated margarine).

MATERIALS AND METHODS

Animals and diets. Male outbred Wistar rats (Small Animal Research Center of the Wageningen Agricultural University) eight weeks old (body weight 192 g) were housed individually at a constant temperature of 21°C. During the experimental period of two weeks, groups of rats (six rats per group) were fed a purified diet which differed only in $CaHPO_4$ content (25 and 225 μmol/g) and in type of dietary fat. Three types of commercially available dietary fats were used: Butter, saturated margarine and a margarine with a high content of polyunsaturated fatty acids (PUFA). The fatty acid composition of the experimental diets measured with gaschromatographic analysis according to Badings & De Jong (1983) is given in Table 1. Correction was made for the cholesterol content of the butter and saturated margarine diets by supplementing the PUFA-margarine diet with 0.05% (wt/wt) cholesterol. The composition of the low $CaHPO_4$ diet was (g/kg diet): casein (acid casein, DMV Veghel, The Netherlands) 200, dextrose 460, corn oil 20, butter/saturated margarine/polyunsaturated margarine (83% fat) 215, cellulose 20, mineral mix 35, vitamin mix 10, $CaHPO_4{\cdot}2H_20$ 4.3 and acid-washed sand 35.7. Cholesterol and $CaHPO_4$ were added in exchange of sand. The compositions of the vitamin and mineral mixtures have been described (Schaafsma & Visser, 1980; AIN, 1977). The control diets mimic Western high-risk diets containing approximately 40 en-% fat (20% wt/wt), low fibre and low calcium. Feed and water were supplied ad libitum. Animal weights were recorded weekly and feed intake was measured every 2 days. Feces were collected quantitatively during days 11-14 of the experiment.

[^{3}H]Thymidine incorporation. After the experimental feeding period of 14 days, rats (non-fasted) were injected i.p. with [methyl-^{3}H]thymidine (Amersham Int., Buckinghamshire, England; s.a. 25 Ci/mmol; dose 100 μCi/kg) in saline. Two hours later they were killed by decapitation after anaesthetization with CO_2 and the colon

was excised and opened. Intestinal contents were removed by rinsing with ice-cold 154 mM KCl. The mucosa of the colon was scraped using a spatula and after homogenization in 154 mM KCl, the scrapings were analyzed exactly as described previously (Lapré & Van der Meer, 1992). Proliferation was expressed as dpm [^{3}H]/µg DNA. It should be noted that for a reliable estimate of proliferation the amount of radioactivity incorporated into DNA must be varying between the experimental groups whereas the amount of DNA per scraping must be constant. In our study no significant differences between the amount of DNA/scraping were observed between the experimental groups (mean ± SE: 231± 9 µg DNA/scraping, n=36).

Table 1 Fatty acid composition of the different experimental diets (mmol/g diet).

	Butter	Sat. margarine	PUFA margarine
Saturated			
$C_{4:0}$-$C_{10:0}$	160	28	3
$C_{12:0}$	33	68	14
$C_{14:0}$	84	67	5
$C_{16:0}$	221	171	68
$C_{18:0}$	65	51	51
$C_{20:0}$-$C_{22:0}$	3	51	7
Mono-unsaturated			
$C_{16:1}$	13	41	1
$C_{18:1}$	165	158	140
Poly-unsaturated			
$C_{18:2}$	47	111	431
Minor fatty acids	47	22	4

Total feces analyses. Fecal bile acid excretion was measured as described elsewhere (Van der Meer *et al.*, 1985). Briefly, freeze-dried feces were extracted with a t-butanol-water (1:1, vol/vol) mixture and subsequently bile acids were assayed enzymatically using a fluorimetric enzymatic kit (Sterognost 3α-FLU, Nycomed AS,

Norway). Total free fatty acids in feces were extracted three times with diethyl ether after acidification with HCl (final concentration: 4 M). After evaporation of the diethyl ether under nitrogen and subsequent resolubilization in ethanol, free fatty acids were assayed enzymatically (NEFA-C kit, Wako Chemicals, Germany). Appropriate standards and reference samples were assayed simultaneously. The recovery of added standards in these procedures to measure bile acids and fatty acids in feces always exceeded 95%. Calcium was measured after extraction with trichloroacetic acid (TCA) (final concentration: 5% wt/vol) in an atomic absorption spectrophotometer and inorganic phosphate was determined in the TCA-extract using the method described by Fiske and Subbarow (1925).

Fecal water preparation. Fecal water was prepared by reconstituting freeze-dried feces with double-distilled water to 35% dry weight, which reflects the wet weight to dry weight condition in the distal rat colon (unpublished data). After homogenizing, the samples were incubated for one hour at 37°C in a shaking waterbath followed by centrifugation for 10 min at 15,000*g (Eppendorf 5415). Centrifugation for 20 or 30 min produced no significant differences in bile acid, calcium and phosphate content of fecal water nor did it affect its cytotoxicity (data not shown). The supernatant was carefully aspirated and pH was measured at 37°C. Samples were stored at -20°C until further use. Control experiments showed that fecal water from freeze-dried feces using this procedure did not differ significantly from fecal water prepared from fresh feces for the parameters studied (Lapré *et al.*, 1991a; Lapré & Van der Meer, 1992).

Cytotoxicity assay. Cytotoxicity of fecal water was determined as described previously (Lapré *et al.*, 1991a) with the following minor modifications. The incubation mixture contained 40, 80, 120 or 160 µl of fecal water, 154 mmol/L NaCl to a total volume of 160 µl and 40 µl of washed human erythrocytes (final hematocrit: 5%). Samples were incubated for two hours at 37°C. Cytotoxicity of each fecal water was quantified as the area under the lytic curve. This cytotoxicity is expressed as a percentage of the maximal area, which implies 100% lysis at each dilution of fecal water.

Fecal water analyses. Calcium and inorganic phosphate were measured as described above. Free fatty acids were assayed using an enzymatic method (NEFA-C kit, Wako Chemicals, Germany). Bile acids in fecal water were determined using a fluorimetric enzymatic assay (Sterognost 3α-Flu, Nyegaard, Norway). Appropriate reference samples and standards were measured simultaneously. Standards added to samples

were always recovered for more than 92%.

Total alkaline phosphatase (ALP) activity was determined according to Bessey *et al.* (1946) using a glycine buffer (final: 100 mM, pH 9.8) in the presence of zinc (final: 2 mM) and magnesium (final: 5 mM). p-Nitrophenyl phosphate was used as substrate and the absorbance of the reaction product p-nitrophenol was determined spectrophotometrically at 405 nm. The concentration p-nitrophenol was calculated using a standard curve of known p-nitrophenol concentrations and ALP activity was expressed as μmol p-nitrophenol/min/ml fecal water (U/ml). Intestinal ALP activity was inhibited using 60 mM L-phenylalanine which acts as a specific uncompetitive inhibitor of the intestinal isozyme in humans and rats (Fishman *et al.*, 1962). The difference in activity between total (non inhibited) and the activity after inhibition with L-phenylalanine is the activity of the intestinal isozyme. This enzyme-kinetic measurement of intestinal ALP correlated highly ($r=0.98$, $y=1.03x-0.01$) with the immunoprecipitation method for determining intestinal ALP (Lapré *et al.*, 1991b).

Statistics. Values are the means of six rats with their standard errors. After analysis of variance the differences between the means of the groups were tested using Fisher's protected least significant difference test (two-sided). Differences were regarded significant if $P<0.05$. Data comparing cytotoxicity of fecal water with intestinal epitheliolysis and epitheliolysis with colonic proliferation were analyzed with single linear regression analysis. Multiple regression analysis of luminal surfactants on cytotoxicity, epitheliolysis and proliferation was done using a commercially available statistical package (SPSS/PC+ v2.0) (SPSS Inc. Chicago, USA).

RESULTS

Feed intake (19.1±0.2 g/day) and weight gain (79.9±1.6 g/14 days) were not significantly affected by the experimental diets. Supplemental $CaHPO_4$ significantly increased fecal mass (g dry/day) on the saturated margarine diet and stimulated the fecal excretion of calcium and inorganic phosphate (Table 2). No significant effects of the experimental diets on the pH of fecal water were observed. Concentrations of calcium and inorganic phosphate in fecal water were not significantly different between the different types of dietary fat. Supplemental $CaHPO_4$ significantly increased both the concentrations of calcium and inorganic phosphate in fecal water on all three types of dietary fat (Table 2).

Table 2 Effects of type of dietary fat and supplemental $CaHPO_4$ on fecal mass and fecal excretion and fecal water concentrations of calcium and inorganic phosphate. Control diets contain 25 μmol $CaHPO_4$/g diet and diets with supplemental calcium contain 225 μmol $CaHPO_4$/g diet.

	Butter		Saturated margarine		PUFA margarine	
	Control	+ Calcium	Control	+ Calcium	Control	+ Calcium
Total feces						
Fecal mass (g dry/day)	1.25±0.08[a,b]	1.32± 0.10[a,b]	1.09± 0.09[a]	1.52± 0.13[b]	1.14±0.13[a]	1.24±0.06[a]
Calcium (μmol/g dry)	33 ±8[a]	2138 ±92[c]	36 ±11[a]	1806 ±83[b]	24±4[a]	1850±77[b]
Inorganic phosphate (μmol/g dry)	46 ±7[a]	1325 ±79[b]	40 ± 7[a]	922 ±40[d]	33±3[a]	1154±35[c]
Fecal water						
pH	7.7 ±0.1	7.3 ±0.2	7.5 ±0.2	7.3 ±0.1	7.5 ±0.1	7.5 ±0.1
Calcium (mM)	0.8 ±0.1[a]	2.5 ±0.3[b]	0.7 ±0.1[a]	2.3 ±0.4[b]	0.6 ±0.1[a]	3.0 ±0.3[b]
Inorganic phosphate (mM)	3.1 ±0.5[a]	12.7 ±1.2[b]	3.6 ±0.6[a]	10.0 ±1.0[b]	3.0 ±0.3[a]	9.9 ±1.4[b]

Values are means of six rats ± SEs. Values in the same row not sharing the same superscript are significantly different: $P<0.05$ (Fisher's protected LSD test).

Total free fatty acid concentration was significantly lower on the PUFA-margarine control diet compared to the butter and saturated-margarine control diets (Figure 1). The free fatty acid excretion was drastically increased by supplemental $CaHPO_4$ with the same fat-type dependency. Supplemental $CaHPO_4$ slightly increased the total fecal concentration of bile acids (Figure 1).

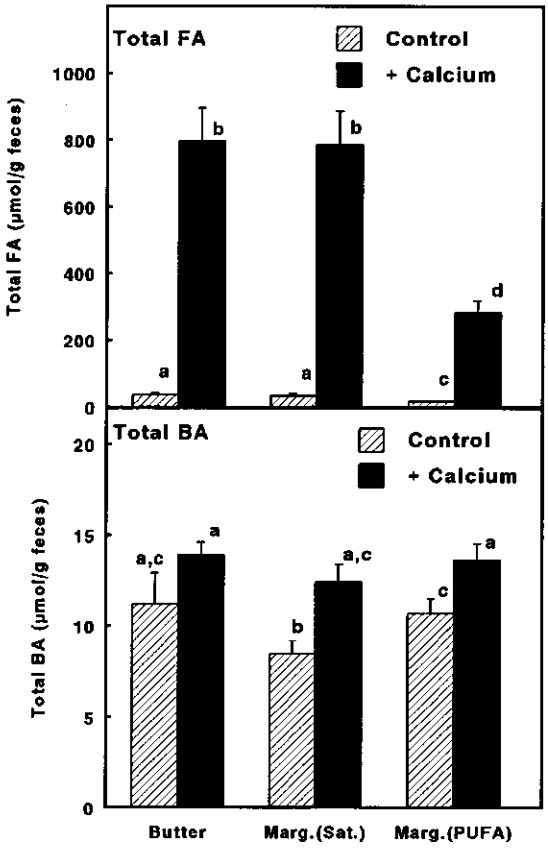


Figure 1

Concentrations of total fatty acids (µmol/g dry wt) and bile acids of rats fed diets differing in type of dietary fat and amount of $CaHPO_4$. Values are means of six rats ± SEs. Bars not sharing the same superscript are significantly different: $P<0.05$. Control diets contain 25 µmol $CaHPO_4$/g diet and diets with supplemental calcium contain 225 µmol $CaHPO_4$/g diet.

Because the cytotoxic effects of surfactants are not mediated by their total fecal concentrations, but by their concentrations in fecal water (Lapré *et al.*, 1991a), we quantified the concentrations of fatty acids and bile acids in fecal water (Figure 2). In contrast to the increases in total fecal fatty acids, the concentrations of soluble fatty acids were decreased by supplemental $CaHPO_4$. Analogous to the total fatty acid concentrations, concentrations of soluble fatty acids were the lowest on the PUFA margarine diets (Figure 2). The concentrations of soluble bile acids were also drastically decreased by supplemental $CaHPO_4$ (Figure 2).These calcium-dependent decreases in soluble surfactant concentrations should according to the hypothesis of Newmark *et al.* (1984) result in a lower cytotoxicity of fecal water. Analogous to the effects on concentrations of soluble surfactants, cytotoxicity of fecal water was drastically inhibited by supplemental $CaHPO_4$ dependent on the type of dietary fat (Figure 2). Multiple regression analysis showed that concentrations of fatty acids and bile acids are important determinants of cytotoxicity (R=0.76).

Luminal cytotoxicity as measured by lysis of erythrocytes reflects the potency of the colonic contents to damage cells and could result in changes in intestinal epitheliolysis. Intestinal ALP activity in fecal water might reflect intestinal epitheliolysis (Lapré *et al.*, 1991b) and therefore we determined this enzyme as a measure for intestinal epitheliolysis. The fat-type dependent effects on luminal surfactants and cytotoxicity are reflected in effects on intestinal epitheliolysis. The calcium-dependent decreases in soluble surfactants and cytotoxicity resulted in comparable decreases in intestinal epitheliolysis (Figure 3). Concentrations of soluble surfactants and intestinal epitheliolysis were highly correlated with multiple regression analysis (R=0.74). The effects on intestinal epitheliolysis should result in changes in the compensatory proliferation of the colonic epithelium. For the butter and the saturated-margarine diets, the calcium-dependent decreases in luminal surfactants, luminal cytotoxicity and intestinal epitheliolysis resulted in a lower colonic proliferation, but no significant decrease in colonic proliferation on the PUFA-margarine diet was observed. Multiple regression analysis showed that luminal surfactants and in vivo colonic proliferation were highly correlated (R=0.84). On the contrary, no significant associations were found between the concentrations of calcium in fecal water and colonic proliferation. Thus, luminal effects of diet cause a response of the colonic epithelium dependent on concentrations of soluble surfactants and luminal cytotoxicity.

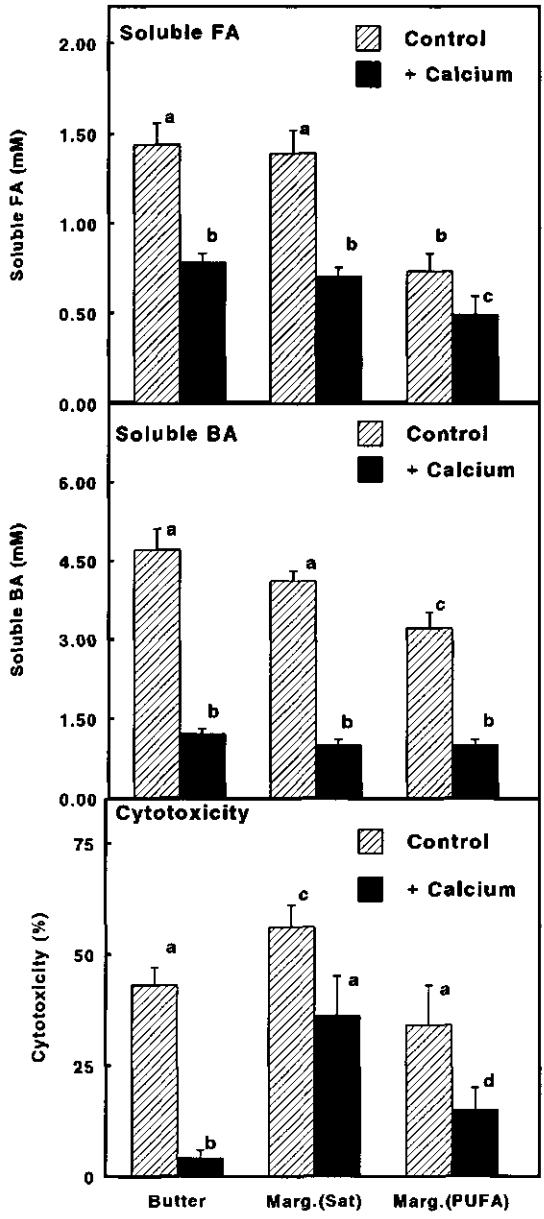


Figure 2

Concentrations of fecal fatty acids and bile acids (mM) in fecal water and fecal water cytotoxicity of rats fed diets differing in type of dietary fat and amount of $CaHPO_4$. Values are means of six rats ± SEs. Bars not sharing the same superscript are significantly different: $P<0.05$. Control diets contain 25 µmol $CaHPO_4$/g diet and diets with supplemental calcium contain 225 µmol $CaHPO_4$/g diet.

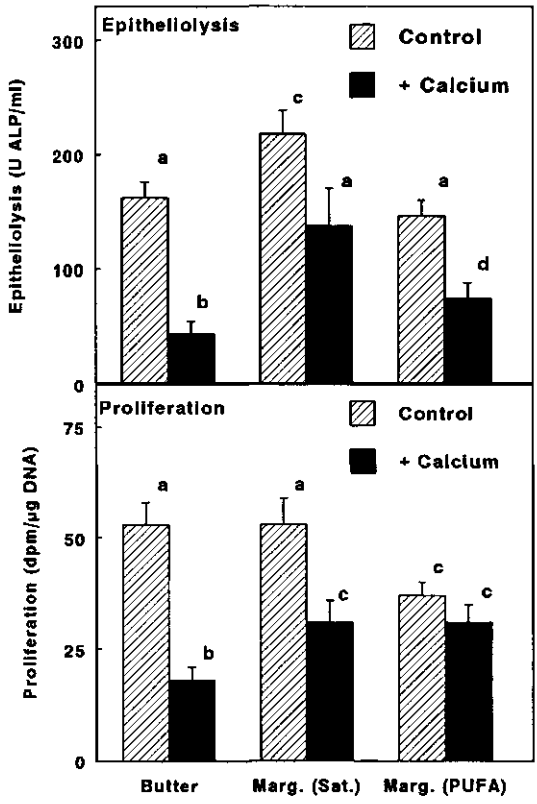


Figure 3 Intestinal epitheliolysis and colonic epithelial proliferation in rats fed diets differing in type of dietary fat and amount of $CaHPO_4$. Values are means of six rats ± SEs. Bars not sharing the same superscript are significantly different: $P<0.05$. Control diets contain 25 µmol $CaHPO_4$/g diet and diets with supplemental calcium contain 225 µmol $CaHPO_4$/g diet.

Because the effects of supplemental $CaHPO_4$ suggest causal relationships between luminal cytotoxicity , intestinal epitheliolysis and colonic epithelial proliferation, we correlated the data for the individual rats (Figure 4). Luminal cytotoxicity, reflecting the potency of the colonic contents to damage cells, is highly correlated ($r=0.97$, $n=36$, $P<0.001$) with intestinal epitheliolysis for control diets as well as $CaHPO_4$ supplemented diets. Intestinal epitheliolysis is also highly correlated with colonic proliferation ($r=0.88$, $n=36$, $P<0.001$) for control and $CaHPO_4$ diets.

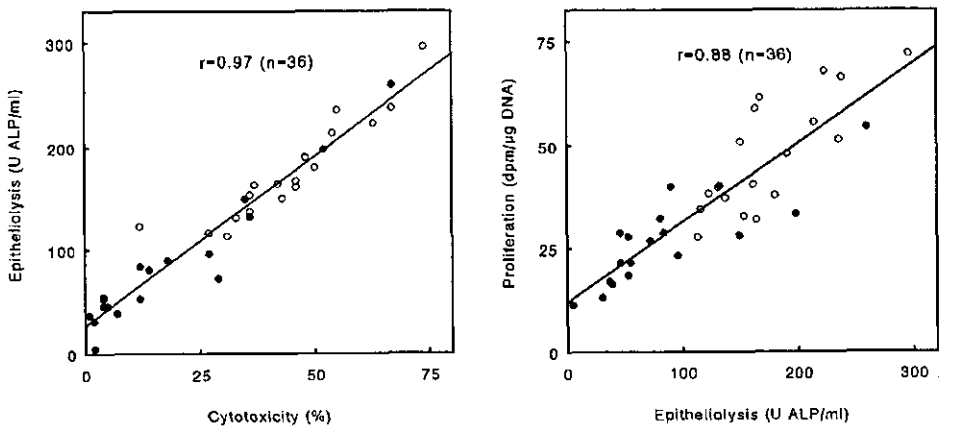


Figure 4 Relationships between cytotoxicity of fecal water and intestinal epitheliolysis, and between intestinal epitheliolysis and colonic epithelial proliferation of rats fed diets differing in type of dietary fat and amount of $CaHPO_4$. Open points represent control diets (25 µmol $CaHPO_4$/g diet) and closed points represent calcium-supplemented diets (225 µmol $CaHPO_4$/g diet).

DISCUSSION

Supplementation of Western high-risk diets with $CaHPO_4$ resulted in a drastic increase in total fatty acids and slight increases in total bile acid concentrations in feces. On the contrary, the concentrations of soluble surfactants were drastically decreased by supplemental $CaHPO_4$. Thus, $CaHPO_4$ supplementation resulted in an increased precipitation of hydrophobic surfactants consistent with the first step in the hypothesis of Newmark *et al.* (1984). The precipitation of fatty acids is probably caused by calcium-fatty acid 'soaps'. Whether phosphate is involved in this complexation (Swell *et al.*, 1956; Richards & Carroll, 1959) is at present not known and requires further investigation. Bile acids seem to be more easily precipitated by insoluble calcium phosphate than by ionized calcium (Van der Meer & De Vries, 1985; Van der Meer *et al.*, 1990a,1991; Qiu *et al.*, 1991). With regard to this, it should be noted that calcium in the colonic lumen is predominantly present as insoluble calcium phosphate (Van der Meer *et al.*, 1990a; Fordtran & Locklear, 1966; Spencer *et al.*, 1984).

Recently we have shown that effects of diet on colonic proliferation could be

mediated by soluble surfactant concentrations and luminal cytotoxicity (Lapré & Van der Meer, 1992). Supplemental calcium lowers proliferation induced by bile acids or fatty acids in rodents when instilled intrarectally (Wargovich *et al.*, 1983, 1984) or supplemented with diet (Bird *et al.*, 1986; Skraastad & Reichelt, 1988). Our present study investigated the combination of the luminal effects of $CaHPO_4$ supplementation and the subsequent response of the colonic epithelium. The lack of association between soluble concentrations of calcium and colonic proliferation precludes a major role of a direct effect of luminal calcium concentrations on in vivo colonic proliferation as has been observed in vitro (Buset *et al.*, 1986; Appleton *et al.*, 1991; Whitfield *et al.*, 1991). On the contrary, concentrations of fatty acids and bile acids were highly correlated with in vivo colonic proliferation suggesting that these surfactants are important determinants of colonic proliferation. Consistent with the hypothesis of Newmark *et al.* (1984), the intermediate steps between soluble surfactants and colonic proliferation consist of cytotoxic effects of the intestinal contents resulting in intestinal epitheliolysis.

The luminal effects of dietary $CaHPO_4$ supplementation i.e. a decrease in soluble surfactants and luminal cytotoxicity, resulted in a decreased intestinal epitheliolysis. For the butter and saturated margarine diets, these effects of supplemental $CaHPO_4$ caused a lower proliferation consistent with the proposed sequence of effects. However, despite the observed decreases of luminal parameters and intestinal epitheliolysis on the PUFA-margarine diet no significant reduction in proliferation was found with supplemental $CaHPO_4$. Whether this lack of effect on colonic proliferation suggests an alternative or additional mechanism by which polyunsaturated fatty acids induce proliferation e.g. by a direct effect on protein-kinase C (Craven & DeRubertis, 1988) is at present not known. Interestingly, the individual data points of rats fed Ca-supplemented diets lie on the same regression lines comparing cytotoxicity with epitheliolysis, and epitheliolysis with proliferation as the points of rats fed the control diets. In our opinion, this indicates that the main effect of a decreased proliferation caused by supplemental $CaHPO_4$ is due to the surfactant-dependent decreases in cytotoxicity and epitheliolysis. To our knowledge, this study with rats is the first one to demonstrate the sequence of effects of calcium on luminal and epithelial parameters. It should be stressed that our study does not prove cause-and-effect relationships, because our study was done under steady-state conditions. However, because of the consistency with the in vitro and in vivo studies cited above,

it is reasonable to speculate that this sequence of effects is causal.

Further information whether the sequence of luminal and epithelial effects of calcium observed in rats are also of relevance for studies with humans can be obtained by comparison of its effect on biomarkers of colonic proliferation with luminal parameters. For instance, preliminary results from our diet-controlled study with healthy volunteers indicate that supplemental calcium decreases soluble fatty acid concentrations and inhibits cytotoxicity of fecal water (Van der Meer *et al.*, 1990b) consistent with protective effects of dietary calcium on colonic proliferation (Lipkin & Newmark, 1985; Rozen *et al.*, 1989). Bruce and coworkers could not demonstrate a decrease in proliferation by supplemental calcium in placebo-controlled studies using patients with partial or subtotal colectomy (Gregoire *et al.*, 1989; Stern *et al.*, 1990). Investigation of solubility of bile acids and fatty acids and determination of luminal cytotoxicity in these studies would probably have provided more information about the nature of these controversional results.

In conclusion, dietary $CaHPO_4$ supplementation of Western high-risk diets decreases the concentrations of soluble bile acids and fatty acids by precipitating these surfactants. Consequently, cytotoxicity of fecal water and intestinal epitheliolysis are decreased. These effects may explain the fat-type dependent decreases in colonic cell-proliferation observed in this study after dietary supplementation with $CaHPO_4$. Ultimately, the interactions of calcium with bile acids and fatty acids may explain how diet could affect the risk of colon cancer.

Acknowledgements: The authors wish to thank J. Haas for expert biotechnical assistance and M. Peters for taking care of the animals. This study was supported by Grant 663/88-1.7 from the European Community and by the Netherlands Organization for Scientific Research (NWO), Medical Sciences by Grant 900-562-078.

REFERENCES

American Institute of Nutrition. Report of the American institute of nutrition ad hoc committee on standards for nutritional studies. *J Nutr* 1977;107:1340-1348

Appleton GVN, Owen RW, Wheeler EE, Challacombe DN, Williamson RCN. Effect of dietary calcium on the colonic luminal environment. *Gut* 1991;32:1374-1377

Badings HT, De Jong C. Glass capillary gas chromatography of fatty acid methyl esters. A study of

conditions for the quantitative analysis of short- and long-chain fatty acids in lipids. *J Chrom* 1983;279:493-506

Bessey OA, Lowry OH, Brock MJ. A method for the rapid determination of alkaline phosphatase with five cubic millimeters of serum. *J Biol Chem* 1946;164:321-323

Bird RP, Schneider R, Stamp D, Bruce WR. Effect of dietary calcium and cholic acid on the proliferative indices of murine colonic epithelium. *Carcinogenesis* 1986;7:1657-1661

Bull AW, Marnett LJ, Dawe EJ, Nigro ND. Stimulation of deoxythymidine incorporation in the colon of rats treated intrarectally with bile acids and fats. *Carcinogenesis* 1983;4: 207-210

Buset M, Lipkin M, Winawer S, Friedman E. Inhibition of human colonic epithelial cell proliferation in vivo and in vitro by calcium. *Cancer Res* 1986;46:5426-5430

Buset M, Galand P, Lipkin M, Winawer S, Friedman E. Injury induced by fatty acids or bile acid in isolated human colonocytes prevented by calcium. *Cancer Lett* 1990;50:221-226

Coleman R, Lowe PJ, Billington D. Membrane lipid composition and susceptibility to bile salt damage. *Biochim Biophys Acta* 1980;599:294-300

Craven PA, DeRubertis FR. Role of activation of protein kinase C in the stimulation of colonic epithelial proliferation by unsaturated fatty acids. *Gastroenterology 1988;95:676-685*

Fiske CH, Subbarow Y. The colorimetric determination of phosphorus. *J Biol Chem* 1925;66:375-400

Fishman WH, Green S, Inglis NI. Organ specific behavior exhibited by rat intestine and liver alkaline phosphatase. *Biochim Biophys Acta* 1962;62:363-375

Fordtran JS, Locklear TW. Ionic constituents and osmolality of gastric and small-intestinal fluids after eating. *Am J Dig Dis* 1966;11:503-521

Garland CF, Comstock GW, Garland FC, Helsing KJ, Shaw EK, Gorham ED. Serum 25-hydroxyvitamin D and colon cancer: eight year prospective study. *Lancet* 1989;ii:1176-1178

Gregoire RC, Stern HS, Yeung KS, Langley S, Furrer R, Bruce WR. Effect of calcium supplementation on mucosal proliferation in high risk patients for colon cancer. *Gut* 1989;30:376-382

Jain M, Cook GM, Davis FG, Grace MG, Howe GR, Miller AB. A case-control study of diet and colorectal cancer. *Int J Cancer* 1980;26:757-768

Lapré JA, Strootman EGE, Van der Meer R. Cytotoxic effects of mixed micelles of bile acids and fatty acids. In: *Abstract Book Sec. Int. Meeting on Pathochemistry, Pathophysiology and Pathomechanics of the Biliary System.* Bologna 1990:177

Lapré JA, Van der Meer R. Diet-induced increase in colonic bile acids stimulates lytic activity of fecal water and proliferation of colonic cells. *Carcinogenesis* 1992;13:41-44

Lapré JA, De Vries HT, Van der Meer R. Dietary calcium phosphate inhibits cytotoxicity of fecal water. *Am J Physiol* 1991a;261:G907-G912

Lapré JA, Kleibeuker JH, Van der Meer R. Intestinal alkaline phosphatase in fecal water reflects epitheliolysis and is decreased by dietary calcium. *Gastroenterology* 1991b;100:A378

Lipkin M. Biomarkers of increased susceptibility to gastrointestinal cancer: a new application to studies of cancer prevention in human subjects. *Cancer Res* 1988;48:235-245

Lipkin M, Newmark H. Effect of added dietary calcium on colonic epithelial cell-proliferation in subjects at high risk for familial colonic cancer. *N Eng J Med* 1985;313:1381-1384

McSherry CK, Cohen BI, Bokkenheuser VD, Mosbach EH, Winter J, Matoba N, Scholes J. Effects of calcium and bile acid feeding on colon tumors in the rat. *Cancer Res* 1989;49:6039-6043

Narisawa T, Magadia NE, Weisburger JH, Wynder EL. Promoting effects of bile acids on colon carcinogenesis after intrarectal instillation of N-methyl-N'-nitro-N-Nitrosoguanidine in rats. *J Natl Cancer Inst* 1974;53:1093-1097

Newmark HL, Wargovich MJ, Bruce WR. Colon cancer and dietary fat, phosphate, and calcium: a hypothesis. *J Natl Cancer Inst* 1984;72:1323-1325

Newmark HL, Lipkin M. Colonic hyperplasia and hyperproliferation induced in rodents by a nutritional stress diet containing 4 factors of the western human diet: high fat and phosphate, low calcium and vitamin D. In: Lipkin M, Newmark HL, Kelloff G, eds. *Calcium, Vitamin D, and Prevention of colon cancer.* Boca Raton: CRC Press Inc. 1991:145-154

Qiu S, Wen G, Hirakawa N, Soloway RD, Hong N, Crowther RS. Glycochenodeoxycholic acid inhibits calcium phosphate precipitation in vitro by preventing the transformation of amorphous calcium phosphate to calcium hydroxyapatite. *J Clin Invest* 1991;88:1265-1271

Reddy BS, Watanabe K, Weisburger JH, Wynder EL. Promoting effects of bile acids in colon carcinogenesis in germ free and conventional F344 rats. *Cancer Res* 1977;37:3238-3242

Richards JF, Carroll KK. Studies on calcium phosphate fatty acid salts occurring in fecal lipids. *Can J Biochem Phys* 1959;37:725-730

Rozen P, Fireman Z, Fine N, Wax Y, Ron E. Oral calcium suppresses increased rectal proliferation of persons at risk of colorectal cancer. *Gut* 1989;30:650-655

Schaafsma G, Visser R. Nutritional interrelationships between calcium, phosphorus and lactose in rats. *J Nutr* 1980;110:1101-1111

Skraastad D, Reichelt KL. An endogenous colon mitosis inhibitor and dietary calcium inhibit the increased colonic cell-proliferation induced by cholic acid. *Scand J Gastroenterol* 1988;23:801-807

Sorenson AW, Slattery ML, Ford MH. Calcium and colon cancer: a review. *Nutr Cancer* 1988;11:135-145

Spencer H, Kramer L, Norris C, Osis D. Effect of calcium and phosphorous on zinc metabolism in man. *Am J Clin Nutr* 1984;40:1213-1218

Stern HS, Gregoire RC, Kashtan H, Stadler J, Bruce WR. Long-term effects of dietary calcium on risk markers for colon cancer in patients with familial polyposis. *Surgery* 1990;108:528-533

Swell L, Trout EC, Field H, Treadwell CR. Effect of dietary fat and fatty acid on fecal excreation of a calcium oleate phosphate complex. *J Biol Chem* 1956;22:613-615

Van der Meer R, De Vries HT, Glatz JFC. *Cholesterol metabolism in health and disease: Studies in the Netherlands.* Wageningen, The Netherlands: Ponsen and Looijen; 1985:113-119

Van der Meer R, De Vries HT. Differential binding of glycine- and taurine-conjugated bile acids to insoluble calcium phosphate. *Biochem J* 1985;229:265-268

Van der Meer R, Termont DSML, De Vries HT. Differential effects of calcium ions and calcium phosphate on cytotoxicity of bile acids. *Am J Physiol* 1991;260:G142-G147

Van der Meer R, Welberg JWM, Kuipers F, Kleibeuker JH, Mulder NH, Termont DSML, Vonk RJ, De

Vries HT, De Vries EGE. Effects of supplemental dietary calcium on the intestinal association of calcium, phosphate and bile acids. *Gastroenterology* 1990a;99:1653-1659

Van der Meer R, Lapré JA, Kleibeuker JH, De Vries EGE, De Vries HT. Effects of supplemental dietary calcium on composition and cytotoxicity of fecal water. *Gastroenterology* 1990b;98:A317

Velardi ALM, Groen AK, Oude Elferink RJP, Van der Meer R, Palasciano G, Tytgat GNJ. Cell-type dependent effect of phospholipid and cholesterol on bile salt cytotoxicity. *Gastroenterology* 1991;101:457-64

Wargovich MJ, Eng VWS, Newmark HL, Bruce WR. Calcium ameliorates the toxic effect of deoxycholic acid on colonic epithelium. *Carcinogenesis* 1983;4:1205-1207

Wargovich MJ, Eng VWS, Newmark HL. Calcium inhibits the damaging and compensatory proliferative effects of fatty acids on mouse colon epithelium. *Cancer Lett* 1984;23:253-258

Weisburger JH. Causes, relevant mechanisms, and prevention of large bowel cancer. *Seminars in oncology* 1991;18:316-336

Whitfield J. Calcium switches, cell cycles, differentiation, and death. In: Lipkin M, Newmark HL, Kelloff G, eds. *Calcium, Vitamin D, and Prevention of colon cancer*. Boca Raton: CRC Press Inc., 1991:31-74

Willett WC, Stampfer MJ, Colditz GA, Rosner BA, Speizer FE. Relation of meat, fat, and fiber intake to the risk of colon cancer in a prospective study among women. *N Eng J Med* 1990;323:1664-1672

CHAPTER 8 EFFECTS OF SUPPLEMENTAL DIETARY CALCIUM ON COMPOSITION AND LYTIC ACTIVITY OF FECAL WATER

John A. Lapré, Hielke. T. De Vries, Denise S.M.L. Termont, Jan H. Kleibeuker, Elisabeth G.E. De Vries and R. Van der Meer. **(Submitted for publication)**

ABSTRACT

Dietary calcium supplementation inhibits hyperproliferation of rectal epithelium, possibly by precipitating luminal surfactants and thus preventing their cell-damaging effects. Therefore we studied the effects of supplemental dietary calcium (35 mmol/day) on the composition and lytic activity of fecal water in twelve healthy volunteers. Fecal water was isolated by low-speed centrifugation. Lytic activity was determined as lysis of human erythrocytes by fecal water. Supplemental calcium increased soluble calcium and decreased soluble inorganic phosphate. The logarithm of the concentration product of calcium and phosphate was linearly dependent on pH. These observations indicate formation of insoluble calcium phosphate. Supplemental Ca did not alter the total bile acid concentration in fecal water, but significantly decreased the ratio of hydrophobic over hydrophilic bile acids from 3.3 to 2.3. Calcium also significantly decreased the concentration of fatty acids (2863 μmol/L to 2126 μmol/L). Consistent with these decreases in hydrophobic surfactants, calcium decreased the lytic activity of fecal water from 47% ± 9 to 27% ± 8 ($n=12$, $P<0.05$). We conclude that supplemental dietary calcium decreases luminal cytotoxic surfactant concentrations and thus inhibits luminal lytic activity. This may explain how dietary calcium decreases epithelial cell-proliferation.

INTRODUCTION

Epidemiological data indicate that colon cancer is associated with a high fat-intake (Weisburger & Wynder, 1987; Willett, 1989; Willett *et al.*, 1990) probably by increasing the concentrations of soluble cytotoxic surfactants like bile acids and fatty acids in the colon (Newmark *et al.*, 1984; Weisburger & Wynder, 1987). Several epidemiological studies have also shown that dietary calcium is negatively associated with the risk of colon cancer (Garland *et al.*, 1989; Sorenson *et al.*, 1988). It has been hypothesized that this effect is due to binding of soluble surfactants by calcium in the intestinal lumen (Newamrk *et al.*, 1984).

In vitro studies have shown that bile acids bind to insoluble calcium phosphate (Van der Meer & De Vries, 1985; Van der Meer *et al.*, 1991) and that this binding decreases their well-known cell-damaging properties (Van der Meer *et al.*, 1991). In nutritional experiments with rodents, dietary calcium supplementation lowered colonic epithelial cell-proliferation (Bird *et al.*, 1986; Reshef *et al.*, 1990). An increased colonic

epithelial cell-proliferation is considered to be a biomarker of an increased susceptibility to colon cancer (Lipkin, 1988). Recent studies have indicated that in rats lytic activity of fecal water and colonic proliferation are highly correlated (Lapré & Van der Meer, 1992) and that dietary calcium supplementation lowers the concentration of soluble bile acids and luminal lytic activity (Lapré *et al.*, 1991a; Van der Meer & Govers, 1991).

Also in humans, dietary calcium has been shown to reduce colonic cell-proliferation (Lipkin & Newmark, 1985; Lipkin *et al.*, 1989; Rozen *et al.*, 1989; Lans *et al.*, 1991). However, other studies did not find a protective effect of supplemental calcium (Gregoire *et al.*, 1989; Stern *et al.*, 1990; Cats *et al.*, 1991). Few human intervention studies have been conducted regarding the mechanism of the protective effects of dietary calcium. For instance, interesting studies by Rafter's group (Rafter *et al.*, 1987; Allinger *et al.*, 1990) showed effects of a low-risk (either low fat, high calcium, high fibre or a lactovegetarian) diet vs a high-risk (either high fat, low calcium, low fibre or a mixed) diet on cellular toxicity of fecal water. However, their study design, using complete diets with multiple dietary variables, could of course not provide information about the mechanism of the protective effects of calcium per se. Therefore, we studied the effects of supplemental calcium on the intestinal association of calcium, phosphate and bile acids in a controlled human intervention trial (Van der Meer *et al.*, 1990). Analogous to the in vitro and animal experiments, we found that intestinal calcium, phosphate and bile acids were closely associated. Because in vitro (Van der Meer *et al.*, 1985; Van der Meer *et al.*, 1991) and animal experiments (Lapré *et al.*, 1991a; Van der Meer & Govers, 1991) indicate that calcium decreases the concentrations of soluble bile acids and lytic activity of fecal water, we have now determined the effects of supplemental calcium on fecal water composition and lytic activity in samples of our calcium intervention trial.

MATERIALS AND METHODS

Experimental procedures

Protocol and participants. The protocol of the study and characteristics of its participants have been published recently (Van der Meer *et al.*, 1990). Briefly, twelve healthy male volunteers strictly maintained their, calcium-constant, habitual diet during

the experimental period of two weeks. After a control period of one week, the subjects augmented their intake of calcium by ingesting with their meals tablets of calcium carbonate to a daily total of 35.5 mmol. Before and at the end of one week of calcium supplementation, the subjects collected feces for periods of three days. Individual stools were stored in tightly closed boxes at 4°C immediately after defecation and were handled within 12 hours afterwards.

Preparation of fecal water. After determination of the wet weight, the individual stools were freeze-dried, weighed again and subsequently homogenized in a Waring blender (Waring Products Division, New Hartford, CT). Fecal water was prepared by reconstituting individually pooled feces with double-distilled water to the original amount of water. After one hour incubation at 37°C in a shaking waterbath, the samples were centrifuged for 10 minutes at 15,000*g (Eppendorf 5415, Eppendorf Gerätebau, Hamburg, Germany). The supernatant was carefully aspirated and pH was measured at 37°C. Samples were stored at -20°C until further use. It should be noted that in rats fecal water from freeze-dried feces using this procedure was not different from fecal water prepared from fresh feces with respect to lytic activity and to the concentrations of calcium, phosphate, bile acids and fatty acids (Lapré & Van der Meer, 1992; Lapré *et al.*, 1991a).

Solubility product analyses. Na_2HPO_4 (final concentration: 8.0 mmol/L) was added to solutions containing $CaCl_2$ (final concentration: 20 mmol/L) and $MgCl_2$ (final concentration: 5.6 mmol/L). These concentrations were chosen to mimic the relative amounts of these minerals in the feces of these volunteers (Van der Meer *et al.*, 1990). The tubes also contained buffer (final concentration: 100 mmol/L) and NaCl to maintain a constant ionic strength of 150 mmol/L. MES (2[N-morpholino]-ethanesulfonic acid) was used in the pH range 5.5-6.5 and MOPS (3-[N-morpholino]-propanesulfonic acid) in the range 6.6-7.4. After 15 minutes of incubation at 37°C, the tubes were centrifuged for 2 minutes at 10,000*g. Supernatants were collected and the pH was measured. After acidification with trichloroacetic acid (final concentration: 5% wt/vol) and subsequent centrifugation (2 minutes at 10,000*g), calcium and magnesium were determined in these supernatants by atomic absorption spectrophotometry (Perkin Elmer 1100, Norwalk, CT). Phosphate was measured as described by Fiske and Subbarow (1925).

Fecal water analyses

Calcium, magnesium and inorganic phosphate. After acidification with

trichloroacetic acid (final concentration: 5% wt/vol) and centrifugation for 2 minutes at 10,000*g, calcium, magnesium and inorganic phosphate were determined in the supernatants as described above.

Bile acids in fecal water. Bile acids in fecal water were determined by capillary gas-liquid chromatography. Briefly, fecal water supplemented with internal standard (7α, 12α,-dihydroxy-5ß-cholanic acid, Calbiochem, San Diego, CA) was hydrolyzed in 1M NaOH and 60% (vol/vol) methanol for two hours at 80°C and subsequently extracted three times with petroleum ether (boiling range 60-80°C) to remove neutral sterols. After acidification with HCl to pH=1, bile acids were extracted three times using diethyl ether. In control experiments this extraction resulted in >95% recovery of the different bile acids. The diethyl ether extracts were dried under a stream of N_2 and subsequently methylated (Shaw & Elliott, 1978) and silylated as described (Setchell *et al.*, 1983). Aliquots of 1 µl were injected into the gas chromatograph (GC) (Carlo Erba Model Mega 5160, Carlo Erba, Milan, Italy) by on-column High Oven Temperature (HOT) injection (AS-550 on-column injector, Carlo Erba, Milan, Italy) using H_2 as carrier gas (flow rate: 1.8 ml/min). The column used was supplied with a retention gap (1 m * 0.53 mm) connected to a CP Sil 19 CB (25 m * 0.25 mm) and a CP Sil 5 CB column (5 m * 0.25 mm) (Chrompack, Middelburg, The Netherlands). Flame-ionization was used as detection and peak areas were measured using a Spectra Physics 4100 computing integrator (Spectra Physics, San José, CA) and compared with those of standard solutions. Differences in response of the flame-ionization detector for the various steroids were corrected for by specific response factors on the basis of the molar composition of a mixture of pure bile acids. This mixture and its components were also measured enzymatically using a spectrofluorimetric method (Sterognost 3α-FLU, Nycomed AS, Oslo, Norway). Molar response factors for the different bile acids ranged from 0.9 to 1.5. The overall recovery of added lithocholate/deoxycholate in this GC procedure was 102 ± 6% (mean ± SD, n=5). In addition to the normal GC procedure, pooled samples were also measured by GC/MS (Quadruple Mass-spectrometer VG 12-50, VG Biotech, Manchester, England) for peak identification using the chromatographic conditions described above. This indicated that in diethyl ether extracts which had not undergone a prior extraction with petroleum ether, the peaks of lithocholate were strongly contaminated by plant sterols like a stereo-isomeric form of stigmastanol. Thus, petroleum ether extraction is essential to prevent overestimation of these bile acids in

fecal water under the chromatographic conditions described.

Neutral sterols. Neutral sterols were determined in the petroleum ether extracts after silylation using 5α-cholestane (Sigma, St. Louis, MO) as internal standard using the chromatographic conditions described above.

Free fatty acids. Free fatty acids in fecal water were determined using a method which is specific for free fatty acids (De Jong & Badings, 1990). Briefly, fecal waters were mixed with ethanol (1:1 v/v) and extracted three times with diethyl ether/heptane (1:1, v/v) after acidification with H_2SO_4 (final concentration: 0.1 mmol/L). The extracts were applied to a conditioned (10 ml heptane) aminopropyl column (Bond-Elut, Analytichem Int. Harbor City, CA). Neutral lipids were removed from the column by eluting with chloroform/2-propanol (2:1 vol/vol) and free fatty acids were eluted with diethyl ether containing 2% (v/v) formic acid. An aliquot of 0.5 µl was injected on the GC (same as above) using on-column injection. A fused silica capillary column (15 m * 0.53 mm) coated with FFAP (= Free Fatty Acid Phase) (d_f = 1.0 µm) (J&W Scientific, Folsom, CA) was used. The flow rate of the carrier gas (H_2) was 13 ml/min. Detection was performed using a flame-ionization detector coupled to a Spectraphysics 4100 computing integrator (same as above). Heptadecanoic acid ($C_{17:0}$) was used as internal standard. This method allowed us to quantitate free fatty acids in the range C_6 to $C_{20:0}$ because in this range the recovery of added pure fatty acids was >90%. Recovery of the short-chain fatty acids was too low to give reliable measurements.

Lytic activity assay. The lytic activity assay described in this paper is a modification of the hemolysis method described by Rafter *et al.* (1987). Apparently because fecal pigments may interfere with the spectrophotometric measurement of hemoglobin, these authors quantified hemolysis indirectly by counting the remainder of intact erythrocytes. Since we found in control experiments that this procedure is liable to artifacts (12), we used Fe-release quantified by atomic absorption spectrophotometry as a measure of hemolysis. Human erythrocytes were isolated exactly as described previously (Van der Meer *et al.*, 1991). The incubation mixture contained 40, 80, 120 and 160 µl fecal water, 154 mmol/L NaCl to a total volume of 160 µl and 40 µl cells (final hematocrit: 5%). In each assay, fecal water without erythrocytes to correct for the Fe-content of the fecal water, erythrocytes in double-distilled water (= 100% lysis) and erythrocytes in 154 mmol/L NaCl (= 0% lysis) were incubated simultaneously. Samples were incubated for 6 hours at 37°C in a shaking waterbath, then centrifuged

for 1 minute at 735*g (Eppendorf 5415). This low value of relative centrifugal force (RCF) was chosen because control experiments showed that at higher RCF values hemoglobin occasionally coprecipitated; this was probably due to a denaturation of hemoglobin during the relatively long incubation time. With a RCF value of 735*g, recovery of added hemoglobin was >95%. After centrifugation and dilution in double-distilled water, Fe-content of the supernatants was measured using atomic absorption spectrophotometry. Lytic activity of each fecal water sample was quantified as the area under the lytic curve. This lytic activity is expresseo as a percentage of the maximal area, which implies 100% lysis at each dilution of fecal water. The mean variation coefficient (SD/mean * 100%) using five different samples each measured in triplicate was 5.7%.

Statistics. Values are given as means ± SEs. Because our in vitro (Van der Meer *et al.*, 1991) and animal studies (Lapré *et al.*, 1991a; Van der Meer & Govers, 1991) have indicated that calcium decreases the hydrophobicity of surfactants and their lytic activity, differences between the control and calcium period were tested for one-tail significance. Differences in composition of fecal water were tested by Student's t-test for paired samples. Data on lytic activity were tested using Wilcoxon's signed rank test. Differences were regarded as significant if $P<0.05$. Data of the solubility product experiments were analyzed using regression analysis.

RESULTS

The input-output analysis of this study has been given earlier (Van der Meer *et al.*, 1990). In summary, during the calcium supplementation period the total (urinary + feces) output of phosphate and magnesium remained constant. Supplemental calcium was recovered for 95%, indicating an excellent dietary compliance. Calcium supplementation stimulated the fecal excretion of calcium, phosphate and bile acids and slightly increased fecal pH.

In fecal water the concentration of calcium was increased by supplemental calcium whereas the concentration of soluble phosphate was drastically decreased (Figure 1).

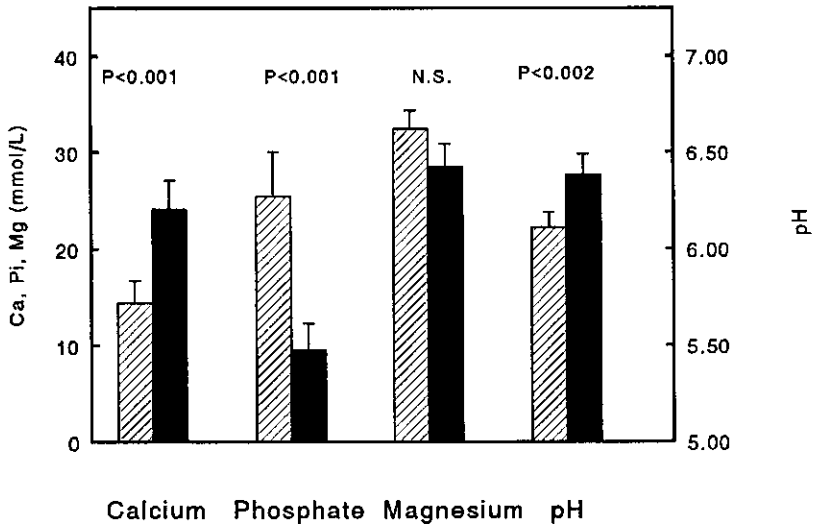


Figure 1 Effects of supplemental dietary calcium on mineral composition and pH of fecal water. Hatched bars represent control period and closed bars represent calcium-supplemented period.

This indicates the formation of insoluble calcium phosphate in the intestine. No effect was seen on soluble magnesium concentrations. Analogous to feces (Van der Meer *et al.*, 1990), the pH of fecal water was slightly increased by supplemental calcium. Subsequently, we ascertained whether fecal soluble calcium and phosphate were in equilibrium with precipitated calcium phosphate. We first quantified this equilibrium in vitro using mineral concentrations comparable with those in feces (Van der Meer *et al.*, 1990).

As shown in Figure 2, in vitro this equilibrium is reflected by a linear relationship between pH and the logarithm of the concentration product of calcium and phosphate in the equilibrated supernatants. This linear relationship for the solubility product versus pH is in accordance with the theory of calcium phosphate precipitation. Magnesium hardly coprecipitated with calcium and phosphate because >90% of the magnesium added was recovered in the supernatant. For calcium and phosphate in fecal water also a linear relationship (r=-0.90) was observed, which paralleled the line for the solubility product of insoluble calcium phosphate in vitro.

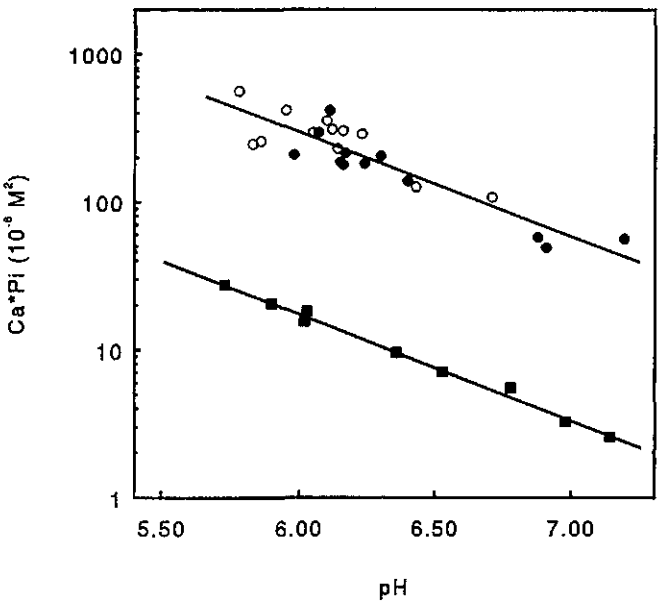


Figure 2 Relationship between the concentration product of soluble calcium and inorganic phosphate versus pH of fecal water (○,●) and of supernatants of in vitro precipitation study (■). Open circles represent the control period and closed circles represent the calcium supplemented period.

In view of the hypothesis that dietary calcium inhibits luminal lytic activity by decreasing the concentrations of soluble surfactants, we quantified the bile acid and fatty acid concentrations in fecal water. Table 1 summarizes the effects of supplemental calcium on the luminal surfactant concentrations. It should be noted that the total bile acid concentration in fecal water was not significantly altered by supplemental calcium. However, there is a trend towards a decrease in hydrophobic cytotoxic bile acids and an increase in hydrophilic, less cytotoxic bile acids. As a consequence the ratio of (mono + dihydroxy) to (keto + trihydroxy) bile acids is very significantly decreased. Because the solubility of neutral sterols is dependent on the hydrophobicity of bile acids (Chijiiwa *et al.*, 1988), we also determined the effects of supplemental calcium on cholesterol and coprostanol in fecal water. As can be seen

in Table 1, the concentrations of cholesterol as well as of coprostanol were very significantly decreased by calcium supplementation.

Subsequently, we determined the effects of supplemental calcium on the fatty acid concentrations in fecal water. Total fatty acid concentration in fecal water was significantly decreased by supplemental calcium. The most predominant fatty acids in fecal water were C_6, C_8, C_{16}, $C_{18:0}$ and $C_{18:1}$, with only trace amounts of the other fatty acids. None of the individual fatty acids showed a significant reduction in concentration after calcium supplementation. Only the decrease in $C_{18:1}$ reached borderline significance.

Table 1 Effects of supplemental dietary calcium on bile acid composition, neutral sterols and fatty acid composition in fecal water.

	Control	Calcium	Difference
Bile acids (μmol/L)	175±58	205±93	30±38
Lithocholate	11± 2	6± 1	-5± 1[c]
Isolithocholate	5± 1	1± 1	-4± 1[b]
Deoxycholate	61±15	55±14	-5± 4
Isodeoxycholate	18± 3	18± 4	0± 2
Chenodeoxycholate	4± 1	6± 2	2± 1[a]
12-Ketolithocholate	26±10	28±10	2± 3
Cholate	13± 4	27±13	14±10
Ketodihydroxy cholanate	7± 5	23±20	16±16
Others	31±21	41±33	10±12
Ratio (mono+dihydroxy) to (keto+trihydroxy) bile acids	3.3±0.5	2.3± 0.4	-1.0±0.3[b]
Neutral sterols (μmol/L)	101±16	59± 8	-42±10[c]
Cholesterol	-15±10	34± 5	-15± 5[b]
Coprostanol	-27±10	24± 6	-27± 7[c]
Fatty acids (μmol/L)	2863±611	2126±374	-737±272[b]
C_6-C_8	2254±580	1733±328	-521±363
C_{16}	156±28	112± 22	-44± 27
C_{18}	149±32	104± 38	-45± 45
$C_{18:1}$	189±66	79± 17	-110± 60[a]
Minor fatty acids	115±64	97± 76	-18± 26

Values are Mean ± SE, n=12. [a]: P<0.05, [b]: P<0.01, [c]: P<0.001

Thus, supplemental dietary calcium lowered the concentrations of potentially lytic surfactants in fecal water and should, according to our in vitro observations (8,30), inhibit the lytic activity of fecal water. In eleven of the twelve persons lytic activity of fecal water was decreased by supplemental dietary calcium (Figure 3). Only in one person was lytic activity increased by extra calcium. This person also showed a decrease in total fecal and fecal water pH whereas the other persons showed an increase in fecal water pH after calcium supplementation. Lytic activity was significantly decreased ($P<0.025$, Wilcoxon) by supplemental calcium from 47% ± 9 to 27% ± 8 for these twelve volunteers (for n=11, calcium decreased lytic activity from 50% ± 9 to 23% ± 7).

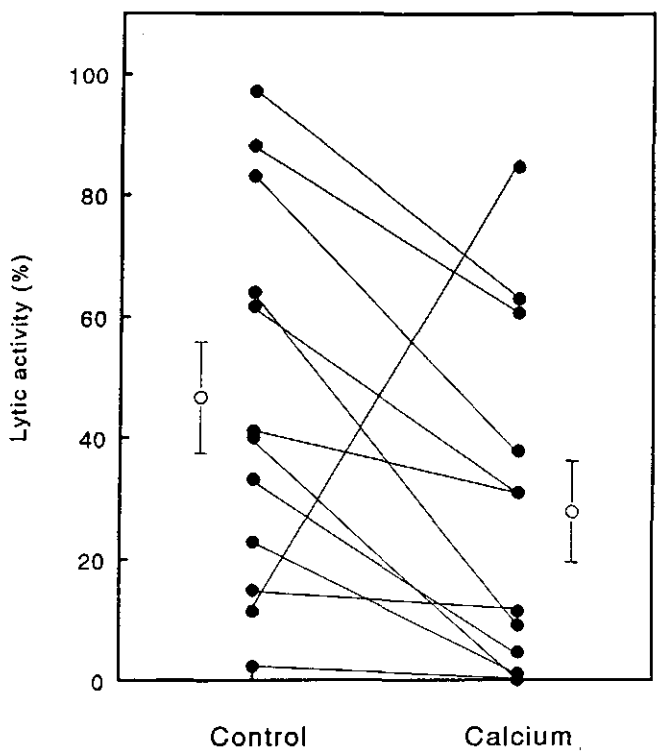


Figure 3. Lytic activity (%) of fecal water in the control period and after supplemental dietary calcium. Open circles represent means ± SEs (n=12).

DISCUSSION

The results of this study concerning the effect of supplemental calcium on fecal water composition are consistent with the effect on intestinal association of calcium, phosphate and bile acids observed in the first part of the study (Van der Meer *et al.*, 1990). For instance, the decrease of the phosphate concentration in fecal water is consistent with the increase in precipitated phosphate concentrations in feces (Van der Meer *et al.*, 1990). Combined with the calcium-phosphate-precipitation experiment, these results strongly suggest the formation of insoluble calcium phosphate in the intestine as also shown in nutritional experiments with rats (Van der Meer & Govers, 1991). It should be noted that the intercept of the regression line for fecal water is different from that for calcium phosphate precipitation in vitro. Thus the concentrations of soluble calcium and phosphate are higher in feces than those observed in vitro. These high concentrations of soluble calcium, magnesium and phosphate have also been found in studies using in vivo dialysis (Wrong *et al.*, 1965). This can be explained by the lower activity coefficients for calcium and phosphate in fecal water compared to the in vitro conditions. This is probably due to the higher viscosity of fecal water, but this requires further investigation.

Supplemental calcium did not affect the low total concentration of bile acids in fecal water, which is in accordance with analogous results observed in our resolubilization study (Van der Meer *et al.*, 1990). Similar low concentrations of soluble bile acids have also been observed in other studies (Rafter *et al.*, 1987; Allinger *et al.*, 1989; Stadler *et al.*, 1988). In our in vitro studies the low, submicellar concentrations of bile acids as measured in fecal water (approximately 200 μM) were also not precipitated by calcium phosphate (Van der Meer & De Vries, 1985; Van der Meer *et al.*, 1991). In contrast to the lack of effect on the total concentration of soluble bile acids, the ratio of hydrophobic to hydrophilic bile acids in fecal water was significantly decreased by supplemental calcium, which is consistent with the effects on the duodenal bile acid composition observed in the first part of our study (Van der Meer *et al.*, 1990). As discussed previously (Van der Meer & De Vries, 1985; Van der Meer *et al.*, 1991), this indicates that supplemental calcium causes precipitation of micellar concentrations of the hydrophobic dihydroxy bile acids. Rafter *et al.* (1987) and Allinger *et al.* (1989) did not specify whether the ratio of hydrophobic to hydrophilic bile acids in fecal water was decreased. Only a decrease in the concentration of deoxycholate was reported (Allinger *et al.*, 1989). Because in these studies the total

soluble bile acid concentration remained constant, this suggests that the ratio of hydrophobic to hydrophilic bile acids was also lowered. With regard to this effect it should be noted that our in vitro studies indicate that a decreased hydrophobicity of bile acids inhibits their lytic activity (Van der Meer *et al.*, 1991). Bile acids are not the only surfactants in fecal water, therefore we also determined the soluble free fatty acid concentrations. The decrease in soluble fatty acids that we found after supplemental calcium can not be directly compared with the concentrations observed in the multivariate studies of Rafter *et al.* (Rafter *et al.*, 1987; Allinger *et al.*, 1989). Moreover, their method of determination of fatty acids did not distinguish between the (lytic) free fatty acids and those derived from tri-, di-, and monoglycerides and of phospholipids. Consequently, the concentrations given by these authors are higher than the concentrations shown in Table 1. In line with the decrease in free fatty acid concentration and the shift from hydrophilic to hydrophobic bile acids in fecal water a decrease in neutral sterol concentration was observed. These changes in fecal water lipids show that supplemental calcium causes a decrease in hydrophobicity of fecal water.

The observed calcium-dependent decreases in fatty-acid concentration and in hydrophobicity of the bile acids during supplemental calcium resulted in a decreased lytic activity of fecal water. This effect of calcium is consistent with the effects observed in our in vitro studies (Lapré *et al.*, 1990). In those studies we showed that fatty acids with a carbon number >10 are lytic to erythrocytes and that bile acids in concentrations measured in fecal water synergistically stimulate this fatty acid-induced lytic activity. This synergistic effect increases with increasing hydrophobicity of the bile acids (Lapré *et al.*, 1990).

The membrane-damaging activity of bile acids and fatty acids is a well-established intrinsic physicochemical property of these surfactants. Lysis, however, is determined by this lytic activity as well as by the susceptibility of the plasma membrane exposed to these surfactants. For this reason we wish to stress that our hemolysis assay is only meant to quantitate the calcium-dependent changes in overall lytic activity of the luminal surfactants to which the colonic epithelium is exposed. Our data should not be interpreted as a quantitative measure of epitheliolysis caused by these surfactants because the determinants of the epithelial susceptibility are at present largely unknown. For instance, individual differences in composition and thickness of the protective mucin layer as well as in lipid composition of the apical

membrane of the colonocyte (Simons & Van Meer, 1988) may modulate the epithelial susceptibility to luminal surfactants. Recent experiments indicate however, that bile-acid-induced lysis of erythrocytes (Van der Meer *et al.*, 1991; Coleman *et al.*, 1987) is similar to that of human colonic epithelial cells in vitro (Velardi *et al.*, 1991). In addition, it should be noted that diet-induced changes of lytic activity of fecal water and of in vivo colonic epithelial cell-proliferation are highly correlated in rats (Lapré & Van der Meer, 1992). This at least indicates that the lytic activity of fecal water is a physiologically relevant intermediate in the mechanism of dietary modulation of colonic proliferation. Precise quantitation of the relationships in humans between lytic activity, epitheliolysis and compensatory hyperproliferation awaits the availability of a quantitative marker of epitheliolysis. This is at present under investigation and preliminary results indicate that release of the epithelial exoenzyme alkaline phosphatase may be of relevance for quantitating epitheliolysis (Lapré *et al.*, 1991b).
These protective effects of supplemental dietary calcium on composition and lytic activity of luminal surfactants offer a molecular explanation for the calcium-dependent inhibition of epithelial hyperproliferation in patients at risk of colon cancer (Lipkin & Newmark, 1985; Lipkin *et al.*, 1989; Rozen *et al.*, 1989;Lans *et al.*, 1991). As mentioned above, this mechanism is consistent with in vitro studies on solubility and cytotoxicity of bile acids and fatty acids (Van der Meer & de Vries, 1985; Van der Meer *et al.*, 1991) and with the protective effect of calcium observed in animal studies (Lapré *et al.*, 1991a; Van der Meer & Govers, 1991; Wargovich *et al.*, 1984). Notwithstanding these findings, some recent studies indicate that the mechanism and efficacy of the protective effect of calcium are not yet fully established. For instance, it has also been shown that soluble calcium can increase the lytic activity of hydrophobic bile acids in vitro (Van der Meer *et al.*, 1991). In addition, it has been hypothesized (Whitfield, 1991) that soluble calcium per se may also stimulate the proliferation of aberrant epithelial cells. Moreover, in other clinical trials calcium did not inhibit epithelial hyperproliferation (Gregoire *et al.*, 1989; Stern *et al.*, 1990; Cats *et al.*, 1991). At present it is not known whether these differences in efficacy of dietary calcium are due to differences in methodology or to pathophysiologically relevant differences in e.g. luminal lytic activity, epitheliolysis and proliferative response. To address these questions, additional combined clinical and biochemical studies are urgently required.

Acknowledgements: The authors wish to thank C. De Jong, R. Poelstra, R. Neeter and H. Teisman for their assistance with the GC/MS analysis. This study was supported by the Dutch Cancer Society (GUKC 89-08), by the Netherlands Organization for Scientific Research (NWO), Medical Sciences (Grant 900-562-078) and by a grant from the European Community (Grant 1001/90-32.1).

REFERENCES

Allinger UG, Johansson GK, Gustafsson JA, Rafter JJ. Shift from a mixed to a lactovegetarian diet: influence on acidic lipids in fecal water - a potential risk factor for colon cancer. *Am J Clin Nutr* 1989;50:992-996

Bird RP, Schneider R, Stamp D, Bruce WR. Effect of dietary calcium and cholic acid on the proliferative indices of murine colonic epithelium. *Carcinogenesis* 1986;7:1657-1661

Cats A, De Vries EGE, Welberg JMW, Hardonk MJ, Zwart N, Harms G, Van der Meer R, Mulder NH, Kleibeuker JH. Increase of epithelial cell-proliferation in the sigmoid during dietary calcium supplementation. *Gastroenterology* 1991;100:A353

Chijiiwa K, Kiyosawa R, Fukudome K, Nakayama F. Differences in the release of cholesterol from taurocholate versus taurochenodeoxycholate micellar solutions. *Biochim Biophys Acta* 1988;962:208-213.

Coleman R. Bile salts and biliary lipids. *Biochem Soc Trans* 1987;15:68S-80S

De Jong C, Badings HT. Determination of free fatty acids in milk and cheese. *J High Res Chrom* 1990;13:94-98

Fiske CH, Subbarow Y. The colorimetric determination of phosphorus. *J Biol Chem* 1925;66:375-400

Garland CF, Comstock GW, Garland FC, Helsing KJ, Shaw EK, Gorham ED. Serum 25-hydroxyvitamin D and colon cancer: eight-year prospective study. *Lancet* 1989;II:1176-1178

Gregoire RC, Stern HS, Yeung KS, Stadler J, Langley S, Furrer R, Bruce WR. Effect of calcium supplementation on mucosal cell proliferation in high risk patients for colon cancer. *Gut* 1989;30:376-382

Lans JI, Jaszewski R, Arlow FL, Tureand J, Luk GD, Majumdar APN. Supplemental calcium suppresses colonic mucosal ornithine decarboxylase activity in elderly patients with adenomatous polyps. *Cancer Res* 1991;51:3416-3419

Lapré JA, Strootman EG, Van der Meer R. Cytotoxic effects of mixed micelles of bile acids and fatty acids. In: *Abstract Book Second Int. Meeting on Pathochemistry, Pathophysiology and Pathomechanics of the Biliary System.* Bologna March 19-21, 1990:177

Lapré JA and Van der Meer R. Diet-induced increase of colonic bile acids stimulate lytic activity of fecal water and proliferation of colonic cells. *Carcinogenesis* 1992;13:41-44

Lapré JA, De Vries HT, Van der Meer R. Dietary calcium phosphate inhibits cytotoxicity of fecal water. *Am J Physiol* 1991a;261:G907-G912

Lapré JA, Kleibeuker JH, Van der Meer R. Intestinal alkaline phosphatase in fecal water reflects epitheliolysis and is decreased by dietary calcium. *Gastroenterology* 1991b;100:A378

Lipkin M, Newmark H. Effect of added dietary calcium on colonic epithelial cell proliferation in subjects at high risk for familial colonic cancer. *N Eng J Med* 1985;313:1381-1384.

Lipkin M, Friedman E, Winawer SJ, Newmark H. Colonic epithelial cell proliferation in responders and nonresponders to supplemental dietary calcium. *Cancer Res* 1989;49:248-254

Lipkin M. Biomarkers of increased susceptibility to gastrointestinal cancer: new application to studies of cancer prevention in human subjects. *Cancer Res* 1988;48:235-245

Newmark HL, Wargovich MJ, Bruce WR. Colon cancer and dietary fat, phosphate, and calcium: a hypothesis. *J Natl Cancer Inst* 1984;72:1323-1325

Rafter JJ, Child P, Anderson AM, Alder R, Bruce WR. Cellular toxicity of fecal water depends on diet. *Am J Clin Nutr* 1987;45:559-563

Reshef R, Rozen P, Fireman Z, Fine N, Barzilai M, Shasha SM, Sholnik T. Effects of a calcium-enriched diet on the colonic epithelial hyperproliferation induced by N-Methyl-N'-Nitro-N-Nitrosoguanidine in rats on a low calcium and fat diet. *Cancer Res* 1990;50:1764-1767

Rozen P, Fireman Z, Fine N, Wax Y, Ron E. Oral calcium suppresses increased rectal proliferation of persons at risk of colorectal cancer. *Gut* 1989;30:650-655

Setchell KDR, Lawson AM, Tanida N, Sjövall J. General methods for the analysis of metabolic profiles of bile acids and related compounds in feces. *J Lipid Res* 1983;24:1085-1100.

Shaw R, Elliott WH. Bile acids. LV. 2,2-Dimethoxypropane: an esterifying agent preferred to diazomethane for chenodeoxycholic acid. *J Lipid Res* 1978;19:783-786.

Simons K, Van Meer G. Lipid sorting in epithelial cells. *Biochemistry* 1988;27:6197-6202

Sorenson AW, Slattery ML, Ford MH. Calcium and colon cancer: a review. *Nutrition and cancer* 1988;11:135-145

Stadler J, Stern HS, Yeung KS, Mcguire V, Furrer R, Marcon N, Bruce WR. Effect of high fat consumption on cell proliferation activity of colorectal mucosa and on soluble faecal bile acids. *Gut* 1988;29:1326-1331

Stern HS, Gregoire RC, Kashtan H, Stadler J, Bruce WR. Long-term effects of dietary calcium on risk markers for colon cancer in patients with familial polyposis. *Surgery* 1990;108:528-533

Van der Meer R, De Vries HT. Differential binding of glycine- and taurine-conjugated bile acids to insoluble calcium phosphate. *Biochem J* 1985;229:265-268

Van der Meer R, Welberg JWM, Kuipers F, Kleibeuker JH, Mulder NH, Termont DSML, Vonk RJ, De Vries HT, De Vries EGE. Effects of supplemental dietary calcium on the intestinal association of calcium, phosphate and bile acids. *Gastroenterology* 1990;99:1653-1659

Van der Meer R, Termont DSML, De Vries HT. Differential effects of calcium ions and calcium phosphate on cytotoxicity of bile acids. *Am J Physiol* 1991;260:G142-G147

Van der Meer R, Govers MJAP. Dietary phosphate does not inhibit the protective effects of calcium on luminal solubility and cytotoxicity of bile acids and fatty acids. *Gastroenterology* 1991; 100:A407

Velardi ALM, Groen AK, Oude Elferink RJP, Van der Meer R, Palasciano R, Tytgat GNJ. Cell-type dependent effect of phospholipid and cholesterol on bile salt cytotoxicity. *Gastroenterology* 1991;101:457-464

Wargovich MJ, Eng VWS, Newmark HL. Calcium ameliorates the damaging and compensatory

proliferative effects of fatty acids on mouse colonic epithelium. *Cancer Lett* 1984;23:253-258

Weisburger JH, Wynder EL. Etiology of colorectal cancer with emphasis on mechanism of action and prevention. In: DaVita VT, Hellman S, Rosenberg SA eds. *Important advances in oncology 1987.* JB Lippincott, Philadelphia, 1987:197-220

Whitfield J. Calcium switches, cell cycles, differentiation, and death. In: Lipkin M, Newmark HL, Kelloff G, eds. *Calcium, Vitamin D, and Prevention of colon cancer.* Boca Raton: CRC Press Inc. 1991:31-74

Willett W. The search for the causes of breast and colon cancer. *Nature* 1989;338:389-394

Willett WC, Stampfer MJ, Colditz GA, Rosner BA, Speizer FE. Relation of meat, fat, and fiber intake to the risk of colon cancer in a prospective study among women. *N Eng J Med* 1990;323:1664-1672.

Wrong O, Metcalfe-Gibson A, Morrison RBF, Ng ST, Howard AV. In vivo dialysis of faeces as a method of stool analysis. *Clin Sci* 1965;28:357-375

CHAPTER 9 SUMMARY AND CONCLUDING REMARKS

SUMMARY

Colon cancer is the second-most common malignancy in both males and females and is strongly related to environmental factors of which diet seems to be the most important one. Dietary fat is positively correlated with the incidence of colon cancer whereas dietary fibre and dietary calcium seem to be negatively associated with the risk of colon cancer. The development of colon cancer can genetically and histologically be characterized in different phases which may offer several oppertunities for dietary intervention. A high intake of dietary fat promotes colon cancer in tumor-induction models whereas dietary calcium counteracts this promotive effect of dietary fat. The promotive effect of dietary fat may be mediated by bile acids and fatty acids in colon which induce hyperproliferation of the colonic epithelium. Colonic hyperproliferation is generally considered as a biomarker of an increased susceptibility to colon cancer. The mechanism by which the surface-active bile acids and fatty acids induce hyperproliferation consists probably of damage of the cell-membrane resulting in cytolysis and cell-death. Luminal binding of these surfactants by insoluble calcium phosphate may decrease the solubility of these surfactants, their cytotoxic effects and the induction of hyperproliferation (Figure 1).

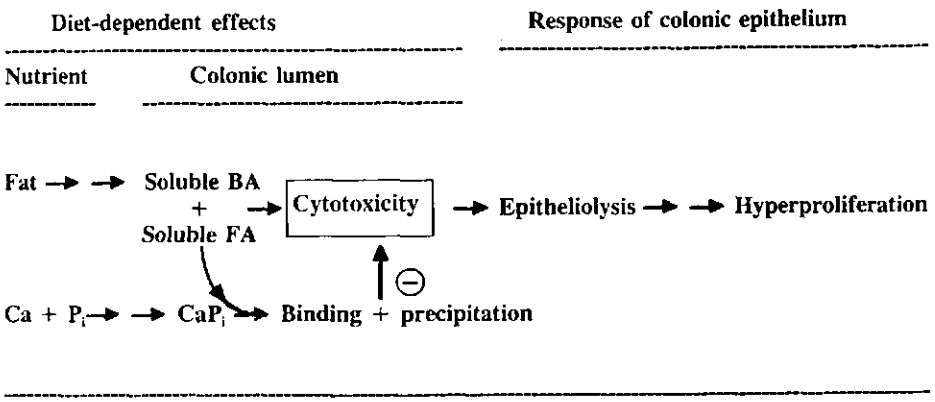


Figure 1 Hypothetical mechanism of the interactions between dietary fat and calcium with regard to the risk of colon cancer.

The experimental studies described in this thesis investigated this hypothesized mechanism by which dietary calcium inhibits proliferation of the colonic epithelium and the risk of colon cancer using the following step-wise approach. First, in in vitro studies (chapter 3) it was shown that bile acids and fatty acids are toxic for erythrocytes and for colonic epithelial cells in vitro (CaCo-2 cells). Moreover, cytotoxicity of these surfactants was similar in both cell-types. Fatty acids were as cytotoxic as bile acids with a similar dependency on hydrophobicity, but in contrast to bile acids, cytotoxicity of fatty acids was apparently not associated with micelle-formation. Low submicellar, sublytic concentrations of bile acids synergistically stimulated fatty acid-induced cytotoxicity. Cytotoxicity of these mixtures of bile acids and fatty acids was, analogous to cytotoxicity of bile acids alone, dependent on micelle formation. The synergistic effect of bile acids on fatty acid-induced cytotoxicity was stimulated by an increasing hydrophobicity of the bile acids. Lowering of the fatty acid concentration of these mixtures drastically decreased their cytotoxic effects. This in vitro study showed that physiologically relevant mixtures of bile acids and fatty acids are toxic to enterocytes as well as erythrocytes.

Secondly, it was investigated in rats whether a diet-induced increase in colonic bile acids stimulated cytotoxicity of fecal water and colonic proliferation (chapter 4). Therefore, fecal water was isolated from feces. Fecal water is that fraction of feces which contains the soluble surfactants which are assumed to damage cells. A diet-induced increase in colonic bile acids drastically stimulated cytotoxicity of fecal water analogous to the increase in concentration of bile acids in fecal water. Also an increase in colonic proliferation measured as [^{3}H]-incorporation into DNA was observed. Cytotoxicity of fecal water and colonic proliferation were highly correlated ($r=0.85$, $n=24$, $P<0.001$) indicating cause-and-effect relationships. Thus, this study showed that diet could affect the risk of colon cancer by modulation of cytotoxicity of the intestinal contents. Subsequently, the next step in the hypothesis, suggesting that dietary supplementation with $CaHPO_4$ stimulated the intestinal formation of insoluble CaP_i, decreased the concentrations of soluble surfactants and lowered cytotoxicity of fecal water, was investigated (chapter 5). Supplemental $CaHPO_4$ resulted in a dose-dependent decrease in bile acid concentration of fecal water and an increased precipitated fraction of bile acids. Consequently a drastic, dose-dependent fall in cytotoxicity was observed. Correlations between precipitated bile acids and cytotoxicity with precipitated calcium and inorganic phosphate ($r>0.90$) showed that these

parameters were closely associated. Thus, dietary supplementation with $CaHPO_4$ decreased cytotoxicity of fecal water by intestinal precipitation of bile acids.

Because bile acids and fatty acids are products of fat digestion, the type of dietary fat may influence the cytotoxic effects of the intestinal contents and the interaction with calcium phosphate. Therefore, three different purified types of dietary fat (*milk fat, palm oil and corn oil*) were used each at a low (25 μmol $CaHPO_4$/g diet) and a high (225 μmol $CaHPO_4$/g diet) level (chapter 6). Milk fat contains rather large amounts of short- and medium-chain triglycerides ($C_{4:0}$-$C_{12:0}$), palm oil contains triglycerides rich in palmitate ($C_{16:0}$) and oleate ($C_{18:1}$) and corn oil contains linoleate ($C_{18:2}$) rich triglycerides. The excretion of fatty acids was dependent on the type of dietary fat and increased in the order corn oil << milk fat << palm oil. Supplemental $CaHPO_4$ drastically stimulated fatty acid excretion with the same fat-type dependency. In contrast, concentrations of soluble fatty acids and bile acids were drastically decreased by supplemental $CaHPO_4$. This effect was dependent on the type of dietary fat with palm oil resulting in the highest concentration of fatty acids. Cytotoxicity of fecal water decreased analogous to the decrease in concentrations of soluble surfactants with the same fat-type dependency. Multiple regression analysis showed that the concentrations of soluble bile acids and fatty acids are equally important determinants of cytotoxicity and explain 80% ($R = 0.89$) of the cytotoxicity of fecal water. Intestinal alkaline phosphatase activity in fecal water as a marker of intestinal epitheliolysis showed that the effects on cytotoxicity of fecal water were reflected in effects on intestinal epitheliolysis ($r=0.92$, $P<0.001$). Thus, despite increases in total fecal concentrations, dietary calcium phosphate decreased concentrations of soluble surfactants dependent on the type of dietary fat. The decrease in soluble surfactants resulted in a decreased cytotoxicity of fecal water and a decreased intestinal epitheliolysis.

To investigate whether the luminal effects of dietary calcium resulted in a change in response of the colonic epithelium, the anti-proliferative effect of calcium was studied in rats fed Western-type high risk diets containing low $CaHPO_4$ (25 μmol Ca/g), low fibre and high fat (40 en-%) levels (chapter 7). It should be noted that this low calcium diet mimicked a human diet with a calcium consumption of 500 mg/day (mean intake in The Netherlands is about 1000 mg/day). Types of fat used were commercially available fats: butter, mimicking a diet with a high saturated medium-chain triglycerides content, saturated margarine, reflecting a diet with a high long-chain

saturated fat content, and polyunsaturated margarine, ressembling a diet with a high polyunsaturated fat content. Diets were also supplemented with $CaHPO_4$ to 225 μmol/g diet. Dietary calcium phosphate decreased soluble surfactant concentrations and cytotoxicity of fecal water. These luminal effects of dietary calcium resulted in a lower intestinal epitheliolysis. On the butter and saturated margarine diets, these protective effects of calcium resulted in a decreased colonic proliferation. On the polyunsaturated diet, proliferation was not decreased by supplemental calcium which may suggest an additional mechanism in the induction of hyperproliferation. Multiple regression analysis of soluble surfactants with cytotoxicity, epitheliolysis and colonic proliferation showed highly significant associations. Cytotoxicity and epitheliolysis as well as epitheliolysis and colonic proliferation were highly correlated (resp. r=0.97 and r=0.88) for control and Ca-supplemented groups. This is consistent with the proposed mechanism and may indicate cause-and-effect relationships. Thus the anti-proliferative effect of dietary calcium is mediated by luminal surfactants and dependent on the type of dietary fat.

Finally, the luminal effects of supplemental dietary calcium were studied in a pilot intervention trial with young healthy volunteers. In this study supplemental calcium complexated with phosphate to insoluble calcium phosphate, but in contrast to the animal studies the (already low) concentration of soluble bile acids was not further decreased. The free fatty acid concentration in fecal water was lowered by supplemental calcium. Gaschromatographic analysis of fecal water showed that the composition of the bile acids in fecal water was altered from hydrophobic to hydrophilic. The overall effect of dietary calcium supplementation was a decrease in hydrophobicity of fecal water measured as a decrease in free fatty acid concentration, hydrophobic bile acids and neutral sterols. This resulted in a significant decrease in cytotoxicity of fecal water in these healthy volunteers. Thus, luminal effects of dietary calcium supplementation in humans are analogous to the effects in rats. These effects may offer a molecular explanation of the protective effects of dietary calcium supplementation on colonic epithelial proliferation in patients at risk for colon cancer.

CONCLUDING REMARKS

The experimental studies described in this thesis lead to the following conclusions:

1. Bile acids and fatty acids are cytotoxic surfactants which cause damage of cellular membranes resulting in cell-death. Lysis of erythrocytes is a convenient and relevant model system to determine cytotoxicity of these surfactants (chapter 3).
2. Diet may modulate the proliferative behavior of the colonic epithelium by luminal effects. Cytotoxicity of fecal water measured as lysis of erythrocytes provides relevant information about the luminal effects of diet (chapter 4 & 5).
3. The type of dietary fat influences the concentrations of luminal surfactants, cytotoxicity, intestinal epitheliolysis (chapter 6 & 7) and colonic proliferation (chapter 7). Dietary calcium phosphate supplementation causes precipitation of luminal surfactants. As a consequence, concentrations of soluble cytotoxic surfactants are lowered, which results in a decreased cytotoxicity of fecal water (chapter 5-7). These luminal effects of dietary calcium phosphate decrease intestinal epitheliolysis (chapter 6 & 7) and lower colonic epithelial proliferation (chapter 7).
4. In humans, supplemental calcium lowers the hydrophobicity of fecal water and consequently decreases cytotoxicity of fecal water.

With regard to these conclusions some remarks have to be made concerning the protective effects of calcium. It should be stressed that cytotoxicity is not a measure of intestinal cell-damage. Luminal cytotoxicity only refers to the potency of the intestinal contents to damage cells. Cytotoxicity is therefore solely determined by the physicochemical characteristics of the luminal surfactants. Lysis of epithelial cells is dependent on this cytotoxicity, but also on the susceptibility of the plasma membrane to the luminal surfactants. Determinants of this epithelial susceptibility are at present largely unknown. In rats consuming identical diets only differing in the amount of $CaHPO_4$ and type of dietary fat, the proposed sequence of effects (figure 1) has been demonstrated. However, in the experiment with human volunteers only the luminal effects of dietary calcium supplementation were studied (chapter 8). Whether the observed protective effects result, analogous to the effects in rats, in a lower colonic proliferation is at present unknown. Therefore, the results of chapter 8

should only be interpreted as a first indication how dietary calcium in humans could decrease the risk of colon cancer.

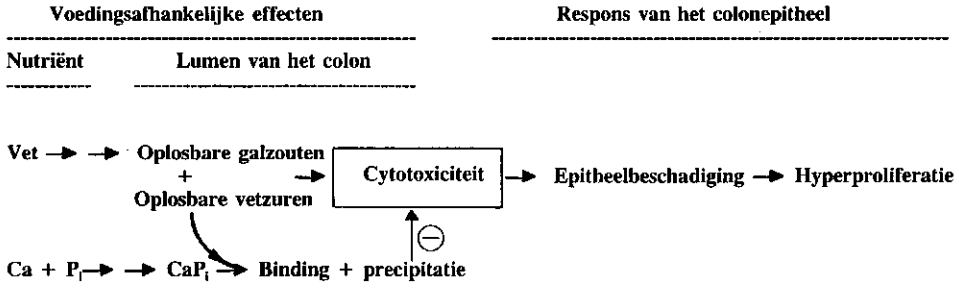
Further investigations regarding calcium and colon cancer should focuss on the effects of calcium supplementation on colonic epithelial proliferation using a combined biochemical and physiological design. Only these combined efforts may shed some light on the complex role of diet in colorectal carcinogenesis. Many questions have still to be answered. Dose-response and time-relationships need to be ascertained. Whether an optimal dose of calcium supplementation exists, is not clear. Whether dairy products, which are rich in calcium, have the same effects should also be ascertained. Possible interactions not only influenced by the the type of dietary fat, but also interactions between the protective agents fibre and calcium in the intestinal lumen may be important. It should be clear that luminal colonic effects of diet are very complex to study, because of intestinal interactions between the different dietary components. However, these interactions are important in the dietary prevention of colon cancer.

In conclusion, the experiments described in this thesis show that dietary supplementation with $CaHPO_4$ decreases cytotoxicity of fecal water resulting in a decreased colonic epithelial proliferation. The relative importance of dietary calcium as an anti-promoter of colon carcinogenesis has to be established in the following years. Several multi-center intervention trials have started during the last few years studying the effects of calcium supplementation on proliferation-markers and recurrence of polyps. However, one should be cautious not to neglect biochemical and animal studies to investigate mechanisms how calcium could affect the risk of colon cancer. Dietary advice with regard to calcium-intake and the prevention of colon cancer has to await knowledge of relevant mechanisms combined with consistent results in in vitro studies, experiments with animals and human intervention trials.

CHAPTER 10 SAMENVATTING EN SLOTBESCHOUWING

SAMENVATTING

Colonkanker is één van de meest voorkomende vormen van kanker in zowel mannen als vrouwen. Het vóórkomen van colonkanker is sterk gekoppeld aan omgevingsfactoren waarvan de voeding één van de belangrijkste lijkt te zijn. Een hoog vetgehalte in de voeding is positief gecorreleerd met het optreden van colonkanker terwijl vezel en calcium in de voeding negatief geassocieerd lijken te zijn. De ontwikkeling van colonkanker kan zowel genetisch als histologisch worden onderscheiden in verschillende fasen, die elk mogelijkheden bieden voor beïnvloeding via de voeding. Een hoge vetinneming bevordert het ontstaan van colonkanker in tumor-inductie modellen terwijl calcium in de voeding dit effect van vet remt. De promoverende werking van vet zou kunnen worden veroorzaakt door in het colon aanwezige galzouten en vetzuren. Deze stoffen zijn in staat om hyperproliferatie van het colonepitheel te induceren. Deze hyperproliferatie wordt in het algemeen beschouwd als een indicator voor een verhoogd risico op het ontstaan van colonkanker. Het mechanisme waardoor de oppervlakte-actieve galzouten en vetzuren hyperproliferatie veroorzaken, bestaat waarschijnlijk uit een beschadiging van de celmembraan gevolgd door cytolyse en celdood. Binding van deze oppervlakte-actieve stoffen door onoplosbaar calciumfosfaat in het darmlumen zou de oplosbaarheid van deze stoffen, hun cytotoxische effecten en de inductie van hyperproliferatie kunnen verlagen (Figuur 1).



Figuur 1 Hypothetisch mechanisme voor de interacties tussen voedingsvet en calcium met betrekking tot het risico voor colonkanker.

In de experimenten beschreven in dit proefschrift is dit hypothetisch mechanisme als uitgangspunt genomen om te onderzoeken hoe calcium in de voeding proliferatie van het colonepitheel en het risico voor colonkanker kan verlagen. Hiertoe is de volgende stapsgewijze aanpak gebruikt. Allereerst is in vitro (hoofdstuk 3) aangetoond dat galzouten en vetzuren toxisch zijn voor erythrocyten en voor colonepitheelcellen in kweek (CaCo-2 cellijn). Bovendien bleek dat de cytotoxiciteit van deze oppervlakte-actieve stoffen in beide celtypen vergelijkbaar was. Vetzuren bleken in dezelfde mate cytotoxisch als galzouten met een overeenkomstig verband tussen cytotoxiciteit en hydrofobiciteit. Echter, in tegenstelling tot galzouten was de cytotoxiciteit van vetzuren niet duidelijk geassocieerd met de vorming van micellen. Lage submicellaire, sublytische concentraties galzouten verhoogden synergistisch de vetzuur-geïnduceerde cytotoxiciteit. De cytotoxiciteit van deze mengsels was wel geassocieerd met de vorming van micellen, overeenkomstig met de cytotoxiciteit van galzouten alleen. Een toenemende hydrofobiciteit van de galzouten die aan vetzuren werden toegevoegd, verhoogde de cytotoxiciteit van deze mengsels. Verlaging van de vetzuurconcentratie resulteerde in een drastische verlaging in cytotoxiciteit van de galzout-vetzuurmengsels. Dit in vitro onderzoek laat zien dat fysiologisch relevante mengsels van galzouten en vetzuren toxisch zijn voor zowel enterocyten als erythrocyten.

Ten tweede is in ratten onderzocht of een voedingsafhankelijke toename van de concentratie galzouten in de dikke darm de cytotoxiciteit van faecaal water en de proliferatie van het colonepitheel stimuleert (hoofdstuk 4). Hiertoe werd faecaal water geïsoleerd uit faeces. Faecaal water is die fractie van de faeces die de oplosbare galzouten en vetzuren bevat, waarvan aangenomen wordt dat ze cellen kunnen beschadigen. Het bleek dat een voedingsafhankelijke verhoging van de totale concentratie galzouten in het colon de cytotoxiciteit van faecaal water drastisch stimuleerde. Deze verhoging kwam overeen met de toename van de galzoutconcentratie in faecaal water. Daarnaast werd ook een verhoging van de proliferatie in het colon gevonden, gemeten door middel van [^{3}H]-thymidine-incorporatie in DNA. Cytotoxiciteit van faecaal water en proliferatie van het colonepitheel bleken sterk gecorreleerd ($r=0,85$, $n=24$, $P<0,001$) wat een indicatie kan zijn voor causale verbanden. Dit experiment geeft aan dat de voeding het risico voor colonkanker kan beïnvloeden door middel van veranderingen in de cytotoxiciteit van de darminhoud. Vervolgens is de volgende stap in de hypothese onderzocht, die

aangeeft dat suppletie van de voeding met $CaHPO_4$ de vorming van onoplosbaar CaP_i verhoogt, de concentraties oplosbare galzouten en vetzuren verlaagt en de cytotoxiciteit van faecaal water vermindert (hoofdstuk 5). Suppletie met $CaHPO_4$ veroorzaakte een dosisafhankelijke verlaging van de galzoutconcentratie in faecaal water en een toegenomen fractie geprecipiteerde galzouten. Als gevolg hiervan werd een drastische, dosisafhankelijke verlaging van de cytotoxiciteit waargenomen. Correlaties tussen fracties geprecipiteerde galzouten en cytotoxiciteit met geprecipiteerd calcium en anorganisch fosfaat ($r>0,90$) gaven aan dat deze parameters nauw waren gecorreleerd. Dus suppletie met $CaHPO_4$ verlaagt de cytotoxiciteit van faecaal water door precipitatie van galzouten in de darm.

Omdat galzouten en vetzuren produkten zijn van de vetvertering, zou het type voedingsvet de cytotoxiciteit van de darminhoud en de interactie met $CaHPO_4$ kunnen beïnvloeden. Hiertoe zijn drie gezuiverde vetten gebruikt (melkvet, palmolie en maïsolie), elk op een laag (25 µmol/g voer) en een hoog (225 µmol/g voer) $CaHPO_4$ niveau (hoofdstuk 6). Melkvet bevat aanzienlijke hoeveelheden korte- en middenketenvetzuren ($C_{4:0}$-$C_{12:0}$), palmolie bevat triglyceriden rijk in palmitinezuur en oliezuur, en maïsolie bevat linolzuurrijke triglyceriden. De excretie van vetzuren was afhankelijk van het type voedingsvet en nam toe in de volgorde: maïsolie << melkvet << palmolie. Suppletie met $CaHPO_4$ verhoogde de vetzuuruitscheiding drastisch met eenzelfde vetafhankelijkheid. De concentraties aan oplosbare galzouten en vetzuren, daarentegen, werden sterk verlaagd door suppletie met $CaHPO_4$. Dit effect was afhankelijk van het type vet, waarbij palmolie in de hoogste concentratie vetzuren in faecaal water resulteerde. Analoog aan de verlaging van de concentraties oplosbare galzouten en vetzuren werd de cytotoxiciteit van faecaal water verlaagd door $CaHPO_4$. Multiple regressie analyse gaf aan dat de concentraties oplosbare galzouten en vetzuren de belangrijkste determinanten van cytotoxiciteit zijn, die 80% ($R=0,89$) van de cytotoxiciteit kunnen verklaren. Intestinaal alkalische fosfatase activiteit als maat voor darmbeschadiging kwam overeen met de effecten op cytotoxiciteit ($r=0,92$, $P<0,001$). Dus ondanks een toename in totale faecale concentraties, verlaagde calciumfosfaat de concentraties oplosbare oppervlakte-actieve stoffen afhankelijk van het type voedingsvet. Deze verlaging resulteerde in een lagere cytotoxiciteit van faecaal water en een afgenomen darmbeschadiging.

Om te onderzoeken of de luminale effecten van calcium in een verandering in respons van het colonepitheel resulteerden, is het anti-proliferatieve effect van calcium

onderzocht in een experiment met ratten gevoerd met een 'Westers, hoog risico' voer. Dit voer bevatte een laag $CaHPO_4$- (25 μmol Ca/g), laag vezel- en hoog vetgehalte (40 en-%) (hoofdstuk 7). Dit lage calciumgehalte weerspiegelt een humane voeding met een calciuminneming van 500 mg/dag (gemiddelde inneming in Nederland is ongeveer 1000 mg/dag). De volgende types commercieel verkrijgbare vetten zijn gebruikt: boter, een margarine met een hoog gehalte aan verzadigde vetzuren en een margarine met een hoog gehalte aan meervoudig onverzadigde vetzuren. daarnaast werden de voeders gesuppleerd tot 225 μmol $CaHPO_4$/g voer. $CaHPO_4$ verlaagde de concentraties oplosbare galzouten en vetzuren en de cytotoxiciteit van faecaal water. Deze luminale effecten van calcium resulteerden in een lagere beschadiging van het darmepitheel. Uiteindelijk mondden deze beschermende effecten van calcium uit in een lagere proliferatie van het colonepitheel op zowel de boter als de verzadigd margarinevoeren. Op het meervoudig onverzadigde margarine werd geen effect op proliferatie gevonden wat een aanwijzing kan zijn voor een additioneel mechanisme m.b.t. de inductie van proliferatie. Multiple regressie analyse van de concentraties oplosbare galzouten en vetzuren met resp. cytotoxiciteit, epitheelbeschadiging en proliferatie resulteerde in zeer significante associaties ($R>0,74$). Zowel cytotoxiciteit en epitheelbeschadiging als ook epitheelbeschadiging en proliferatie vertoonden hoge correlaties (resp. $r=0,97$ en $r=0,88$) voor de controle en calciumgesuppleerde voeren. Dit is consistent met het voorgestelde mechanisme en is een aanwijzing voor een causaal verband. Het anti-proliferatieve effect van calcium verloopt dus via luminale effecten en is afhankelijk van het type voedingsvet.

Uiteindelijk zijn de luminale effecten van calciumsuppletie bestudeerd in een voedingsinterventie met jonge gezonde vrijwilligers. In dit experiment complexeerde calcium met fosfaat tot onoplosbaar calciumfosfaat, maar in tegenstelling tot de rattenexperimenten werd de (reeds lage) concentratie van oplosbare galzouten niet verder verlaagd. De concentratie vrije vetzuren in faecaal water nam wel af door suppletie met calcium. Via gaschromatografische analyse van faecaal water werd gevonden dat de samenstelling van de galzouten veranderde van hydrofoob naar meer hydrofiel. Het uiteindelijke effect van calciumsuppletie was een afname in de hydrofobiciteit van het faecale water gemeten door een afname in de concentraties vetzuren, hydrofobe galzouten en neutrale sterolen. Dit resulteerde in een significante verlaging van de cytotoxiciteit van het faecale water. Dus, de luminale effecten van calciumsuppletie in vrijwilligers zijn vergelijkbaar met die in ratten. Deze effecten

kunnen een moleculaire verklaring vormen hoe calciumsuppletie de proliferatie van het colonepitheel in patiënten met een hoog risico voor colonkanker kan verlagen.

SLOTBESCHOUWING

De experimenten beschreven in dit proefschrift leiden tot de volgende conclusies:

1. Galzouten en vetzuren zijn cytotoxische oppervlakte-actieve stoffen die de celmembraan beschadigen wat resulteert in celdood. Lysis van erythrocyten is een eenvoudig en relevant modelsysteem om de cytotoxiciteit van deze stoffen te bepalen (hoofdstuk 3).
2. De voeding is in staat om de proliferatie van het colonepitheel te beïnvloeden via luminale effecten. Cytotoxiciteit van faecaal water gemeten als lysis van erythrocyten geeft relevante informatie over de luminale effecten van de voeding (hoofdstuk 4).
3. Suppletie van de voeding met calciumfosfaat veroorzaakt precipitatie van oppervlakte-actieve stoffen in het lumen van de darm. Hierdoor worden de concentraties oplosbare galzouten en vetzuren verlaagd wat een verminderde cytotoxiciteit van het faecale water tot gevolg heeft (hoofdstuk 5-7). Deze luminale effecten van calciumfosfaatsuppletie verlagen de darm-epitheelbeschadiging (hoofdstuk 6 & 7) en de proliferatie van het colonepitheel (hoofdstuk 7). Deze effecten van calciumfosfaat zijn afhankelijk van het type voedingsvet (hoofdstuk 6 & 7).
4. In vrijwilligers verlaagt calciumsuppletie de hydrofobiciteit van faecaal water en vermindert daardoor de cytotoxiciteit.

Met betrekking tot deze conclusies moeten enkele opmerkingen worden gemaakt over het beschermende effect van calcium. Benadrukt moet worden dat cytotoxiciteit geen directe maat is voor darmepitheelschade. Luminale cytotoxiciteit is slechts een afspiegeling van de potentiële celbeschadigende capaciteit van de darminhoud. Deze cytotoxiciteit wordt slechts bepaald door de fysisch-chemische karakteristieken van de luminale oppervlakte-actieve stoffen. Lysis van epitheliale cellen is afhankelijk van deze cytotoxiciteit, maar ook van de gevoeligheid van het plasmamembraan. Determinanten van deze gevoeligheid zijn momenteel grotendeels onbekend. In ratten die bijna identieke voeders slechts verschillend in de hoeveelheid

$CaHPO_4$ en type voedingsvet kregen, is de in figuur 1 voorgestelde sequentie van effecten aangetoond (hoofdstuk 5-7). Echter, in het experiment met gezonde vrijwilligers zijn slechts de luminale effecten van calciumsuppletie bestudeerd (hoofdstuk 8). Of de beschermende effecten van calciumsuppletie analoog aan de effecten in ratten resulteren in een verlaagde proliferatie van het colonepitheel is momenteel onbekend. Derhalve moeten de resultaten beschreven in hoofdstuk 8 slechts worden geïnterpreteerd als een eerste indicatie hoe calcium in de voeding in mensen het risico voor colonkanker zou kunnen verlagen.

Verdere onderzoekingen met betrekking tot calcium en colonkanker moeten zich richten op de effecten van calciumsuppletie op de proliferatie van het colonepitheel met behulp van een gecombineerde biochemische en fysiologische aanpak. Alleen deze gecombineerde aanpak kan resulteren in meer begrip over de complexe rol van voeding in de coloncarcinogenese. Vele vragen moeten nog worden beantwoord. Of er een optimale calciumdosis bestaat, is niet duidelijk. Of de effecten van melk- en zuivelprodukten, die rijk zijn aan calcium, vergelijkbaar zijn met die van calcium moet worden onderzocht. Mogelijke interacties, niet alleen beïnvloed door het type voedingsvet, maar ook interacties tussen de beschermende componenten vezel en calcium in het darmlumen kunnen belangrijk zijn. Het moge duidelijk zijn dat de effecten van de totale voeding in het colon moeilijk te bestuderen zijn vanwege mogelijke interacties tussen de verschillende componenten uit de voeding. Echter, juist deze interacties kunnen van belang zijn voor de preventie van colonkanker via de voeding.

De experimenten beschreven in dit proefschrift laten zien dat suppletie van de voeding met $CaHPO_4$ de cytotoxiciteit van faecaal water verlaagt wat uiteindelijk resulteert in een lagere proliferatie van het colonepitheel. Het relatieve belang van calcium als anti-promotor van de coloncarcinogenese moet de komende jaren blijken. Verschillende interventiestudies, elk uitgevoerd door verscheidene instituten, zijn de laatste jaren gestart waarbij de effecten van calciumsuppletie op proliferatiemerkers en terugkeer van poliepen worden bestudeerd. Echter, van belang is dat biochemische en diermodelstudies, die informatie moeten verschaffen volgens welk mechanisme calcium het risco voor colonkanker beïnvloedt, niet worden verwaarloosd. Voedingsadvies met betrekking tot calcium in de voeding en de preventie van colonkanker zal moeten wachten op kennis aangaande relevante mechanismen gecombineerd met consistente resultaten in in vitro experimenten, dierexperimenten en interventiestudies.

Curriculum vitae

John Lapré werd op 29 september 1964 geboren te Groningen. In 1982 behaalde hij het gymnasium-ß diploma aan de R.S.G. 'Koning Willem II' te Tilburg. In september 1982 begon hij aan de studie 'Voeding van de mens' aan de toenmalige Landbouwhogeschool Wageningen. Het propaedeutisch examen werd behaald in september 1983. In maart 1988 studeerde hij af met als hoofdvakken Toxicologie (Prof. Dr J.H. Koeman) en Humane voeding (Prof. Dr J.G.A.J. Hautvast). Zijn stage vervulde hij op het Rijksinstituut voor Volksgezondheid en Milieuhygiëne (RIVM) te Bilthoven. Per 1 maart 1988 begon hij als tijdelijk wetenschappelijk medewerker op de afdeling Voedingsfysiologie van het Nederlands Instituut voor Zuivelonderzoek (NIZO) te Ede aan het in dit proefschrift beschreven onderzoek. In 1988 behaalde hij het Certificaat Onderzoeker ex artikel 9 van de Wet op de Dierproeven. Sinds 1 maart 1992 is hij als wetenschappelijk medewerker in dienst van NIZO te Ede.