Cytotoxicity of Fecal Water Is Dependent on the Type of Dietary Fat and Is Reduced by Supplemental Calcium Phosphate in Rats

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ABSTRACT The effects of the type of dietary fat (180 g/kg diet) and of calcium phosphate (CaHPO₄) supplementation (25 vs. 225 mmol/kg diet) on luminal solubility of fatty acids and bile acids, cytotoxicity of fecal water and intestinal epitheliosis were studied in rats. In rats fed the low and high calcium phosphate diets, fecal excretion of fatty acids diminished in the order palm oil > milk fat > corn oil. Palm oil also caused the highest concentration of fatty acids measured in fecal water followed by milk fat and corn oil when fed at both calcium phosphate levels. The differences in concentrations of luminal surfactants in fecal water of rats fed the three fat diets resulted in a fat type-dependent cytotoxicity of fecal water, with that of palm oil-fed rats the most cytotoxic. The concentrations of fatty acids as well as bile acids in fecal water were, however, significantly lowered by calcium phosphate supplementation in rats fed all types of dietary fat. This reduction in concentration of fecal water surfactants resulted in a lower cytotoxicity of fecal water. The concentration of surfactants in fecal water and cytotoxicity were correlated by multiple regression analysis (R = 0.89). Intestinal epitheliosis measured as alkaline phosphatase activity in fecal water was lowered comparably to the reduction in cytotoxicity by supplemental calcium phosphate. Intestinal epitheliosis and cytotoxicity of fecal water were correlated (r = 0.92, P < 0.001). The type of dietary fat and the amount of dietary calcium phosphate influence the concentrations of surfactants in fecal water and consequently affect cytotoxicity of fecal water and intestinal epitheliosis. These interactions between dietary fat and calcium may partially explain how diet affects the risk of colon cancer. J. Nutr. 123: 578-585, 1993.

INDEXING KEY WORDS:
- colon cancer  •  colonic epithelium
- bile acids  •  fatty acids  •  rats

Epidemiologic studies suggest that a high incidence of colon cancer is positively associated with a high fat intake [Weisburger 1991, Willett et al. 1990] and negatively associated with the intake of dietary calcium [see Sorenson et al. 1988 for review]. Higher levels of dietary fat (200 g/kg compared with 50 g/kg) strongly promote experimental carcinogenesis in rodents [e.g., Reddy 1981]. Polyunsaturated fats may induce higher tumor incidences compared with saturated fats [Reddy et al. 1985]. However, Nicholson et al. [1990] found that fats high in linoleic acid produced lower tumor yields compared with saturated fats. High levels of dietary calcium counteract the promotive effects of high fat (200 g/kg diet) diets for several types of dietary fat [Behling et al. 1990, Wargovich et al. 1990]. It should be noted that these experiments using tumor inducers are difficult to interpret because of methodological differences, as discussed by Nicholson 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 complex 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 and 1984].

Previous studies from our laboratory have shown that calcium phosphate (CaHPO₄) is capable of binding bile acids [Van der Meer and De Vries 1985] in vitro and that this binding decreases their cytotoxicity [Van der Meer et al. 1991]. In rats, dietary supplementation with calcium phosphate decreases

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TABLE 1
Composition of the diets

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Milk fat diet</th>
<th>Palm oil diet</th>
<th>Corn oil diet</th>
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<td>Low</td>
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<td>180</td>
<td>—</td>
</tr>
<tr>
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<td>—</td>
<td>180</td>
</tr>
<tr>
<td>Corn oil&lt;sup&gt;2&lt;/sup&gt;</td>
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<td>20</td>
<td>20</td>
</tr>
<tr>
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<td>—</td>
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</tr>
<tr>
<td>CaHPO₄·2H₂O</td>
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<td>38.7</td>
<td>4.3</td>
</tr>
<tr>
<td>Acid-washed sand</td>
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<td>1.3</td>
<td>35.3</td>
</tr>
<tr>
<td>Constant components&lt;sup&gt;3&lt;/sup&gt;</td>
<td>760</td>
<td>760</td>
<td>760</td>
</tr>
</tbody>
</table>

<sup>1</sup>Diets contained low levels (25 mmol/kg diet) or high levels (225 mmol/kg diet) of calcium phosphate.
<sup>2</sup>The average fatty acid composition of the different dietary fats (g/100 g fatty acids) calculated from the Netherlands Normalisation Institute (Netherlands Normalisation Institute 1987). Milk fat: saturated fatty acids: 4:0-10:0, 11; 12:0, 4; 14:0, 11; 16:0, 27; 18:0, 11; monounsaturated fatty acids: 16:1, 3; 18:1, 25; polyunsaturated fatty acids: 18:2, 2; minor fatty acids: 6. Palm oil: saturated fatty acids: 14:0, 1; 16:0, 43; 18:0, 5; monounsaturated fatty acids: 18:1; polyunsaturated fatty acids: 18:2, 11; minor fatty acids: 1. Corn oil: saturated fatty acids: 16:0, 11; 18:0, 2; monounsaturated fatty acids: 27; polyunsaturated fatty acids: 57; minor fatty acids: 3.
<sup>3</sup>Constant components consisted of the following (g/kg diet): casein, 200; dextrose, 495; cellulose, 20; mineral mix, 35; vitamin mix, 10. The composition of the vitamin premix was as follows (g/kg): thiamin, 0.3; riboflavin, 0.4; pyridoxin, 0.3; nicotinamide, 2.5; p-biotin, 0.012; cyanocobalamin, 0.004; folic acid, 0.084; inositol, 8.4; menadione, 0.1; p-calcium panthetate, 2.0; choline-HCl, 150; all-rac-a-tocopheryl acetate, 0.1; sucrose, 835.8. The mineral premix consisted of the following (g/kg): NaCl, 74; KCl, 75; K₂HPO₄, 100; K₃PO₄, 52; MgO, 24; MnSO₄·H₂O, 6.9; FeSO₄·7H₂O, 5; ZnCO₃, 1.6; CuCO₃·Cu(OH)₂H₂O, 0.3; KIO₃, 0.01; K₃[Fe(CN)₆]·6H₂O, 0.55; Na₂SeO₃, 0.01; sucrose, 660.65.

cytotoxicity of fecal water by lowering the concentration of bile acids in fecal water (Lapré et al. 1991a). Cytotoxicity of fecal water is correlated (r = 0.85, n = 24, P < 0.001) with proliferation of the colonic epithelium (Lapré and Van der Meer 1992) and can be considered an intermediate step in the induction of hyperproliferation of the colonic epithelium.

Studies conducted in vitro showed that fatty acids are also important cytotoxic surfactants (Lapré et al. 1992) whose cytotoxicity may be blocked with supplemental calcium (Buset 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 fat on the concentrations of surfactants in fecal water, cytotoxicity of fecal water, and intestinal epitheliolysis, and their interaction with calcium phosphate in rats.

**MATERIALS AND METHODS**

**Animals and diets.** Eight-week-old male outbred Wistar rats (Small Animal Research Center of the Wageningen Agricultural University) were housed individually at a constant temperature (21°C) and relative humidity (60%) and with a 12-h light:dark cycle (lights on 0600–1800 h). The experimental protocol was approved by the animal ethics committee of the Agricultural University, Wageningen, The Netherlands, and its execution was supervised by the animal welfare officer. During the 2-wk experimental period, groups of rats (seven rats per group) were fed purified diets that differed in calcium phosphate concentration (25 and 225 mmol/kg diet) and in type of dietary fat (Table 1). Three types of dietary fat (180 g/kg diet) were used: milk fat (anhydrous butter oil), palm oil and corn oil. The fatty acid composition of the dietary fats is given as a footnote to Table 1. Correction was made for the cholesterol content of milk fat by supplementing the other diets with 0.4 g cholesterol/kg diet, because cholesterol supplementation is known to stimulate bile acid synthesis and excretion in rats but not in humans. Because our studies were designed to mimic human conditions we decided to correct for the cholesterol content of milk fat. The low calcium control diets mimic Western high risk diets containing 40% energy as fat, low fiber (20 g/kg diet) and low calcium (250 μmol/kg diet). This low calcium level is lower than the AIN recommendation (AIN 1977), but did not affect animal growth in our earlier study (Lapré et al. 1991a). Food and water were freely available. Animal weights were recorded weekly, and food intake was measured every 2 d. Feces were collected quantitatively during d 11–14.

**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
mixture of t-butanol and water [1:1, v/v], and subsequently bile acids were assayed enzymatically using a fluorimetric enzymatic kit (Sterognost 3α-FLU, Nycomed AS, Oslo, Norway). Results obtained with this method correlated \( r = 0.95 \) with those obtained by the standard gas-liquid chromatographic procedure (Van der Meer et al. 1985). Total free fatty acids in feces were extracted three times with diethyl ether after acidification with HCl (final concentration, 4 mol/L). After evaporation of the diethyl ether under nitrogen and subsequent resolubilization in ethanol, free fatty acids were assayed enzymatically (NEFA-C kit, Wako Chemicals). 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 (final concentration, 50 g/L) in an atomic absorption spectrophotometer, and inorganic phosphate was determined in the trichloroacetic acid-extract using the method described by Fiske and Subbarow (1925).

**Fecal water preparation and analysis of solubilized contents.** 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 ground feces with double-distilled water, the samples were incubated for 1 h at 37°C in a shaking water bath followed by centrifugation for 10 min at 15,000 × g (Eppendorf 5415, Eppendorf Gerätebau, Hamburg, Germany). Centrifugation for 20 or 30 min produced no significant differences in bile acid, calcium and phosphate concentration of fecal water, nor did it affect its cytotoxicity. The supernatant was carefully aspirated. 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 variables studied (Lapré and Van der Meer 1992, Lapré et al. 1991a).

Cytotoxicity of fecal water was tested as described previously (Lapré et al. 1991a) with the following minor modifications. Instead of using a single point measurement of cytotoxicity, lytic curves were constructed using different dilutions of fecal water. Using this method, cytotoxicity can be more reliably determined, because the method can discriminate between samples that would have resulted in a cytotoxicity of 100% in a single point measurement. 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, 0.05). The incubation time was 2 h 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.

Free fatty acids were assayed using an enzymatic method (NEFA-C kit, Wako Chemicals). Bile acids in fecal water were determined using a fluorimetric enzymatic assay (Sterognost 3α-FLU, Nycomed AS). Appropriate reference samples and standards were measured simultaneously. Recovery of standards added to samples was always >92%.

Total alkaline phosphatase (EC 3.1.3.1) activity (ALP) was determined according to the method of Bessey et al. (1946) using a glycine buffer (final concentration, 100 mmol/L, pH 9.8) in the presence of zinc (final concentration, 2 mmol/L) and magnesium (final concentration, 5 mmol/L). p-Nitrophenyl phosphate was used as substrate, and the absorbance of the reaction product p-nitrophenol was determined spectrophotometrically at 405 nm. The concentration of p-nitrophenol was calculated using a standard curve of known p-nitrophenol concentrations, and ALP activity was expressed as mmol p-nitrophenol/(min·L fecal water) [U/L]. Intestinal ALP activity was inhibited using 60 mmol/L L-phenylalanine, which acts as a specific and competitive inhibitor of the intestinal isozyme in humans and rats (Fishman et al. 1962). The difference in total activity (non-inhibited) and the activity after inhibition with L-phenylalanine is the activity of the intestinal isoenzyme. This enzymatic measurement of intestinal ALP correlated \( r = 0.98, y = 1.03x - 0.01 \) with the immunoprecipitation method for determining intestinal ALP (Lapré et al. 1991b).

**Statistics.** Values are the means ± SEM for seven rats. After ANOVA the differences between the means of the groups were tested using Fisher's protected least significant difference test [two-sided] (Steel and Torrie 1980). Differences were regarded as significant if \( P < 0.05 \). Data comparing the enzymatic inhibition of intestinal ALP with immunoprecipitation and the comparison of cytotoxicity of fecal water with intestinal epithelial cells were analyzed with single linear regression analysis. Multiple regression analysis of luminal surfactants on cytotoxicity and intestinal epithelial cells was performed with a commercially available statistical package (SPSS/PC+ v2.0).

**RESULTS**

Food intake [mean, 20.7 g/d] and body weight gain [mean, 5.8 g/d] were not significantly affected by the type of dietary fat or by calcium phosphate supplementation. Fecal output in rats fed the milk fat and palm oil diets was significantly higher when a diet with a high calcium phosphate level was fed (Table 2). Significantly greater fecal calcium excretion was observed in the rats fed the low calcium phosphate, palm oil diet compared with rats fed the low calcium phosphate milk fat or corn oil diet. Calcium
and inorganic phosphate excretions were drastically stimulated by calcium phosphate supplementation in rats fed all three types of dietary fat, as would be expected (Table 2).

Fatty acid excretion in feces was stimulated dramatically by calcium phosphate supplementation. It should be noted that the total fecal excretion of fatty acids was directly related to the type of dietary fat. The palm oil diet produced the highest excretion of fatty acids when fed at both levels of calcium phosphate, followed by the milk fat diet. The corn oil diet resulted in the lowest fecal fatty acid excretion. Fecal bile acid excretion was significantly lower in rats fed the corn oil diet and was slightly stimulated by calcium phosphate supplementation in rats fed the corn oil diet only.

The effects of dietary calcium phosphate supplementation on total fecal fatty acids and fatty acids in fecal water (soluble fatty acids) clearly showed that despite greater total fecal fatty acid concentration, the concentration of fatty acids in fecal water was lowered by supplementation (Fig. 1). The palm oil diet resulted in the highest concentrations of both total fecal fatty acids and fatty acids in fecal water compared with the other two dietary fats fed with the low and high calcium phosphate levels, respectively.

Supplemental calcium phosphate did not affect the total fecal bile acid concentration when milk fat and corn oil were fed (Fig. 2). Feeding the palm oil diet with the high level of calcium phosphate resulted in a lower total fecal bile acid concentration than feeding the palm oil diet with the low calcium phosphate level. This effect may have been due to the greater fecal mass in rats fed the high calcium phosphate, palm oil diet, because excretion of bile acids (μmol/d) was unaffected by calcium phosphate supplementation in rats fed either the milk fat or palm oil diet (Table 2). The higher fecal mass might be caused by the stimulated excretion of free fatty acids when rats were fed the high calcium phosphate, palm oil diet (Table 2). In contrast to the total bile acid concentration, the concentration of soluble bile acids (fecal water bile acids) was significantly lowered by supplemental calcium phosphate (Fig. 2). The corn oil diet produced the lowest concentration of bile acids in total feces and in fecal water.

Because cytotoxicity of the intestinal contents seems to be dependent on the concentrations of surfactants in fecal water [Lapré and Van der Meer 1992, Lapré et al. 1991a], we measured cytotoxicity of fecal water using lysis of erythrocytes. Palm oil induced the highest cytotoxicity, followed by milk fat and corn oil when fed at the low calcium phosphate level (Fig. 3). Calcium phosphate supplementation inhibited cytotoxicity of fecal water of rats fed all the diets. However, the palm oil diet still resulted in greater cytotoxicity than the milk fat and corn oil diets. To ascertain whether cytotoxicity of fecal water is determined by the concentrations of fatty acids and bile acids in fecal water, we compared the concentrations of calcium, inorganic phosphate, bile acids and fatty acids in fecal water by multiple regression analysis. This resulted in significant associations with a multiple correlation coefficient of 0.89. The regression equation [% cytotoxicity = 1.97Fa<sub>sol</sub> + 1.91Ba<sub>sol</sub> + 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 between cytotoxicity and total fecal concentrations of fatty acids and bile acids.

Because we have shown in studies conducted in vitro that lysis of colonic epithelial cells results in the release of the apical membrane-marker ALP [Lapré et al. 1992], we used ALP activity in fecal water as a possible marker for intestinal cell damage [Lapré et al. 1991b]. This resulted in effects similar to those for cytotoxicity (Fig. 3). Comparison of the individual
data for cytotoxicity and epitheliolysis of the same rats resulted in a significant correlation ($r = 0.92, n = 28, P < 0.001$) (Fig. 4). The samples that showed no cytotoxicity ($n = 14$) were excluded from this comparison to prevent formation of a cluster around cytotoxicity of 0%. Inclusion of these samples resulted in a correlation coefficient of 0.94. Thus the higher concentrations of surfactants in fecal water from rats fed the palm oil diet were associated with a higher cytotoxicity of fecal water and resulted in a higher lysis of epithelial cells. Dietary calcium phosphate supplementation dramatically decreased cytotoxicity of fecal water and intestinal epitheliolysis in rats fed 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 concentrations in fecal water, are the mediators of intestinal cytotoxicity. In this study, both fatty acids and bile acids in fecal water explained 80% ($R^2$) 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 caused 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 when the high calcium phosphate diets were fed 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 and De Vries 1985, Van der Meer et al. 1990), is at present not known and requires further investigation. With regard to this, it should be mentioned that several studies suggest the presence of fatty acid-calcium-phosphate complexes in feces (Richards and Carroll 1959, Swell et al. 1956).

Analogous to the effects observed in our earlier study in rats (Lapré et al. 1991a), dietary calcium did not increase total fecal bile acid excretion ($\mu$mol/d), but decreased the concentration of bile acids in fecal water. Appleton et al. (1991) found a significant decrease in fecal bile acid concentration (mg/g dry feces) caused 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 effects observed in our previous study (Lapré et al. 1991a).

Not only the total fatty acid concentration but also the concentration of fatty acids in fecal water were directly influenced by the type of dietary fat and by supplemental calcium phosphate. The differences in the concentrations of fatty acids in fecal water might be responsible for the higher cytotoxicity observed in rats fed the palm oil diets compared with the milk fat diets, because no differences in the concentrations of bile acids in fecal water of these two groups were observed. The concentrations of bile acids in fecal water were significantly lower when corn oil was fed compared with the other diets, but the cytotoxicity of fecal water was comparable to that of rats fed the milk fat diets. This effect can be explained by our in

**FIGURE 1** Total and soluble fecal free fatty acid (FA) concentrations ($\mu$mol/g dry wt) of rats fed diets differing in type of fat (milk fat, palm oil and corn oil) and level of calcium phosphate [25 mmol CaHPO$_4$/kg diet (Control) and 225 mmol CaHPO$_4$/kg diet (+Calcium)]. Values are means with their SEM for seven rats. Bars not sharing the same character are significantly different ($P < 0.05$).
vitro studies, which have shown that the combination of linoleic acid with bile acids is highly cytotoxic (Lapré et al. 1992). Supplemental calcium phosphate lowered the concentrations of fatty acids in fecal water in our present study, and this decline combined with the reduction in the bile acid concentration in fecal water might be responsible for the calcium phosphate–induced decrease in 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 (Buset et al. 1990, Lapré et al. 1992) and in combination with bile acids (Lapré et al. 1992) 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 result in damage of the colonic epithelium (Wargovich et al. 1984) and in induction of hyperproliferation (Bull et al. 1983, Wargovich et al. 1990). This effect can be abolished by simultaneous administration of calcium (Wargovich et al. 1984).

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 mmol CaHPO₄/kg diet. They found no significant effect on total fecal bile acid concentration and an increase in fecal fatty acid concentration with the more saturated fats; these results are
in agreement with those of our study. They also found that the beef fat diet resulted in greater loss of protein bands from plasma membranes of colonocytes as compared with the butter fat and safflower oil diets. In another study (Awad et al. 1990) they showed that high level of dietary calcium (375 mmol/kg diet) reduced the concentrations of bile acids and fatty acids in highly diluted water extracts of feces and decreased the loss of protein bands of colon mucosal membranes compared with the control diet (125 mmol Ca/kg diet).

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

In conclusion, our study with rats fed Western-type diets shows that the type of dietary fat affects the concentrations of soluble bile acids and fatty acids as well as cytotoxicity of fecal water and intestinal epitheliolysis. Independent of the type of dietary fat, supplemental calcium phosphate causes a decline in the solubility of bile acids and fatty acids as well as luminal cytotoxicity and intestinal epitheliolysis.

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LITERATURE CITED


