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Effects of Autohydrolyzed Lignin and Lactulose on Gallbladder Bile Composition in Hamsters

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Autohydrolyzed lignin was found to bind bile acids in vitro, an effect which was enhanced at low pH. Lignin with and without lactulose was fed to Golden Syrian hamsters to determine whether it could improve gallbladder bile composition by inducing small increases in bile acid excretion/synthesis. Lignin-supplemented diets increased daily fecal lithocholate excretion in association with a significant reduction in biliary cholesterol saturation. Lignin and lactulose did not affect daily neutral steroid excretion, but the combination decreased cholesterol degradation in the intestine. Further experiments are required to assess the efficacy of autohydrolyzed lignin and lactulose in the prevention of cholesterol gallstone disease.

Cholesterol gallstones may be dissolved by lowering the cholesterol saturation of the bile. Agents presently in use such as chenodeoxycholic acid accomplish this by reducing cholesterol synthesis (Coyne et al., 1976) and cholesterol secretion into the bile (LaRusso et al., 1974). Cholesterol secretion into the bile may also be diminished by controlled stimulation of bile acid synthesis, presumably by diverting cholesterol into the bile acid synthetic pathway (Strasberg et al., 1976). Several forms of fiber mildly stimulate bile acid synthesis by causing an increase in fecal bile acid loss (Kay and Strasberg, 1978), and gallstones are rare in populations with high fiber intake (Burkitt and Painter, 1974). The purpose of this experiment was to determine whether lignin, a component of dietary fiber, could reduce the cholesterol saturation of bile in the hamster by increasing fecal bile acid excretion and bile acid synthesis. A highly purified lignin extracted from aspen wood chips by autohydrolysis was used (Lora and Wayman, 1978). This lignin binds bile acids avidly in vitro, an effect enhanced by lowering the pH (Kay et al., 1979). In vivo acidification of the cecum can be induced by lactulose, a nonabsorbable carbohydrate (Bown et al., 1974). Lactulose was added to lignin in one group to determine if it augmented the effects of lignin on fecal bile acid excretion in vivo. In this initial study, the effects of these agents were tested in hamsters on normal diets with low cholesterol content.

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EXPERIMENTAL SECTION

Experimental Protocol. Twenty four mature male Golden Syrian hamsters were caged singly and randomly divided into four groups. The animals were allowed free access to food and water and were maintained in this manner for 21 days. All animals were fed a nutritionally complete dry semisynthetic diet containing cholesterol (0.2 g/kg of diet) supplemented with either cellulose (5% w/w; control), lignin (5% w/w; Lig), lignin (4.1% w/w) plus lactulose (13.6% w/w; Lig–Lac), or lactulose (13.6% w/w; Lac). This amount of lactulose approximates the upper limit of the usual dose in humans. Water was permitted ad libitum.

Food intake and weights were recorded. Total fecal collection was performed from day 16 to day 21.

At the end of the experimental period, after an overnight fast, the animals were anesthetized with nitrous oxide and halothane, and a laparotomy was performed. The cystic duct was ligated with a metal clip and the gallbladder bile was aspirated. A sample of venous blood was taken from the inferior vena cava, and the pH of the cecal contents

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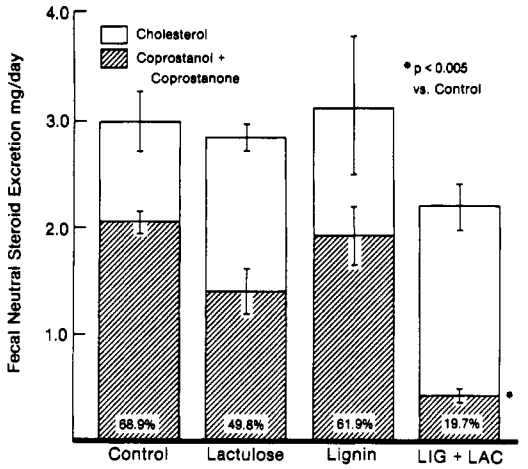

Figure 1. Daily neutral steroid excretion pattern. Note that cholesterol degradation is significantly reduced in the lignin plus lactulose group.

was tested with pH test paper (pHydrion paper, Micro Essential Laboratory, Brooklyn, NY), a method shown to be valid by Conn and Lieberthal (1979). The animals were sacrificed by exsanguination while still anaesthetized.

Chemical Methods. *Fecal Steroids.* Feces from randomly paired hamsters in each group were pooled for analysis so that three samples were measured in each group. The feces were freeze-dried for 24 h and ground into a fine powder. Aliquots were taken for steroid analysis.

Fecal bile acids in a 0.5-mg aliquot were extracted in 1 N NaOH in 90% ethanol. Bile acids were subjected to solvolysis and enzymatic hydrolysis (cholylglycine hydrolase, Sigma Co., St. Louis, Mo). Following removal of neutral steroids by hexane extraction, the free bile acids were exhaustively extracted and analyzed as their methyl acetate derivatives by gas–liquid chromatography (GLC) as described previously (Kay et al., 1980; Grundy et al., 1965).

Fecal neutral steroids were extracted with acetic acid–toluene and saponified at 85 °C for 1 h in 1 N NaOH in 90% ethanol. The neutral steroids are then extracted with hexane and the trimethylsilyl derivatives were analyzed by GLC (Kay et al., 1980; Miettinen et al., 1965).

Biliary Lipid Composition. The volume of bile aspirated from the gallbladder was recorded and added to 2.0 mL of isopropyl alcohol. Aliquots were dried down and used for measurement of total bile acids by the modified hydroxysteroid method (Talalay, 1960), for phospholipid by using the method described by Bartlett (1959), and for cholesterol by GLC using stigmasterol as an internal standard (Morin and Elms, 1975). Biliary cholesterol saturation was expressed as the "lithogenic index" (LI) calculated from the Thomas and Hofmann equation for the Hegardt and Dam line of maximum cholesterol solubility (Thomas and Hofmann, 1970; Hegardt and Dam, 1971).

Plasma Cholesterol. Plasma cholesterol was measured by GLC using stigmasterol as the internal standard (Morin and Elms, 1975).

Statistical Methods. Data were treated by one-way analysis of variance and differences between groups were tested by the Student's t test (Snedecor and Cochran, 1967).

RESULTS

Effects of Diet on Fecal Steroid Excretion. *Neutral Steroid Excretion.* Neutral steroid excretion did not differ from that of the control in any of the study groups. The ratio of coprostanol and coprostanone to cholesterol was significantly decreased in the lignin plus lactulose groups as compared to the other groups (Figure 1).

Acidic Sterol Excretion. Measurement of total fecal bile acid excretion in this experiment was hampered by low sterol concentrations and high background readings ascribed by mass spectroscopy analysis of unidentified peaks to be due to the presence of fatty acids. The lithocholate peak, however, was discrete, and the results presented are those of daily lithocholic acid excretion. We have subsequently modified our method by including a purification step using a Florisil column and have shown that alterations in total fecal bile acid excretion induced by lignin are reflected by changes in lithocholate excretion.

Daily lithocholate excretion was significantly increased over control in both the lignin and the lignin plus lactulose groups ($p < 0.05$) (Table I). Excretion in the lactulose alone group, although slightly lower than that of the control, was not significantly reduced. The lignin plus lactulose group did not significantly differ from the lignin group.

Table I. Effect of Autohydrolyzed Lignin and Lactulose upon Lithogenic Index and Fecal Lithocholic Acid Excretion[a]

	control	lactulose	lignin	Lig-Lac
lithogenic index	0.32 (0.04)	0.36 (0.004)	0.25 (0.03)	0.26 (0.03)
lithocholic acid excretion, mg/day	0.144 (0.032)	0.065 (0.012)	0.348[b] (0.057)	0.301[b] (0.065)

[a] Mean (SEM). [b] $p < 0.05$ vs. control.

Table II. Effect of Autohydrolyzed Lignin and Lactulose upon Gallbladder Bile Composition and Relative Composition[a]

	bile acid	phospholipid	cholesterol	total lipid
	Gallbladder Bile Concentration, mM			
control, $n = 5$	150.2 (15.2)	22.0 (2.4)	2.53 (0.46)	174.8 (17.9)
lactulose, $n = 6$	126.2 (20.5)	14.9 (3.3)	2.09 (0.40)	143.2 (24.1)
lignin, $n = 5$	98.5 (22.0)	13.4[b] (2.6)	1.14[b] (0.12)	113.0 (24.6)
Lig-Lac, $n = 6$	110.7 (13.6)	10.9[b] (1.8)	1.25[b] (0.24)	122.8 (15.4)
	Relative Composition, mM %			
control, $n = 5$	86.0 (0.3)	12.6 (0.2)	1.41 (0.20)	
lactulose, $n = 6$	88.7[c] (0.8)	9.9[b] (0.7)	1.42 (0.04)	
lignin, $n = 5$	86.8 (0.6)	12.0 (0.4)	1.11 (0.13)	
Lig-Lac, $n = 6$	90.3[c] (0.7)	8.7[b] (0.6)	0.99[b] (0.12)	

[a] Mean (SEM). [b] $p < 0.05$ vs. control. [c] $p < 0.01$ vs. control.

Effect on Gallbladder Bile Composition (Table II). *Concentration of Lipids.* Total lipid concentration did not differ among groups. Statistically significant reductions in phospholipid and cholesterol concentrations were found in the lignin and lignin plus lactulose groups.

Relative Composition of Bile. Lactulose produced an increase in bile acid and a decrease in phospholipid without change in cholesterol relative composition when compared to the control diet. The cholesterol molar percent fell on the lignin diet but was not significantly different from control values. Cholesterol molar percent was significantly lower in animals on lignin-containing diets ($n = 11$) vs.

lignin-free diets ($n = 11$) (1.41 ± 0.08 (SEM) mM % vs. 1.04 ± 0.08 mM %, $p < 0.05$).

Lithogenic Index. As shown in Table I, there was a tendency for a decreased lithogenic index in the lignin alone group. Lignin plus lactulose reduced, but not significantly, the lithogenic index as compared to control. Lactulose did not differ significantly from control. The lithogenic index was significantly lower in the animals on lignin-containing diets (LI = 0.27 ± 0.02) than in animals on lignin-free diets (LI = 0.34 ± 0.02) ($p < 0.025$). Notably it was only in the lignin and lignin plus lactulose groups that the fecal bile acid excretion was increased.

Cecal pH. Cecal pH was significantly lower ($p < 0.01$) in the lignin plus lactulose group (7.8 ± 0.1) and the lactulose group (7.8 ± 0.2) than in the control (8.8 ± 0.1) or lignin group (8.7 ± 0.1).

Serum Cholesterol. The serum cholesterol concentrations did not differ among groups.

Effect of Diets on Weights. There were no significant differences among groups in the initial or final weights. None of the animals had diarrhea, and all appeared healthy at the conclusion of the study.

DISCUSSION

Autohydrolyzed Aspen Lignin. The production of this material has been described in detail previously (Lora and Wayman, 1978). Following successive extractions with benzene, 95% alcohol, and water, the wood meal was subjected to autohydrolysis by heating with steam at 195 °C for 40 min. The lignin was then extracted in 90% dioxane in water and purified by precipitation into diethyl ether. The methoxyl content of this lignin is 16.7% with a number-average molecular weight of 1490 (Chua and Wayman, 1979a,b). In in vitro studies it was found to bind bile acids effectively (Kay et al., 1979). The effect was augmented at a low pH, mainly due to a preferential increase in the binding of trihydroxy bile acids (Kay et al., 1979). Increased trihydroxy bile acid binding is theoretically a desirable attribute as it could result in the conversion of the bile acid pool to one of predominantly chenodeoxycholic acid, a dihydroxy bile acid. This would simulate the effect of feeding chenodeoxycholic acid, an accepted method of gallstone dissolution (Pearlman et al., 1979).

Fecal Steroid Excretion. *Neutral Steroids.* Neutral steroid excretion did not differ among groups. However, in the lignin plus lactulose group there was a large decrease in the breakdown of cholesterol to its bacterial metabolites, coprostanol and coprostanone. In this model it appears that the combination of lignin plus lactulose has altered the colonic environment such that either the bacteria present cannot metabolize cholesterol or they no longer have access to it.

It is noteworthy that increased cholesterol to coprostanol and coprostanone ratios have been described in populations with a decreased incidence of colonic cancer (Hill and Aries, 1971).

Acidic Steroids. Both lignin and lignin plus lactulose increased lithocholic acid excretion over control levels. The expected augmentation of fecal bile acid excretion with the addition of lactulose to lignin did not occur. This is probably because the cecal pH was not sufficiently reduced to affect bile acid adsorption to lignin. In the lactulose-containing diets, the cecal pH observed was close to the p*Ka* values of the free bile acids, thus increasing the percentage of bile acids in a nonionized form. Mekhjian et al. (1979) have demonstrated a 2-fold increase in cholic acid absorption following a similar colonic pH decrease in man (pH 8 to pH 7). It is likely that rapid reabsorption of bile acids by passive nonionic diffusion resulted in a slight decrease in fecal bile acid excretion in the lignin plus lactulose group compared to the lignin group. A similar tendency is noted when comparing the lactulose alone group to the control.

Effect on Gallbladder Bile Composition. The reduction in the cholesterol molar percent on the lignin diet was predicted by previous experiments in which small controlled increases in bile acid synthesis reduced cholesterol secretion into bile (Strasberg et al., 1976). Presumably this occurred by diversion of biliary bound cholesterol into the bile acid synthetic pathway. In our experiment, the lignin-induced decrease in lithogenic index was small and statistically significant only when animals receiving lignin-containing diets were compared to animals not receiving lignin in their diets. These results are in keeping with human experiments in which the effect of fiber on gallbladder bile composition in individuals with unsaturated gallbladder bile was minimal (Wickes et al., 1978; McDougall et al., 1978; McK. Watts et al., 1978). In patients with supersaturated bile, however, fiber has consistently improved gallbladder bile composition (McDougall et al., 1978; McK. Watts et al., 1978). Initial studies on the effect of autohydrolyzed lignin on a supersaturated bile model suggests that it effectively reduces the lithogenic index (Rotstein et al., 1980).

The results of this experiment have provided some insight into the possible mechanism by which dietary fiber may prevent gallstone formation. This experiment is preliminary. Autohydrolyzed lignin requires further testing to optimize its beneficial effect on bile composition.

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Digestion of Larch Arabinogalactan by a Strain of Human Colonic *Bacteroides* Growing in Continuous Culture

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Bacteroides thetaiotaomicron, a polysaccharide-degrading bacterium from the human colon, was grown in continuous culture, with arabinogalactan as the limiting substrate, at growth rates similar to those which are probably experienced by these organisms in vivo. Growth yields were respectively 80, 82, 58, and 50 g of cells/mol of utilized substate (as galactose) at growth rates of 3.5, 6.3, 11.6, and 27.7 h/generation. These yields were comparable to those attainable when glucose or galactose was the substrate. However, affinity for arabinogalactan was lower than affinity for galactose. As the rate of growth decreased, the pattern of fermentation products changed: the concentration of acetate and propionate increased and the concentration of succinate decreased. The ability of the bacteria to produce the inducible enzyme α-glucosidase also decreased with decreasing growth rate, indicating that slowly growing bacteria may be less able to adapt to new sources of carbohydrate.

Many of the bacteria which reside in the human colon require a fermentable carbohydrate for growth (Holdeman et al., 1977). A number of these organisms can ferment polysaccharides (Salyers et al., 1977a,b), and polysaccharides from the host's diet or from the host's secretions may be the only fermentable carbohydrates available to these organisms in their natural environment. There is considerable evidence to indicate that extensive bacterial digestion of some types of dietary polysaccharides occurs in the colon (Van Soest, 1978). However, little is known about the factors which affect the extent of this digestion, the types of polysaccharides most likely to be attacked, and the effect of this microbial digestion on the host. Some understanding of these factors is necessary in order to answer such questions as (a) whether colon bacteria live mainly off their host or off their host's diet, (b) whether the composition of the host's diet (especially its fiber content) affects the extent to which bacteria utilize host-produced substances as a source of carbohydrate, (c) in what ways bacterial digestion affects the properties of dietary fiber and thus the action of fiber in the colon, and (d) whether products from bacterial fermentation of substances which are indigestible by the host are absorbed and whether this absorption contributes substantially to the host's nutrition.

Since the bacterial flora of the colon is an extremely complex community of interdependent organisms (Moore and Holdeman, 1974; Wolin, 1974), there are undoubtedly a large number of factors which influence its composition and metabolic activities. The nature of available carbohydate is likely to be a major factor affecting the functioning of this community because so many bacteria rely either directly on carbohydates or on the products of carbohydate fermentation by other organisms. At any one time, the concentration of any particular type of polysaccharide in the colon is probably quite low. Moreover, the polysaccharide pool, which is composed of a complex mixture of different types of polysaccharides, is constantly changing. Because concentrations of substrates are low, growth rates are probably low as well (Brock, 1971). The transit time of most materials through the colon is around 30–40 h. Thus, the bacterial mass, which is stable and accounts for nearly one-third of the volume of colon contents, can replace itself only once during this time. Bacteria which are located in the ascending colon near the ileocecal valve where dietary material enters the colon probably experience much higher growth rates than bacteria which are further along in the colon. However, even these growth rates are likely to be much slower than the growth rates of 30 min–2 h/generation which are customary in most in vitro systems used by microbiologists to study the metabolic activities of bacteria. To date, no direct measurements have been made of growth rates of human colonic bacteria, but measurements have been made of bacterial growth rates in the cecum and colon of rodents. Growth rates of the organisms tested were found to be on the order of 10 h/generation or longer (Gibbons and Kapsimalis, 1967).

Virtually nothing is known about the effect of very slow growth rates on the ability of colon bacteria to utilize complex substrates such as polysaccharides or on their ability to produce new degradative enzymes in order to shift from one substrate to another as the composition of available carbohydrate changes. To obtain some insight into the effect of slow growth rates on bacterial digestion of polysaccharides, we have investigated the utilization of larch wood arabinogalactan by a strain of *Bacteroides thetaiotaomicron* growing in continuous culture with arabinogalactan as the limiting nutrient. Larch wood arabinogalactan is a mixture of two component polysaccharides, one having a molecular weight of around 160 000 and the other having a molecular weight of around

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