# Unconventional Sources of Dietary Fiber

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# Unconventional Sources of Dietary Fiber

## Physiological and in Vitro Functional Properties

**Ivan Furda,** EDITOR
*General Mills, Inc.*

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# FOREWORD

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# PREFACE

THE IDEA OF ORGANIZING THIS SYMPOSIUM was born at the 179th Annual ACS meeting in Houston in 1980, from discussions with the previous chairman of the Agricultural and Food Chemistry Division, Dr. Robert Ory. It was felt that the subject of dietary fiber should be viewed in a broader spectrum that should not cover excessively the current common, almost classical types, of fiber such as wheat and corn bran or cellulose. It should also encompass less conventional sources of dietary fiber which would be the main topic of this symposium. Because soluble nondigestible plant polysaccharides are now included in the definition of dietary fiber, and because numerous nondigestible polymers of nonplant origin exist, greater attention should be paid to these alternatives. These polymers have a variety of unique physiological, chemical, and functional properties that need to be better understood and utilized. This type of understanding is frequently achieved when different points of view are presented and a variety of scientists engage in discussion.

Presentations in this volume come from chemists, medical researchers, and microbiologists, as well as nutritionists and food scientists. In a few cases, rather exotic fiber types such as tobacco fiber, wheat straw lignin, or shellfish aminopolysaccharides are discussed. Other sources include psyllium, different legumes, and vegetable and fruit fibers. There is no doubt that additional unconventional fiber sources will be continuously identified and increasingly used.

It is my hope and belief that this publication will help in the expansion and utilization of fiber sources in foods, as well as in pharmaceutical and medical preparations. After all, we should recognize that the chance to accomplish this is quite favorable because "complex carbohydrates," which are the main constituents of dietary fiber, represent the only group of major food components for which increased daily intake is repeatedly being recommended.

I would like to express my appreciation and thanks to all participants of this symposium and to the writers of the individual chapters for their efforts and contributions. Without them, this undertaking and publication



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would not be possible. Finally, I am grateful to Akiva Pour-El for his administrative help, and to General Mills and The Quaker Oats Company for their generous financial support.

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# 1

# Effect of Conventional and Unconventional Dietary Fibers in Colon Carcinogenesis

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Epidemiologic and animal model studies suggest a protective effect of certain dietary fibers against colon cancer. This protection by the dietary fibers may be mediated through (a) the dilution of tumorigenic compounds in the gut,(b) the binding of tumorigenic compounds to the fiber in the gut, and (c) an indirect effect on the metabolism of carcinogens. Experiments were conducted in animal models to study the effect of certain non-conventional dietary fibers on colon carcinogenesis. Rats were fed the diets containing alfalfa, citrus pulp, pectin, wheat bran, undegraded carrageenan or cholestyramine and treated with azoxymethane (AOM), 3,2'-dimethyl-4-aminobiphenyl (DMAB), or methylnitrosourea (MNU) to induce tumors. Animals fed the wheat bran or citrus pulp and treated with AOM or DMAB had a lower colon tumor incidence than did those fed the control diet and treated with respective carcinogens. Alfalfa diet had no effect on AOM- or MNU-induced colon tumors, whereas the pectin diet inhibited AOM-induced but not MNU-induced colon tumors. Animals fed the diets containing undegraded carrageenan or cholestyramine developed more AOM and/or MNU-induced colon tumors than did the rats fed the control diet. Thus, the protective effect of various dietary fibers in colon carcinogenesis depends on the type of fiber fed, as well as the type of carcinogen used to induce tumors.

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Rapid progress has been made in basic concepts concerning carcinogenesis (1,2). There are agents which are genotoxic, which by definition interact with the gene to yield an abnormal genetic material and would be considered initiating agents (3). The second broad class of agents act by epigenetic mechanisms and tend to increase the development of lesions initiated by genotoxic carcinogens. Thus, this list includes co-carcinogens which operate at the same time as genotoxic carcinogens and can alter the metabolism of a genotoxic agent with an increased ratio of activation/detoxification metabolites. Such agents can also act as more classic tumor promoters which exhibit their effect after the action of a genotoxic carcinogen.

Current concepts of colon cancer etiology stem from multidisciplinary research efforts based on three major approaches, namely: (1) the variation in incidence of colon cancer as a function of area of residence, with particular regard to migrant population; (2) the changes in incidence as a function of time; and (3) detailed laboratory studies in humans, in animal models, and through *in vitro* systems. The consistency of these findings suggests that environmental factors in general, and dietary factors in particular, play a dominant role in the development of colon cancer in humans.

Epidemiologic studies suggest that diets particularly high in total fat and low in fiber and in certain vegetables as well as high intake of beef are generally associated with an increased incidence of large bowel cancer in man (4-10). Dietary fat may be a risk factor in the absence of factors that are protective, such as use of high fibrous foods and fiber (11,12). As an example, in Finland, where the dietary intake of fat is similar to many of the Western countries and the fiber intake is higher, the incidence of colon cancer is lower than all of the Western countries.

This brief review evaluates current research on the relation between dietary fiber and large bowel cancer in humans, including the use of animal models. This brief review also presents an evaluation of the mechanism whereby certain dietary fibers including conventional and unconventional fibers modify the risk for the development of colon cancer.

## Correlation and Case-Control Studies

Cross-national correlations between the incidence of colon cancer and dietary habits have been used to select hypotheses for testing in case-control and cohort studies. These studies have shown that certain food preferences appear to be associated with either a high- or a low-risk for colon cancer. When such correlations are supported by experimental evidence from animal studies, the hypothesis could be attractive.

Burkitt (10,13) first observed the rarity of large bowel cancer in most African populations and suggested that populations consuming a diet rich in fiber have a lower incidence of this type of cancer, while those eating refined carbohydrates

and little fiber have a higher incidence. A recent study comparing low-risk populations in Kuopio, Finland, with those at high risk in Copenhagen indicated that dietary fiber intake is higher in Finland compared with Copenhagen (14). The Finnish population is unique in this respect because in this country the total dietary fat is similar to countries with high rate of colon cancer. The crucial difference in dietary intake between Finland and Denmark and other Western countries may relate to dietary fiber and meat. Our data also suggest that one of the factors contributing to the low risk of large bowel cancer in Kuopio appears to be that a high intake of dietary fiber (mainly cereal fiber) leads to increased stool bulk, in effect diluting tumorigenic compounds in the colon (12). The results are consistent with a possible role for dietary fiber in the prevention of colon cancer in humans.

Case control studies have been conducted to study the possible relation of dietary fiber to large bowel cancer. Recently, Dales *et al.* (15) found that among American blacks significantly more colon cancer patients than controls reported that their diet was high in saturated fat and low in fibrous foods. Investigating many dietary constituents, Modan *et al.* (16) discovered that those contributing less to the diets of patients with colon cancer than to the diets of controls were those containing fiber. Bjelke (17) who interviewed hospitalized patients and controls in Minnesota and in Norway, learned that colorectal cancer patients less frequently ate vegetables, in particular the Minnesota patients ate less cabbage. Similarly, Graham *et al.* (18) found that individuals who ate vegetables such as cabbage, broccoli and Brussels sprouts had a lower risk of colon cancer.

These studies indicate that diets with a high intake of total fat and a low intake of certain fibers and certain vegetables are generally associated with an increased incidence of colon cancer in humans. Even in populations consuming high amount of fat, high dietary fiber acts as a protective factor in colon carcinogenesis.

## Possible Mechanism of Protective Effect of Dietary Fiber In Colon Carcinogenesis

Although the concept of fiber involvement in colon carcinogenesis is attractive, the data often appear contradictory and confusing. Discrepancies may have arisen from the general misuse of fiber terminology. As well, experimental design has failed to account for the possible subtle effect of inhibitors, especially in relation to the promoting process. Evaluations of the biologic function of dietary fiber have often lacked complete information on the nature of the fiber.

Dietary fiber comprises a heterogenous group of carbohydrates, including cellulose, hemicellulose and pectin, and a noncarbohydrate substance, lignin (19). According to Van Soest (20), fibers can be classified into three groups: vegetable

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fibers, which are highly fermentable and have little indigestible residue; brans, which are less fermentable; and chemically purified fibers, such as cellulose, which are relatively nonfermentable. Pectins and gums, soluble substances that are not true fibers, are considered part of the dietary fiber complex because of the similar effects they can elicit in the diet. Wheat bran and vegetable and fruit fibers have different percentages of cellulose, hemicellulose and lignin.
Carrageenan, a broad generic class of sulfated polysaccharides derived from a wide range of seaweed species, can be classified as non-conventional fiber and is used in food as an emulsifier, stabilizer, thickener and gelling agent.

The protective effect of dietary fiber may be due to adsorption, dilution or metabolism of cocarcinogens, promoters and yet-to-be-identified carcinogens by the components of the fiber (12,21,22). There is evidence that alfalfa, wheat straw and some other fibers can bind considerable amounts of bile acids *in vitro* (23). This indicates that the different types of nonnutritive fibers possess specific binding properties. Dietary fiber could also affect the enterohepatic circulation of bile salts (24) which act as colon tumor promoters (6). Fiber not only influences bile acid metabolism (12,22) thereby reducing the formation of potential tumor promoters in the colon, but also exerts a solvent-like effect in that it dilutes potential carcinogens and cocarcinogens by its bulking effect (12) and is able to bind bile acids and certain carcinogenic compounds (23,25,26). On the other hand, dietary undegraded carrageenan markedly enhances the bile acid content in the colon, thereby increasing the potential colon tumor promoters in the gut (6).

Smith-Barbaro *et al.* (26) in our laboratory determined the capacity of various fibers to bind the colon carcinogen 1.2-dimethylhydrazine (DMH) in vitro. The percent of DMH bound to wheat bran, corn bran, alfalfa fiber and dehydrated citrus pulp was dependent on pH of the medium as well as the type of fiber examined. Results from this study show that at colonic pH, a greater percent of DMH was bound by wheat bran than by citrus pulp. Therefore, it is possible that certain fibers bind carcinogen at colonic pH, thus making it unavailable for contact with the colonic mucosa. Other fibers such as pectin (soluble fiber) do not bind DMH at colonic pH, but may modify the metabolism of carcinogen via activation/deactivation steps either in the liver and/or in the colonic mucosa.

Investigations have been carried out in several laboratories to determine whether there are differences in fecal constituents between populations at high and low risk of colon cancer, and whether changes in the fiber content of the diet would alter the concentration of fecal bile acids that act as colon tumor promoters and the activity of fecal microflora. Recently, we studied healthy individuals in Kuopio, Finland, an area of low risk for the development of colon cancer (12). Dietary

histories indicated that the total fat consumption is similar to that in the United States but the intake of cereal fiber in Finland is higher and the daily output of feces three times higher than that of healthy individuals in the United States. The concentration of fecal secondary bile acids, mainly deoxycholic acid and lithocholic acid is less in Kuopio than in the United States, but the total daily output is the same in the two populations because of the threefold greater daily output of feces in Kuopio. This suggests that increased fecal bulk dilutes suspected carcinogens and promoters that may be in direct contact with the large bowel mucosa. Cummings (27) demonstrated that fiber from carrot, cabbage, apple, bran and guar gum produces different responses in fecal weight in humans related to the intake of pentose-containing polysaccharides in the fiber. The fecal weight increased by 127% when bran was added to the diet and 20% when guar gum was added; carrot, cabbage and apple produced intermediate changes.

In another study, Cummings (28) reported that an increase in cereal fiber intake from 17 to 45 g/d increased the fecal weight from 79 to 228 g/d and diluted the fecal bile acids. Kay and Truswell (29) showed that adding wheat fiber to the diet decreased the concentration of fecal bile acids and neutral steroids because of the bulking effect of fiber, whereas the addition of pectin to the diet increased the fecal steroid and bile acid output. These results suggest that the effect on fecal bile acid excretion may depend on the type of fiber consumed.

The effect of dietary wheat bran and alfalfa at 15% level on the composition of fecal bile acids was studied in rats fed a semipurified diet (30). Diets containing wheat bran and alfalfa caused a significant increase in stool weight. The concentration of fecal bile acids, particularly hyodeoxycholic acid, β-muricholic acid, deoxycholic acid and lithocholic acid was lower in rats fed wheat bran, compared to those fed a control diet,but the daily output of these bile acids was the same in both groups. Alfalfa had no effect on the concentration of fecal bile acids, but the daily excretion of deoxycholic acid, lithocholic acid and 12-ketolithocholic acid was increased compared to the control diet. It is apparent from this study that the fecal excretion of bile acids varies with the type and amount of dietary fiber.

Until recently, the nature of the carcinogens responsible for colon cancer not only was obscure, but there were no real leads. Because of potential importance of fecal mutagens in the genesis of large bowel cancer and of possible role of dietary factors in the induction of colon cancer, the fecal mutagenic activity of various population groups with distinct dietary habits and varied colon cancer incidences was determined by several investigators. Ehrich et al. (31) have demonstrated that the stools of South African urban whites who consume a high-fat, low-fiber diet and who are at high risk for colon

cancer development were higher (17% of the individuals) in mutagenic activity with Salmonella typhimurium strains TA98 and TA100 without microsomal activation compared to South African urban and rural blacks (0-5% of the individuals) who consume a low-fat, high-fiber diet and who are at low-risk. Bruce et al. (32) were the first to show that the feces of some normal humans consuming a high-fat, low-fiber diet contained compounds that caused direct mutagenesis of TA98 and TA100 in the Ames assay. They have also demonstrated that increased dietary fiber, -tocopherol or vitamin C reduced fecal mutagens. Kuhnlein et al. (33) compared fecal mutagens (water extracts) from a group of vegetarians consuming high-fiber diets with those from persons on typical North American diets containing meat. On TA100 and TA98, ovo-lactovegetarians and strict vegetarians had lower levels of fecal mutagens than non-vegetarians. Correlation studies between the pH of the fecal homogenate and mutagenicity indicate the presence of several fecal mutagens.

Recently, we have studied fecal mutagens of 3 populations with distinct risk for the development of colon cancer, a high-risk population in New York (non-SDA) consuming a high-fat, low-fiber, mixed-Western diet, a low-risk vegetarian SDA (Seventh-Day Adventists) and a low-risk population in Kuopio, Finland consuming a high-fat, high-fiber diet (34). Fecal samples of non-SDA were highly mutagenic in TA98 without microsomal activation, followed by TA100 without activation and TA100 with activation. None of the samples of SDA tested showed mutagenic activity in any of the tester systems, whereas Kuopio samples exhibited activity only in TA98 with microsomal activation.

## Animal Model Studies in Colon Carcinogenesis: Effect of Conventional and Unconventional Fibers

Research on the mechanisms of cancer causation in the large bowel has been assisted by the discovery of several animal models that mirror the type of lesions seen in man. These models include (a) induction of large bowel cancer in rats through chemicals such as 3-methyl-4-aminobiphenyl, or 3-methyl-2-naphthylamine; (b) derivatives and analogs of cycasin and methylazoxymethanol (MAM) such as azoxymethane (AOM and 1,2-dimethylhydrazine (DMH), which work well in rats and mice of selected strains; and (c) intrarectal administration of direct-acting carcinogens of the type of alkylnitrosoureas, such as methylnitrosourea (MNU) or N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) which lead to cancer of the descending colon in every species tested so far.

The relation between dietary fiber consumption and colon cancer has been studied in experimental animals. Wilson et al. (35) found that Sprague-Dawley rats fed a diet containing 20% corn oil or beef fat and 20% wheat bran had fewer benign colon tumors induced by DMH given by gastric intubation than rats fed a control diet containing 20% fat and no bran. It is possible that with additional time, a number of tumors that were classi-

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fied as benign might have developed a more invasive character. There was no difference in the incidence of colon cancer between the rats fed corn oil and those fed beef fat. In another study, Clapp et al. (36) found that DMH-induced colon tumors were increased in BALB/C mice fed a semisynthetic diet containing 5% fat and 20% W/W corn bran, soybean bran and soft winter wheat bran. DMH-induced colon tumor incidence in mice fed the control diet was surprisingly low, about 12%. In these studies, the experimental diets containing high-fat/no bran and high-fat/high bran, (35) or low-fat/no-bran and low-fat/high bran (36) differed substantially in caloric density. Animals on high bran diets might have consumed quantitatively different levels of vitamins and minerals compared to those fed high fat-no bran diet or low fat-no bran diets because the rats adjust their food intake to compensate for non-digestible fiber in high bran diet. It is also possible that certain fibers may cause abnormal histology and damage to colonic mucosa, thereby altering the absorptive capacity of the intestinal tract as well as bile acid-binding capacity (37,38) which have bearing on colon carcinogenesis.

A recent study by Fleiszer et al. (39) indicated that the incidence of colon tumors induced by DMH in rats decreases as the dietary intake of fiber increases. The number of animals used in this study was small. The diets in that study, namely high bran diet (28% fiber), special chow (15% fiber), rat chow (5% fiber) Flexical diet (0% fiber) differed not only in consistency (that is, solid or liquid) but also in the proportions of protein and fats, which have been shown to have an independent effect on colon carcinogenesis induced by DMH. However, the colon cancer incidence in fiber-free (Flexical) group was lower than in rat chow group. Some reduction in tumor incidence in the rats ingesting a high-fiber diet might be expected on the basis of reduced energy intake. Although the study's findings suggest that reduced intake alone cannot account for the significant protective effect of dietary bran, a better experimental design might have strengthened the results.

In another study, Cruse et al. (40) found that a diet containing 20% wheat bran had no effect on colon carcinogenesis induced by DMH in rats. Not only were the number of animals used in this study small (10 rats/group), but also the doses of the chemical in their experiment were so high (40 mg/kg body weight-/week for 13 weeks) that any protective effect of bran might have been unobservable. In a study of the effect of diet on chemical carcinogenesis, it is important to avoid exposing the animal to an excessive level of carcinogen for a long period, as this may obscure more subtle changes induced by certain dietary modifications. In addition, differences in caloric density of experimental diets attributable to the dilutional effect of added fiber further complicated the interpretation of the data. In fact, the data presented by Cruse et al.(40) suggest that a high-fiber diet reduces the frequency of death due to DMH in rats.

The effect of a diet containing 15% alfalfa, pectin or wheat bran on colon carcinogenesis by MNU or AOM was studied in F344 rats by Watanabe et al. (41). In this study, the experimental diets were not adjusted isocalorically. The addition of pectin or wheat bran to the diet greatly inhibited colon tumor incidence induced by AOM, a carcinogen requiring host-mediated metabolic activation (Table I). However, the incidence of AOM-induced colon tumors was not influenced by the addition of alfalfa to the diet. The diets containing wheat bran and pectin did not protect against MNU-induced colon carcinogenesis.

Table I

Colon Tumor Incidence in Female F344 Rats Fed Diets Containing Pectin, Alfalfa, or Wheat Bran and Treated with Azoxymethane or Methylnitrosourea

| Diet | % Animals Colon Tumors | |
|---|---|---|
| | Azoxymethane treated | Methylnitrosourea treated |
| Control | 57 | 69 |
| Pectin | 10[a] | 59 |
| Alfalfa | 53 | 83[b] |
| Wheat Bran | 33[a] | 60 |

[a] Significantly different from the groups fed the control diet or alfalfa diet by $X^2$ test, $P<0.05$.

[b] Significantly different from the other groups, $P<0.05$.

Thus, modifying effect of pectin on AOM-induced colon carcinogenesis might be explained in terms of diet-dependent intestinal mucosal, as well as hepatic, microsomal carcinogen-metabolizing enzyme inhibitors or inducers in the diet that modify the capacity of the animal to metabolize the carcinogen (42). However, this factor would presumably not play a role in MNU-induced carcinogenesis because MNU does not require metabolic activation either in the liver or in the intestine. These results thus indicate that the protective effect of fiber in colon carcinogenesis depends on the type of carcinogen and the source of fiber.

The effect of alfalfa, wheat bran and cellulose on the incidence of intestinal tumors induced by AOM was further studied in Sprague-Dawley rats fed diets containing 10% alfalfa, wheat bran or cellulose and 30% beef fat, 20% alfalfa, bran or cellulose and 6% beef fat, or 30% alfalfa, bran or cellulose and 6% beef fat (21). The presence of 10% fiber in the high fat diet did not reduce the frequency of intestinal tumors. However, in the same study, presence of 20% bran or cellulose or 30% of any

fiber in a diet containing 6% fat significantly reduced the frequency of intestinal tumors. All the groups, except those with a diet containing 20% alfalfa, had a lower frequency of tumors in the proximal half of the large bowel than the groups not ingesting fiber. The concentration, but not the total daily excretion, of fecal steroids was significantly lower in the groups with a lower tumor frequency. Therefore, the inhibition of tumor formation by dietary fiber may be due to the dilution of bile steroids in the lumen of the intestine by the additional bulk (21).

Bauer et al. (43) have demonstrated that the protective effect of dietary fiber against colon carcinogenesis probably occurs at the promotional stage rather than in the initiating period. Rats were fed a fiber-free diet with 20% corn oil or diets containing 20% corn oil and 20% wheat bran, 20% carrot fiber or 6.5% citrus pectin from 3 days before the first injection of DMH (15 mg/kg body wt/week for 12 weeks) until 14 days after the last injection. They were then transferred to a standard rat pellet diet for 10 to 12 weeks and autopsied. The fiber-containing diets were fed only for about 15 weeks out of a total experimental period of 25 weeks.There was no difference in the incidence of colorectal tumors between the groups fed a fiber-free diet and those fed a diet containing wheat bran or carrot fiber. The citrus pectin group had a higher incidence of colorectal tumors. However, it is possible that the high tumor yield resulting from large doses of the carcinogen in this study masked any protective effect of dietary fiber. In addition, these results and those of others reported above have suggested that the continual feeding of a high fiber diet protects against colon carcinogenesis, while a switch from a high-fiber to a low-fiber diet after administration of the carcinogen has no observable effect. These observations imply that dietary fiber protects against tumorigenesis during the promotional phase.

The effect of dietary wheat bran and dehydrated citrus fiber at 15% level and 5% dietary fat on intestinal carcinogenesis induced by AOM and DMAB was studied in male F344 rats (44,45). Composition of diets was adjusted so that all animals in different experimental groups consumed approximately the same amount of protein, fat, minerals and vitamins. The animals fed the wheat bran or citrus fiber and treated with AOM had a lower incidence (number of animals with tumors) and multiplicity (number of tumors/tumor bearing rat) of colon tumors and tumors of the small intestine than did those fed the control diet and treated with AOM (Table II; 15). Although 15% purified pectin in the diet (41) inhibited the colon tumor incidence better than did 15% dehydrated citrus fiber, in this study the inhibition of colon tumor multiplicity was more pronounced with the dehydrated citrus fiber compared with purified pectin. Because dehydrated citrus fiber contains about 20% pectin, the pectin content of this diet was considerably lower than that of the diet used in

Table II

Colon Tumor Incidence in F344 Male Rats fed Diets Containing Wheat Bran or Citrus Fiber and Treated with Azoxymethane

| Diet | Animals with colon tumors | | | | | | Colon tumors per tumor bearing rat | | |
|---|---|---|---|---|---|---|---|---|---|
| | Total | | Adenoma | | Adenocarcinoma | | Total | Adenoma | Adenocarcinoma |
| | No. | % | No. | % | No. | % | | | |
| Control (96)[b] | 86 | 90 | 83 | 86 | 60 | 63 | $3.45 \pm 0.16$[a] | $2.37 \pm 0.16$ | $1.08 \pm 0.18$ |
| Wheat Bran (51) | 36 | 71[d] | 24 | 47[d] | 20 | 39[d] | $1.55 \pm 0.12$[e] | $0.94 \pm 0.13$[e] | $0.61 \pm 0.11$[e] |
| Citrus Pulp (51) | 32 | 63[d] | 21 | 41[d] | 20 | 39[d] | $1.67 \pm 0.18$[e] | $0.90 \pm 0.14$[e] | $0.88 \pm 0.16$ |

a Total represents animals with adenomas and/or adenocarcinomas

b Effective number of animals in each group is shown in parenthesis

c Mean $\pm$ SEM

d Significantly different from the group fed the control diet by $X^2$ test ($P<0.05$ or better)

e Significantly different from the group fed the control diet by Student's t-test ($P<0.05$ or better)

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the study by Watanabe et al. (41). Therefore, the modifying effect of dehydrated citrus fiber in intestinal tumor incidence may be due to the pectin content of the citrus fiber, its fecal bulking effect, and its non-fiber components.

The animals fed the wheat bran and treated with DMAB had a lower incidence and multiplicity of colon and small intestinal tumors compared to those fed the control diet and treated with DMAB (Tables III and IV; 44). Animals fed the diet containing citrus fiber developed fewer DMAB-induced small intestinal tumors than did the rats fed the control diet.

Recently, Freeman et al. (46) compared the incidence of colon tumors induced by DMH in Sprague-Dawley rats fed either a fiber-free diet or a diet containing 4.5% purified cellulose. Among the animals ingesting cellulose, fewer had colonic neoplasms, and the total number of colon tumors in this group was lower. This protective effect appeared to be associated with a shift in tumor distribution from the proximal colon to a more distal site. Although the mechanism for this apparent redistibution of tumors within the colon remains obscure, some change in the luminal physiochemical environment or some inherent difference in the mucosa of the two areas may be responsible.

Carrageenans are complex polysaccharides prepared from the red marine algae and are widely used in the food industry. A series of studies with degraded carrageenan showed that this product could induce ulceration of the cecum and the proximal colon in laboratory animals (47-49). Although some species of animals fed undegraded carrageenan appeared to be healthy with no adverse effect (50,51), guinea pigs had ulceration in the colon (52). More recently, the carcinogenicity of food-grade carrageenan has been evaluated in a lifetime animal study in rats fed 4g/kg body wt. and in guinea pigs fed 3.7g/kg body wt. (53). There was no significant difference in mammary tumor yield between the control diet and carrageenan-fed animals.

The effect of dietary undegraded carrageenan on AOM- and MNU-induced colon carcinogenesis was studied in F344 rats in our laboratory (54). The animal fed the carrageenan diet and treated with AOM or MNU had a higher incidence of colon tumors than did those fed the control diet (Table IV), suggesting a promoting effect of undegraded carrageenan in colon carcinogenesis.

Agar, a sulfated polysaccharide derived from the red-purple seaweed, is used in food products as a gelling agent and as a laxative and is not digested by humans. Dietary agar enhances DMH-induced colon carcinogenesis in male $CF_1$ mice (55). In another study, cholestyramine added to the diet at 2% level enhanced colon tumors induced by AOM (56). The tumors in the cholestyramine fed rats were larger, appearing more like human colonic tumors.

Table III

Colon and Small Intestinal Tumor Incidences in F344 Male Rats Fed Diets Containing Wheat Bran or Citrus Fiber and Treated with 3,2'-dimethyl-4-aminobiphenyl

| Diet | Animals with colon tumors (tumor incidence) | | | | | | Animals with small intestinal tumors (tumor incidence) | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Total[a] | | Adenoma | | Adeno-carcinoma | | Total | | Adenoma | | Adenocarcinoma | |
| | No. | % | No. | % | No. | % | No. | % | No. | % | No. | % |
| Control[b] (94) | 43 | 46 | 38 | 40 | 13 | 14 | 52 | 56 | 12 | 13 | 45 | 48 |
| Wheat bran (50) | 13[d] | 26 | 10[d] | 20 | 4 | 8 | 17[d] | 34 | 2 | 4 | 15[d] | 30 |
| Citrus Pulp (50) | 22 | 44 | 18 | 36 | 7 | 14 | 14[d] | 28 | 3 | 6 | 12[d] | 24 |

a Total represents animals with adenomas and/or adenocarcinomas

b Effective number of animals in each group is shown in parenthesis

c Mean $\pm$ SEM

d Significantly different from the group fed the control diet by $X^2$ test, $P<0.05$ or better

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The aforementioned limited results suggest that the protection against colon carcinogenesis afforded by dietary fibers, both conventional and unconventional, depends on the source of fiber and the type of carcinogen.

Table IV

Colon Tumor Incidence in Female F344 Rats fed a Control or Undegraded Carrageenan Diet and Treated with AOM or MNU

| | Carcinogen | No. of rats | % | Tumors/rat |
|---|---|---|---|---|
| Control diet | AOM | 30 | 57[a] | 1.5 |
| Carrageenan diet | AOM | 26 | 100 | 11.3 |
| Control diet | MNU | 29 | 69[a] | 1.5 |
| Carrageenan diet | MNU | 29 | 100 | 4.4 |
| Control diet | | 15 | 0 | 0 |
| Carrageenan diet | | 15 | 7 | 1 |

[a] Significantly different from the carrageenan diet by $X^2$ test ($P<0.01$, or better)

## Conclusions

During the last decade, as this review indicates, some progress has been made in the understanding of the role played by the dietary constituents in general and specifically the role of fibers in cancer of the colon. The population with high incidences of cancer of the colon are characterized by consumption of a high level of dietary fat. Furthermore, dietary fat may be a risk factor for the colon cancer in the absence of factors that are protective, such as use of certain high fibrous foods and fiber. Thus, alteration of dietary habits leading to a higher intake of certain fibers would be indicated to decrease the risk of this important cancer. Beginning this dietary pattern early in life may prove most beneficial.

The demonstration of two stages in experimentally induced cancer in animal models suggests that there are two stages in environmentally induced cancer in humans (57). Most human cancers probably result from a complex interaction of carcinogens, co-carcinogens, and tumor promoters. Most of nutritional or dietary factors act at the promotional phase of carcinogenesis.

Because promotion is a reversible process, in contrast to the rapid, process of initiation by carcinogens, manipulation of promotion would seem to be the best method of cancer prevention.

In addition to pinpointing harmful environmental agents and their elimination, which is also one of the practical methods of preventing cancer, there seems to be on the horizon the promise of select methods of prevention. Although there is much need for further research to understand the modification of carcinogenic process, the successes thus achieved have expanded into some understanding of the numerous steps involved in the activation and detoxification of various chemical carcinogens and promoters by a variety of means. Application of a number of such basic tools, elimination of harmful agents from the environment, reduction of biochemical activation processes and a concomitant increase in detoxification reactions, as well as trapping of active intermediates by harmless nucleophilic reagents point to a promising future for preventive efforts. Thus, there is hope that these important types of cancer can be controlled by modifying not only environment with respect to genotoxic carcinogens, but also that of epigenetic carcinogens and, thus, humans cancer risk feasibly can be reduced (58).

Acknowledgments

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2

# Leguminous Seed Fiber

## Uses in Disease States and Effects on Carbohydrate Digestion in Vitro and Absorption in Vivo

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The fiber hypothesis suggested that diabetes and heart disease are the result, at least in part, of a lack of fiber in the diet. This paper examines some of the recent evidence concerning the mechanism by which dietary fiber affects carbohydrate metabolism, and how this, in turn, may be of use in the treatment of these disorders. It is suggested that the rate of digestion and absorption of carbohydrate from the small intestine is a major determinant of the physiological response to a food. Both the rate of diffusion of glucose *in vitro* from glucose/fiber mixtures, and the rate of *in vitro* digestion of foods, are proportional to the *in vivo* blood glucose responses seen after feeding the fibers or the foods. In this context dietary fiber derived from leguminous seeds, taken either purified, or in its natural state (i.e. eating the bean itself) has been shown to be most effective in reducing blood glucose and serum lipid concentrations. This strongly supports the increased consumption of beans for health reasons.

Current interest in dietary fiber began about ten years ago when Burkitt and Trowell hypothesized that diabetes and heart disease were fiber deficiency disorders (1). Since then there has been increasing research on the effects of dietary fiber on carbohydrate and lipid metabolism. Such work was pioneered and stimulated in large part by Dr. J. Anderson of Lexington, Kentucky who first used high carbohydrate/high fiber diets to treat diabetes (2) and Dr. D. Kritchevsky who has examined the effects of fiber on the metabolism of cholesterol (3).

Our own interest in dietary fiber was stimulated by the originally suggested mechanism by which primitive diets affect carbohydrate metabolism. According to the hypothesis, the

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fiber-rich energy dilute primitive diet travelled the full length of the small intestine releasing its products of digestion slowly (Fig.1a). By contrast, the refined Western diet was seen to be rapidly absorbed in the upper small intestine (Fig.1b). The consequences of a fiber-rich meal, it was supposed, would be a slow, prolonged and relatively small rise in the blood glucose level, compared with a rapid rise after a refined, fiber depleted meal (Fig.2).

## Effect of Leguminous Fiber on Gut Physiology

To see whether fiber might slow the absorption of material from the intestine we used a simple dialysis model. Solutions of glucose were placed inside semipermeable dialysis bags with and without the addition of equal amounts of different types of dietary fiber; wheat bran, pectin, methyl cellulose, gum tragacanth and guar gum. Compared with the fiber-free control, all types of fiber produced some impedance to the outward passage of glucose, but the most effective were the most viscous substances guar and tragacanth.

When these same five fiber preparations were added in 12 g dietary fiber equivalents to 50 g oral GTT taken by normal volunteers there was a flattening of both the glucose and insulin responses (4). Again guar and tragacanth, the most viscous materials, were the most effective. The question obviously arose as to whether the flatter blood glucose responses after guar were the result of malabsorption of glucose caused by a much reduced rate of absorption. However studies using urinary xylose excretion as a measure of carbohydrate absorption suggested that after a guar containing GTT there was delayed but complete absorption of the carbohydrate load (4). The lack of symptoms of carbohydrate malabsorption also strongly supported this.

In addition to slowed absorption in the small intestine, a reduced rate of gastric emptying and decreased upper gastrointestinal motility may also be factors responsible for reduced glucose levels after fiber containing meals. Guar and pectin have been shown by isotopic studies to delay gastric emptying in both normals (5) and postgastric surgery patients (6,7). In addition breath hydrogen studies have demonstrated that viscous fiber increases the mouth to cecum transit time (4). However as a model of the effect of fiber in the small intestine, the % reduction of diffusion of glucose from dialysis bags by each of the four viscous dietary fibers (guar gum, tragacanth, pectin and methyl cellulose) was proportional to the degree to which each fiber reduced the blood glucose response ($r = 0.9938$) (Fig.3). The coefficient of determination for this regression line suggests that over 95% of the reduction in the blood glucose response by each fiber may be accounted for by impaired diffusion of glucose from the small intestine (8,9).

The dynamic viscosity of 1% solutions of the fibers were

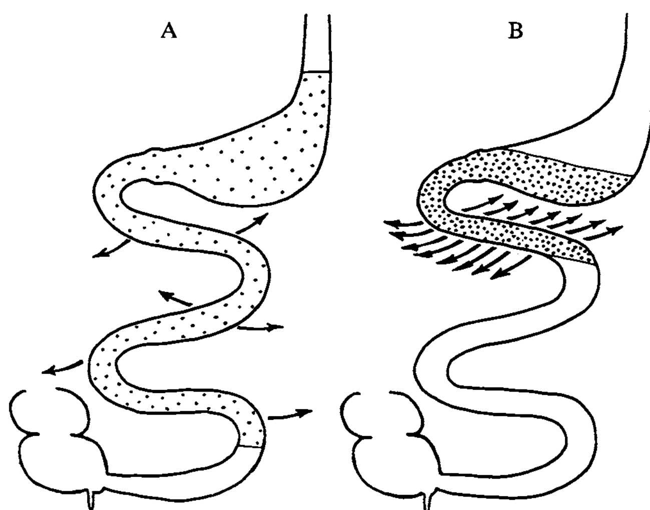


*Figure 1. Hypothetical effect on upper gastrointestinal function of high-fiber diets containing low energy density foods (A) and low-fiber diets of high energy density foods (B).*

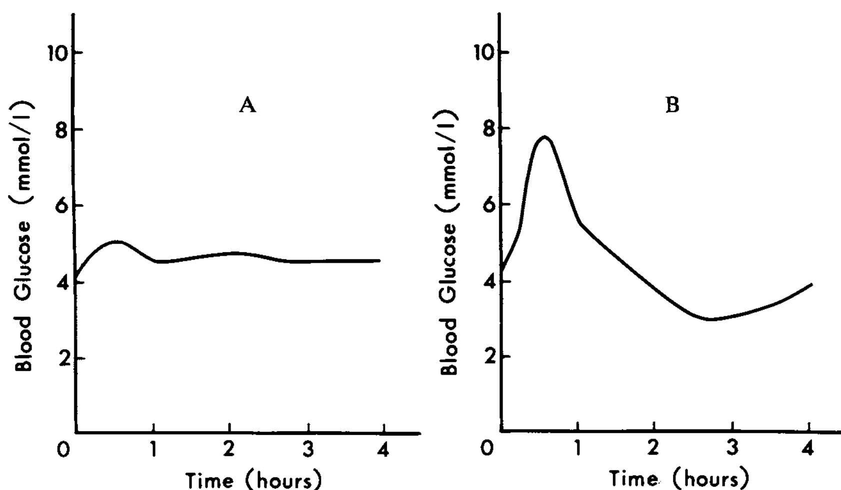


*Figure 2. Hypothetical effect on postprandial blood glucose of high-fiber diets containing low energy density foods (A) and low-fiber diets of high energy density foods (B).*

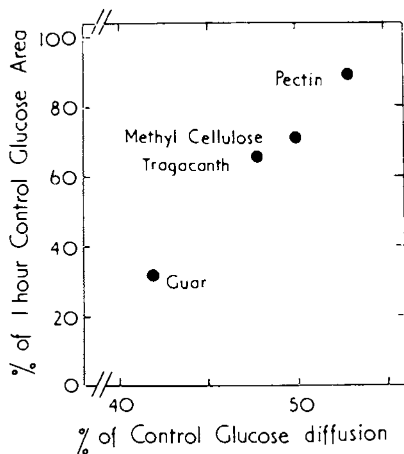


*Figure 3. Mean (n = 6 normal subjects) blood glucose area (calculated geometrically for the 0, 15, 30, 45, and 60 min blood glucose concentrations) of 50 g glucose tolerance tests containing 12 g dietary fiber from guar, gum tragacanth, pectin, or methyl cellulose. The mean blood glucose area is expressed as a percent of the control (fiber-free) blood glucose area and plotted against the concentration at 3 h of glucose in the dialysate surrounding a dialysis bag that contains 30 mL 0.1 M glucose solution and 1 g of guar, gum tragacanth, pectin, or methyl cellulose expressed as a percent of the control (fiber-free) dialysate glucose concentration ($r = 0.99$, $p < 0.01$).*

measured by Stokes method and were as follows: guar, 1.3 N.sec/m; gum tragacanth, 0.52 N.sec/m; pectin, 0.21 N.sec/m; and methyl cellulose, 0.07 N.sec/m (4). Because of the small numbers the relationships between viscosity of the individual fibres and the *in vitro* reduction of diffusion of glucose from dialysis bags (r = 0.917), and viscosity and the *in vivo* reduction of blood glucose responses (r = 0.913) did not reach significance.

## Use of Fiber in Treatment of Diabetes

Similar reductions of the postprandial glucose rise after a meal have been seen in diabetics given guar and pectin (10). Moreover, in metabolic ward studies on 18 diabetics, the majority of whom were taking insulin, the addition of 14 to 26 g guar per day to the diet for 5 days resulted in a 50% reduction in urinary glucose output compared with a 5 day control period (11) (Fig.4). In these studies guar was given in divided doses with each meal, mixed into fruit juices, soups and mashed potato, or baked into breads and crispbreads. Others have also used viscous fibers to treat diabetics and found decreased fasting blood glucose levels and urinary glucose excretion with Konjac Mannan (12) and guar (13).

Particulate fiber (i.e. bran and cellulose) has not produced such marked or consistent effects. Nevertheless two studies have shown reduced postprandial blood glucose, daily glycosuria, insulin requirement and frequency of hypoglycemic episodes in diabetics under metabolic ward conditions (14,15). In addition wheatbran has been shown to improve glucose tolerance on longer term supplementation of newly diagnosed non-insulin requiring diabetics, (16) and in non-diabetics with diverticular disease (17). Nevertheless the effects of wheat bran on blood glucose responses to glucose and starch, glucose kinetics and plasma insulin concentrations have not always been confirmed (4,18,19).

## Importance of Mixing Fiber Supplements with Food

Guar has not always been used successfully in diabetes; (20,21) the reason here is probably that guar was not given pre-mixed with the carbohydrate portion of meals. To illustrate this, we found in a group of healthy volunteers that guar given just 2 min before a glucose tolerance test was without effect on the resulting blood glucose response (Fig.5), while the same amount of guar mixed with the glucose resulted in the expected flattening (22).

## Therapeutic use of Fiber in Diabetes: Need for Fiber Supplemented Foods

Long term use of viscous fiber is only possible if palatable

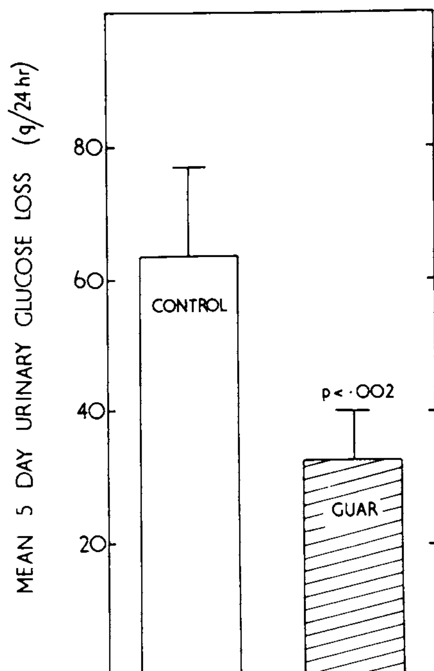


*Figure 4. Overall mean (± SEM) daily urinary glucose loss in 18 diabetics (22 studies) over 5 d without (control) and with 14–26 g guar per day added to metabolically controlled diets.*

supplements are available. A guar crispbread produced by Speywood Laboratories (Bingham, Nottingham, England) is the only preparation known to the authors where fiber has been effectively and palatably pre-mixed with the dietary carbohydrate . Each slice of crispbread contained 1 g guar, and diabetic patients consumed 14-26 slices per day depending upon their calorie intake (1 slice per 100 kcal). In addition to the 50% reduction of urinary glucose excretion seen using this product in metabolic ward studies, (23) it has allowed a 20% reduction in insulin dose over the first three mouths of use to be maintained in a group of 6 insulin requiring diabetics for over a one year period (24). No reductions in serum $Ca^{++}$, $PO_4$, $Zn^{++}$ or $Cu^{++}$ or hemoglobin concentration were observed in 8 patients over a six month period (24). On the other hand total and LDL cholesterol were decreased significantly (5.0 ± 0.2 to 4.5 ± 0.3 mmol/l p 0.05; and 3.2 ± 0.2 to 2.6 ± 0.3 mmol/l p 0.01 respectively) and HDL and TG showed no change (1.2 ± 0.2 to 1.3 ± 0.1 mmol/l and 1.2 ± 0.2 to 1.3 ± 0.3 mmol/l respectively) (24). These findings confirm similar effects of guar crispbread on serum lipids of hyperlipidemic patients (25).

## Slowly Digested Carbohydrate in Whole Foods

In the same way that dietary fiber may alter absorption of carbohydrate from foods with which it is mixed, so may different foods make their carbohydrate available for absorption at different rates. When portions of 14 differnet foods (wholemeal bread, white and wholemeal rice and spaghetti, millet, buckwheat, instant mashed potato, porridge oats, sweet potato, kidney and soya beans, marrowfat peas and red lentils) each containing 2 g carbohydrate were mixed with human digestive juices (obtained from duodenal aspiration) and placed in dialysis bags there was a wide spectrum of rates of release of the carbohydrate they contained (26). Bread and grain products released their carbohydrate at over twice the rate as did the leguminous seeds ($p < 0.01$). When 50 g carbohydrate portions of these foods were fed to groups of normal subjects the beans gave 49% ($p < 0.01$) lower blood glucose responses than did the other foods (26). The estimated % carbohydrate digested *in vitro* after 5 hr correlated significantly with the total dietary fiber content of the 14 foods ($r = 0.53$ $p < 0.05$). The *in vivo* glycemic responses of these 14 foods (expressed as the glycemic index - see below) also correlated significantly with their fiber content (r = .62 p 0.02).

## The Glycemic Index

Both fiber and food form can modify the glycemic response to "available" carbohydrate. Available carbohydrate, as distinguished from unavailable carbohydrate, was first

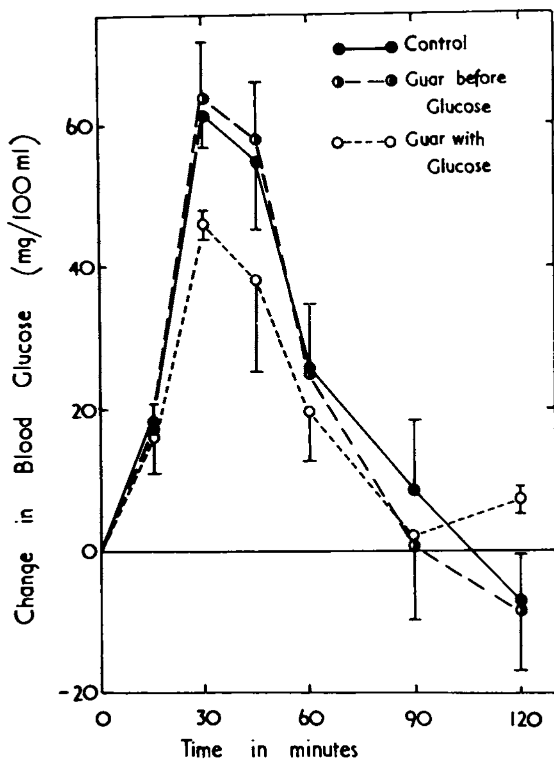


*Figure 5. Mean change in blood glucose in 5 normal individuals after consuming 50 g glucose in 600 mL lemon flavored water, 50 g glucose and 14.5 g guar in 600 mL lemon flavored water, or 14.5 g guar in 300 mL lemon flavored water followed 2 min later by 50 g glucose in 300 mL lemon flavored water.*

determined for a large number of foods by Lawrence and McCance in 1929 in order to accurately determine the carbohydrate content of Lawrence's diabetic diets (27). It now seems important for diabetic diets that foods should be classified also in respect of their rates of biological availability or the extent to which they raise the blood glucose. We have therefore carried out tests of this nature on over 60 foods (28). For the purpose of comparisons between foods we have calculated the glycemic index as:

$$\frac{\text{area under 2 hr blood glucose curve after food}}{\text{area under the 2 hr blood glucose curve after equivalent amount of glucose}}$$

With glucose as 100%, common foods varied from a glycemic index of almost 100% to less than 15% (Fig.6). The importance of the rate of digestion of food in determining the blood glucose response is suggested by the significant correlation between rate of digestion in vitro and the glycemic response in vivo to the 14 foods for which data is available ($r = 0.86$ $p < .001$) (Fig.7).

There was no correlation for 62 foods between glycemic index and food fiber content ($r = 0.220$), or between glycemic index and food sugar content ($r = 0.022$), suggesting that these were not major factors responsible for the glycemic response. Stronger negative correlations were observed between glycemic index and food fat content ($r = 0.386$, $p < 0.01$) and glycemic index and food protein content ($r = 0.532$, $p < 0.001$) (28). The lack of correlation with fiber may have been due to the small effect cereal and vegetable fibers had in reducing blood glucose responses (18). For example there was no difference between wholemeal and white breads, rice or spaghetti, and despite the high fiber content of fresh broad beans, carrots and parsnips, the blood glucose responses of these foods were respectively 79%, 92% and 97% that of glucose (28). Similarly the correlation of glycemic index with food sugar content may have been due to the contribution of sugars such as galactose and fructose to the total food sugar content. These sugars, although presumably rapidly absorbed, would not appreciably raise the blood glucose level (28).

Food tables therefore do not allow one to predict the glycemic response to a food. For this reason selection of foods should be based on physiological testing in man.

## High Fiber Foods and Diabetic Treatment

The results of studies where the cereal fiber content of the diabetic diet were raised have been variable. Their interpretation is also difficult because in many cases the carbohydrate intakes were also raised (2,29,30). It has been suggested that the higher carbohydrate intake may have contributed to the improved diabetic state, (31-34) however this

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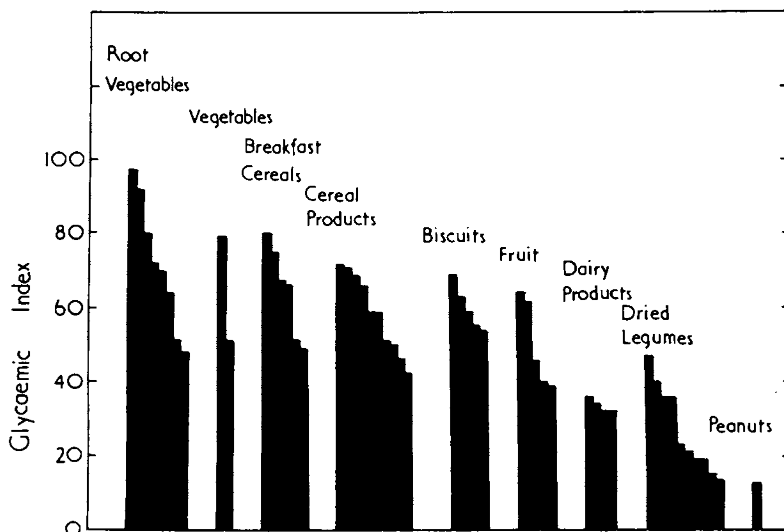


*Figure 6. Glycemic index of foods (i.e., area under 2 h blood glucose response curve of 50 g carbohydrate food portions, 50 g glucose itself being 100%). Each bar in each block represents the mean result for one food tested by 5–10 normal individuals.*

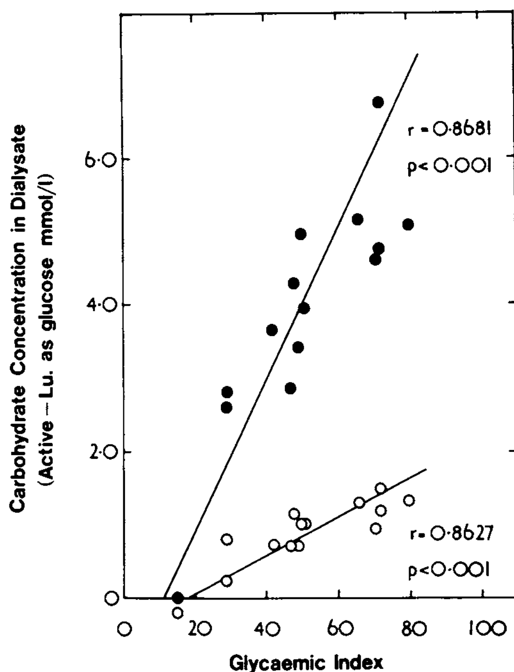


*Figure 7. The glycemic index (*see *text* or *Figure 6) of 14 foods plotted against the concentration of sugars, in the dialysate at 1 and 5 h, released during in vitro digestion of the same foods.*

*Carbohydrate concentration in the dialysate is expressed as the difference between the sugar concentration in the dialysate that surrounds the dialysis bag containing the digestive juices plus food (active) and the sugar concentration surrounding the dialysis bag containing the digestive juices (Lu = Lundh juice) alone. Key: 0, 1 h; and ●, 0.5 h.*

is still a matter of debate (35). In our metabolic ward studies with guar 9 patients had less than 40% carbohydrate in the diets and 14 had over 40%. Supplementation of the diets with guar produced a significantly greater reduction of the urinary glucose loss in the high carbohydrate group (64 $\pm$ 7% vs. 33 $\pm$ 10% $p < 0.02$) (36). This improvement in carbohydrate tolerance with high carbohydrate diets may in part explain the success reported in some studies using wheat bran (37). The validity of high carbohydrate/high fiber diets has been established by the pioneer work of Dr. J. Anderson using diets high in both fiber and carbohydrate taken in unprocessed cereals, cereal products, leguminous seeds and leafy vegetables (2,38-40). Of particular interest to us is the fact that such high carbohydrate diets might also be classed as low glycemic index diets. Patients originally consuming 43% carbohydrate diets were switched to 70% carbohydrate high fiber diets and showed improved control and greatly reduced insulin requirements. Those on less than 20 units of insulin daily could have it withdrawn. These benefits were maintained longterm on an outpatient basis if patients kept to 60% carbohydrate high fiber diets.

The advantages of low glycemic index diets for diabetics may not be confined to improved blood glucose control. Beans have been used successfully to treat hypercholesterolemic individuals in Sishuan province, China (41). In addition it has been shown that locust bean gum reduced serum lipids in hyperlipidemic individuals (42). Reduction of blood lipid levels is important to diabetic individuals who are at a greatly increased risk of developing cardiovascular disease than the general population (43). Effective dietary means for achieving this are welcome at a time when increasing side effects of hypolipidemic drug therapy with, for example clofibrate, are being discovered (44-47).

## Conclusion

Dietary fiber is only one of several possible pharmacologically active substances found in foods, and present in high concentrations in leguminous seeds which may be responsible for the different rates of digestion and blood glucose responses of different meals. Enzyme inhibitors, lectins and saponins are other so called antinutritional factors, also associated with dietary fiber which are able to alter small intestinal function (48). The gastrointestinal tract evolved to deal with these constituents in foods so that while toxic in large amounts (as in uncooked beans) small amounts may have beneficial effects.

These antinutrients have now largely been eliminated from the Western diet by refining and highly processing our foods. However, as with dietary fiber, we are likely in the future to see specific combinations of them reincorporated in small amounts into our diet for reasons of health. The studies on dietary fiber have opened the way for this with their demonstrations of

health benefits for diabetics, and possible relevance to the general population for the prevention of disease. It is also likely in the future, as more knowledge becomes available, that we shall see the food industry playing a role in the pharmacological manipulation of the diet for health using plant derived substances.

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# 3

# Structural Chemistry of Some Nonstarchy Polysaccharides of Carrots and Apples

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Problems in the extraction, isolation, fractionation, and subsequent analysis of non-starchy polysaccharides, which may function as dietary fibre components, are discussed for such substances from carrots and apples. Structural studies on polysaccharides from carrots have resulted in the characterization of a branched arabinan, an esterified galacturonan (pectin), and an acidic xylan as individual components, and further evidence points to the presence of a 4-linked galactan and a 4-linked glucomannan.

The non-starchy polysaccharides from plant sources fall into a limited number of structural families (1) which include cellulose, other β-D-glucans of the 3-linked type (callose) and of the mixed 3- and 4-linked type from cereals, xyloglucans, the various xylans, the pectic substances, i.e. galacturonans and rhamnogalacturonans, and associated arabinans, galactans and arabinogalactans of type I (4-linked), the arabinogalactans of type II (3,6-linked), galactomannans and glucomannans. For investigations on dietary fibre components which seek to establish correlations between individual polysaccharide species and particular physiological and *in vitro* functional properties, it is essential to establish the structural identities of these substances.

In our studies on the polysaccharide constituents of carrots and apples the following considerations have been borne in mind: (i) that account must be taken of the readily soluble as well as of the more obviously fibrous non-starchy polysaccharides; (ii) that cell wall materials must be prepared with minimum loss of these more readily soluble polysaccharides; (iii) that 'broad spectrum' analyses of 'pectic substances' and 'hemicelluloses', even when supported by determinations of individual sugar components, are rarely sufficient to establish the nature of these substances since the same sugar constituents occur in quite

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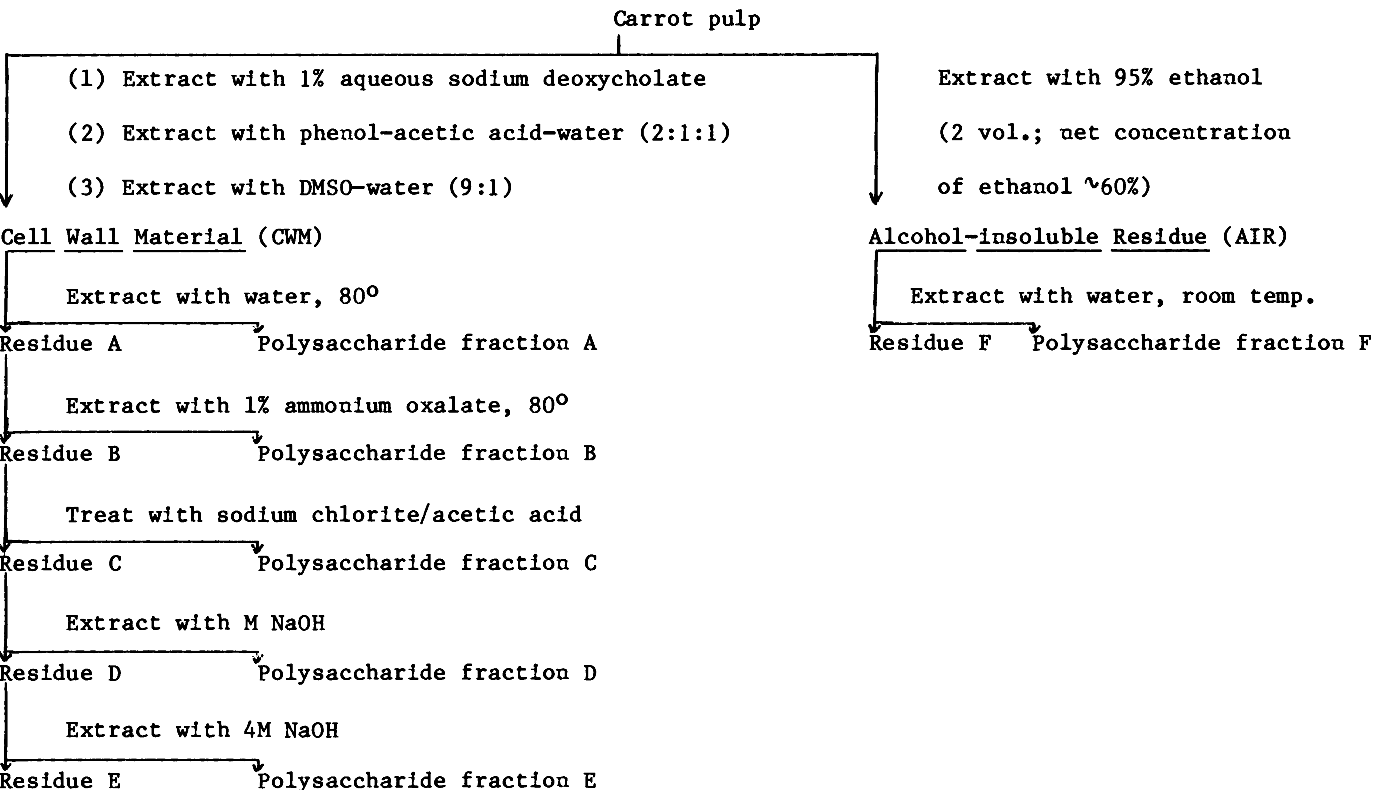
different species; and hence (iv) that individual polysaccharides should be examined in sufficient detail to establish their identities. The fractionation of all polysaccharides from a single source is not easily achieved, but with information on characteristic linkage types (from methylation analysis) in addition to sugar composition it is frequently possible to identify polysaccharides in incompletely separated mixtures.

Selvendran and his collaborators (2) in their extensive studies on dietary fibre polysaccharides have stressed the need for careful treatment of plant material so as to avoid changes in the ease of extraction of polysccharides through modifications in physical properties, and to account for all those polysaccharides which may survive passage through the upper gastrointestinal tract regardless of their natural fibrous or non-fibrous nature. Although no one procedure is likely to prove suitable for all types of plant material, the aim is to achieve maximum solubilization of starch and cytoplasmic material with minimum loss of the non-starchy cell wall polysaccharides. The procedure developed by Selvendran (2) involves successive extractions with cold aqueous sodium deoxycholate, phenol-acetic acid-water, and dimethylsulfoxide (DMSO) containing 10% of water, and has been used to prepare carrot cell wall material (CWM) (Scheme 1) for the graded extraction of polysccharide fractions on which preliminary structural studies have been carried out. An alcohol-insoluble residue (AIR) has also been prepared by extraction of carrot pulp with 95% ethanol (but net concentration ∿60% due to water in carrots) for use as an alternative source for the more readily soluble polysaccharides.

## Results and Discussion

Sugar analyses for carrot CWM and the corresponding AIR are shown in Table I and, as appropriate, indicate sugars formed on hydrolysis with trifluoroacetic acid (3) or after digestion with 72% sulfuric acid followed by dilution (4) [and thus provide by difference an estimate of the insoluble cellulose component], together with uronic acid, estimated as galacturonic acid using the 3-hydroxydiphenyl reagent (5). The similar relative proportions of sugars indicate that, aside from the cellulose, the main polysaccharides are pectins and associated neutral polysaccharides (arabinans, galactans and/or arabinogalactans). Compositional data alone do not differentiate between the differently linked arabinogalactans, nor do they indicate whether xylose residues occur as pectin constituents [as in soybeans (6)], or more probably in xylans or xyloglucans.




Scheme 1. Flow sheet for polysaccharide extractions from carrot pulp

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TABLE I. Sugar compositions (%) of carrot alcohol-insoluble residue (AIR), cell wall material (CWM) and CWM residue E

| Sugar | AIR | | CWM | | CWM residue E | |
|---|---|---|---|---|---|---|
| | TFA[a] | $H_2SO_4$[b] | TFA | $H_2SO_4$ | TFA | $H_2SO_4$ |
| Rhamnose | 3.5 | 3.0 | 2.5 | 2.0 | 1 | 1 |
| Arabinose | 6 | 8 | 5.5 | 5.5 | 1.5 | 2 |
| Xylose | 1.5 | 2 | 1 | 1 | 0.1 | 0.3 |
| Mannose | 1 | 2.5 | 0.5 | 1 | n.d. | 0.3 |
| Galactose | 11 | 13.5 | 9.5 | 9.5 | 4 | 5 |
| Glucose | 2 | 32 | 1 | 22 | 3 | 33 |
| Uronic acid[c] | | 19 | | 15 | | 5 |

[a] Hydrolysis with trifluoroacetic acid.

[b] Digestion with 72% sulfuric acid, followed by dilution and hydrolysis.

[c] Estimation with the 3-hydroxydiphenyl reagent.

CWM was then extracted sequentially with water at 80° and with ammonium oxalate at 80°, delignified with acidified sodium chlorite at 70°, and then further extracted with M and 4M sodium hydroxide. The crude polysaccharide extracts were likewise analyzed for constituent sugars and uronic acid (Table II). In order to obtain defined polysaccharide fractions the following separations were attempted. Polysaccharide fraction A was separated by ion-exchange chromatography on diethylaminoethyl(DEAE)-Sephadex A-50 and the neutral fraction afforded a virtually pure arabinan (1 in Scheme 2) after selective precipitation with cetyltrimethylammonium hydroxide (7). Linkage analysis by methylation confirmed that this arabinan was of the highly branched type associated with pectins for which the representative, but not unique, structure (1) may be advanced.

Polysaccharide fraction B, isolated from ammonium oxalate extraction, was a pectin of low degree of esterification. This material could not be fractionated satisfactorily by ion-exchange chromatography since only a small proportion was eluted from DEAE-Sephadex with neutral buffers. A more characteristic pectin sample, which was possibly lost during CWM preparation, was

TABLE II. Sugar composition (%) of polysaccharide fractions from graded extraction of carrot CWM or AIR

| Source | CWM | | | | | AIR |
|---|---|---|---|---|---|---|
| Polysaccharide fraction | A | B | C | D | E | F |
| Extractant | $H_2O$ | $NH_4$ oxalate | $HClO_2$ | M NaOH | 4M NaOH | $H_2O$ |
| <u>Sugar</u>[a] | | | | | | |
| Rhamnose | 4 | 5 | 6 | 1.5 | 1.5 | 3 |
| Arabinose | 27 | 8 | 14 | 4 | 6 | 4 |
| Xylose | 0.5 | 0.5 | 0.5 | 19 | 14 | 0.2 |
| Mannose | 0.5 | n.d. | 0.2 | 9 | 5 | n.d. |
| Galactose | 16 | 12 | 19 | 8 | 10.5 | 9.5 |
| Glucose | 1 | 0.2 | 0.5 | 16.5 | 7 | 1 |
| Uronic acid[b] | 17 | 69 | 27 | 9.5 | 8.5 | 77 |

[a] Hydrolysis with trifluoroacetic acid.

[b] Estimation with the 3-hydroxydiphenyl reagent.

obtained by extraction of AIR with water. Kirtschev and Kratchanov (8) have reported the isolation of carrot pectin with a variety of aqueous reagents, and our extraction of water-soluble pectin is based on the extraction of a similar carrot AIR with water at room temperature to minimize the possibilities of inadvertent acid hydrolysis or base-catalyzed β-elimination. The aqueous extract afforded a pectin preparation (uronic acid, 77%; degree of esterification, 59%) which was remarkably free from extraneous material and contained the characteristic neutral sugar constituents of many galacturonans, i.e. galactose, arabinose and rhamnose. Two fractions were obtained when this pectin was subjected to ion-exchange chromatography on DEAE-Sephacel using potassium acetate buffered at pH 5. Although neutral sugars predominated in the minor fraction, the major polysaccharide fraction still contained the same neutral sugar constituents, but in reduced proportions.

Linkage analysis of pectins by methylation, designed to avoid the twin problems of base-catalyzed degradation during methylation and sugar decomposition under conditions required for

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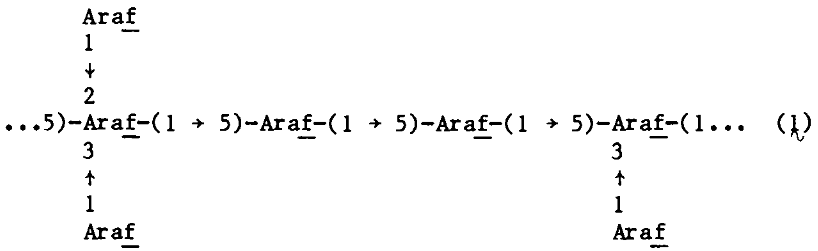
Hydrolysis of methylated arabinan → 2,3,5-$Me_3$ Ara (40)

2,3-$Me_2$ Ara (33)

2-Me Ara (13)

Ara (12)




Scheme 2. Arabinan - methylation analysis and structure (1)

complete hydrolysis of the methylated derivative, was performed by (1) methylation using a minimum excess of base and hydrolysing the methylated pectin to identify neutral sugar constituents, and (2) methylation of the carboxyl-reduced polysaccharide (9). The latter procedure is limited only in that most of the galactose derivatives are those arising from carboxyl-reduced galacturonic acid rather than original galactose residues. The results of these analyses are shown qualitatively rather than quantitatively in Table III, and are interpreted in terms of a linear galacturonan structure with deviations arising from arabinose, galactose and rhamnose as minor neutral sugar constituents (1). Rhamnose residues probably interrupt the galacturonan chain at intervals. Arabinofuranose residues are present in branched chains of the same type as in the arabinan (1) and probably constitute a covalently attached sub-unit. In addition to a small proportion of 4-linked galactopyranose units, other residues of this sugar are present as 6- or 3,6-linked units, whose origin is presently unexplained.

The alkali-soluble 'hemicellulose' fractions contained an array of neutral sugars with small proportions of uronic acid residues. Attempts to separate individual polysaccharides from fraction D by ion-exchange chromatography on DEAE-Sepharose CL-6B and by adsorption chromatography on microcrystalline cellulose (10) were only partially successful. Nevertheless, sugar analyses of these sub-fractions (Table IV), coupled with linkage analysis by methylation (Table V), provided evidence for the nature of the main polysaccharide components. Fraction D4 was an acidic xylan with only small proportions of sugar constituents arising from other polysaccharides. Methylation analysis was consistent with the presence of 4-linked β-D-xylopyranose

TABLE III. Methylation analyses of carrot pectin and the carboxyl-reduced polysaccharide

| | Methylated pectin | Methylated carboxyl-reduced polysaccharide | |
|---|---|---|---|
| Sugar | Sugars detected as alditol acetates | Sugars detected as alditol acetates | Structural unit |
| Galacturonic acid | | 2,3,6-$Me_3$ Gal | -4 Gal*p*A 1- |
| Galactose | 2,3,4,6-$Me_4$ Gal | 2,3,4,6-$Me_4$ Gal | Gal*p* 1- |
| | 2,3,6-$Me_3$ Gal | 2,3,6-$Me_3$ Gal | -4 Gal*p* 1- |
| | 2,3,4-$Me_3$ Gal | 2,3,4-$Me_3$ Gal | -6 Gal*p* 1- |
| | 2,4-$Me_2$ Gal | 2,4-$Me_2$ Gal | -3,6 Gal*p* 1- |
| Arabinose | 2,3,5-$Me_3$ Ara | 2,3,5-$Me_3$ Ara | Ara*f* 1- |
| | 2,3-$Me_2$ Ara | 2,3-$Me_2$ Ara | -5 Ara*f* 1- |
| | 2-Me Ara | 2-Me Ara | -3,5 Ara*f* 1- |
| Rhamnose | 3,4-$Me_2$ Rha | 3,4-$Me_2$ Rha | -2 Rha*p* 1- |
| | 3-Me Rha | 3-Me Rha | -2,4 Rha*p* 1- |

residues with attached side chains of single 4-*O*-methyl-α-D-glucopyranosyluronic acid units approximately every 12-15 units (**3** in Scheme 3). [Enantiomeric (D or L) and anomeric (α or β) configurations commonly encountered in plant polysaccharides are assumed.] The nature of the side chains was shown by (a) hydrolysis of the carboxyl-reduced polysaccharide to give 4-*O*-methylglucose from the corresponding uronic acid, and (b) methanolysis of the methylated xylan followed by acetylation to yield *inter alia* anomeric acetylated methyl ester methyl glycosides of the partially methylated aldobiouronic acid (**2**), whose identities were established by comparison (glc-mass spectrometry) with samples similarly generated from methylated alfalfa xylan (11). Other polysaccharide sub-fractions from the alkaline extract contined 4-linked residues of galactose, glucose and manose, together with arabinofuranose residues of the same type as in the above-mentioned arabinan (**1**). The association of 4-linked glucose and mannose residues points to the probable presence of a glucomannan. Linear chains of 4-linked galactose residues likewise point to the presence of a galactan of the type frequently associated with pectins.

TABLE IV. Composition of sub-fractions from polysaccharide fraction D

| Fractionation | | Ion-exchange | | | | Adsorption on cellulose | | | Cellulose followed by ion-exchange | |
|---|---|---|---|---|---|---|---|---|---|---|
| Eluant | | $H_2O$ | 0--0.5M KOAc | | 0.5M NaOH | $H_2O$ | 7M Urea | 0.5M NaOH | $H_2O$ | M KOAc |
| Fraction | D | D1 | D2 | D3 | D4 | D5 | D6 | D7 | D7a | D7b |
| <u>Sugar</u>[a] | | | | | | | | | | |
| Rhamnose | 5 | | 3 | 3 | 1 | 2 | | | | |
| Fucose | | 2 | | | | | | | | |
| Arabinose | 15 | 11 | 22 | 33 | 3 | 28 | 2 | 2 | | 2 |
| Xylose | 29 | 8 | 45 | 22 | 80 | 29 | 70 | 47 | 15 | 70 |
| Mannose | 13 | 28 | 1 | 1 | 4 | 1 | 9 | 18 | 38 | 12 |
| Galactose | 17 | 7 | 20 | 16 | 5 | 26 | 1 | | | |
| Glucose | 21 | 42 | 4 | 7 | 7 | 13 | 10 | 32 | 48 | 14 |
| Uronic acid[b] | 10 | | | | 8 | | | | | |

[a] Neutral sugars formed on hydrolysis with trifluoroacetic acid are expressed in relative proportions.

[b] Estimation (wt. %) with the 3-hydroxydiphenyl reagent.

TABLE V. Relative proportions of methylated sugars formed on hydrolysis of methylated polysaccharide fractions and identified by glc alone or glc-ms* of partially methylated alditol acetates.

| Fraction | D* | D1 | D2 | D3 | D4* | D7b |
|---|---|---|---|---|---|---|
| Sugar[a] | | | | | | |
| 2,3,5-$Me_3$ Ara | 7 | 5 | 5 | 7 | | |
| 2,3-$Me_2$ Ara | 4 | 1 | 3 | 4 | | |
| 2-Me Ara | 3 | p | 2 | 5 | | |
| Ara | 2 | p | 2 | 2 | | |
| 2,3,4-$Me_3$ Xyl | 3 | 3 | 1 | 1 | 3 | 1 |
| 2,3-$Me_2$ Xyl[b] | 36 | 4 | 47 | 40 | 84 | 25 |
| 2/3-Me Xyl | | | | | 12 | 3 |
| 2,3,6-$Me_3$ Gal | 7 | 6 | 25 | 18 | | |
| 2,3,6-$Me_3$ Man | 11 | 30 | 2 | 2 | | 16 |
| 2,3,6-$Me_3$ Glc | 14 | 27 | 2 | 2 | | 26 |

[a] A number of minor components whose structural identities were not confirmed are omitted.

[b] May include small proportions of 2,3,4,6-$Me_4$ Gal.

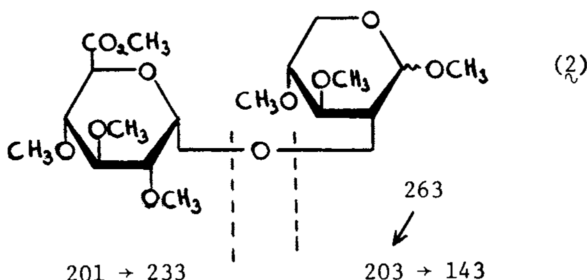
These results provide evidence for the nature of these carrot polysaccharides for which reasonably unambiguous structural information has been obtained. Pectin (with an associated arabinan) and cellulose are the main non-starchy polysaccharides. An acidic xylan is present in smaller amounts together with a 4-linked galactan and probably a 4-linked glucomannan. The CWM and AIR from different batches of carrots differed in total carbohydrate composition, although much less in the relative proportions of sugar constituents, so that only limited quantitative conclusions may be drawn. However, since these results were obtained, analyses of CWM and AIR from the same batch of carrots have shown rather similar carbohydrate compositions. The relatively small compositional differences point to some loss of the more soluble pectic substances during

Hydrolysis of methylated xylan → 2,3,4-$Me_3$ Xyl (1)

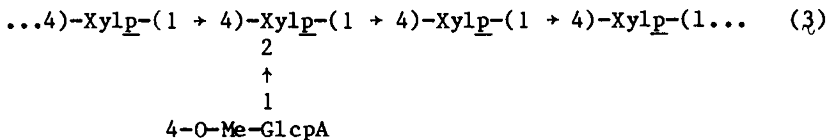
2,3-$Me_2$ Xyl (25)

3-(+ some 2-) Me Xyl (3)

Methanolysis and acetylation to give partially methylated aldobiouronic acid derivatives (2) - m/e values are shown for characteristic fragment ions in the mass spectrum




(2)




(3)

Scheme 3. Acidic Xylan (polysaccharide fraction D4) - methylation analysis, partial fragmentation to methylated aldobiouronic acid derivatives (2), and general structure (3)

CWM preparation, a conclusion supported by the isolation of smaller quantities of these polysaccharides on extraction with water and aqueous ammonium oxalate.

The results of sugar analyses of the CWM and AIR (Table VI) from parallel batches of apples likewise indicate similar overall carbohydrate compositions with only small losses of pectic substances from the CWM, a conclusion again supported by the isolation of smaller quantities of these materials on subsequent extraction. Structural studies on apple polysaccharides will be reported elsewhere.

TABLE VI. Sugar composition (%) of apple CWM and AIR, and pectin fractions extracted with (A) cold water, (B) hot water, and (C) ammonium oxalate

| Source | CWM | | | | AIR | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Extract | | | B | C | | | A | B | C |
| Hydrolysis[a] | TFA | $H_2SO_4$ | TFA | TFA | TFA | $H_2SO_4$ | TFA | TFA | TFA |
| <u>Sugar</u> | | | | | | | | | |
| Rhamnose | 2.5 | 2.5 | 3 | 3.5 | 2 | 1 | 2.5 | 2.5 | 3 |
| Fucose | 1.5 | 1.5 | n.d. | 0.5 | 1.5 | 1 | 1.5 | 0.5 | 2 |
| Arabinose | 12.5 | 11.5 | 23 | 22 | 12.5 | 12.5 | 9 | 23.5 | 21 |
| Xylose | 6.5 | 6 | 2 | 3.5 | 7.5 | 5 | 2 | 1 | 3 |
| Mannose | 1 | 4.5 | n.d. | 0.5 | 1 | 4 | 1 | 0.5 | n.d. |
| Galactose | 6 | 6 | 5 | 14 | 6 | 4 | 6 | 5 | 8 |
| Glucose | 2 | 23 | 1 | 3 | 2 | 18.5 | 2.5 | 2.5 | 1 |
| Uronic acid[b] | | 15 | 55 | 37 | | 16.5 | 54 | 58 | 64 |

[a] Hydrolysis with trifluoroacetic acid or after digestion with 72% sulfuric acid followed by dilution.

[b] Estimation with the 3-hydroxydiphenyl reagent.

## Experimental

General Methods. Gas-liquid chromatography was performed with a Perkin-Elmer Sigma 3B gas chromatograph fitted with a flame ionization detector using (a) a packed column of 3% of silicone gum Silar 10 CP on Chromosorb W-HP (100/200 mesh) or (b) a S.C.O.T. column coated with silicone gum OV-225. A Perkin-Elmer Data System Sigma 10 B was used for peak integration. For glc-mass spectrometry columns (b) or (c) W.C.O.T. coated with silicone gum SP-2330 were used in a Pye-Unicam series 204 gas chromatograph connected by a jet separator to a VG Micromass 16F mass spectrometer and VG data system 2000, operated with an inlet temperature of $\sim 250^{o}$, an ionization potential of 70 eV, and an ion-source temperature of $\sim 250^{o}$. Evaporations were carried out under diminished pressure at temperatures of $40^{o}$ or less.

Sugar Analyses. Hydrolyses of polysaccharide fractions and cell wall preparations were performed (a) directly with trifluoroacetic acid (3) and (b) after digestion with 72% sulfuric acid and dilution, with M-sulfuric acid (4). D-Allose was added as an internal standard and the sugar mixtures were converted into alditol acetates (12) for analysis by glc on column (a). Uronic acid determinations were carried out spectrophotometrically with the 3-hydroxydiphenyl reagent (5). In the case of insoluble materials determinations were performed after digestion with 72% sulfuric acid and appropriate dilution.

Methylation Analyses. Polysaccharide samples were methylated by the Hakomori procedure as described by Lindberg (13). In the case of acidic polysaccharides, especially for pectins, de-esterification was first carried out at $4^{o}$ with sodium hydroxide at pH 12 and, in order to minimize base-catalyzed degradation, methylations were performed with restricted proportions of reagents (14). Completeness of methylation was checked by the absence of hydroxyl bands in the ir spectra. Neutral methylated sugars formed on hydrolysis were converted into the derived partially methylated alditol acetates for analysis by glc alone using column (b) with confirmations of identities by glc-mass on columns (b) or (c). Methylated acidic polysccharides were methanolyzed with methanolic 3% hydrogen chloride for 12h at $100^{o}$ in sealed tubes, and the resulting methyl glycosides were acetylated for glc examination on column (b).

Preparation of Carrot CWM. Carrots (250 g per batch) were cut into small pieces and blended with aqueous 1% sodium deoxycholate (SDC) (200 mL) for 5 min in a Waring blender with addition of 2-octanol (2 mL) as an anti-foaming agent. The blended carrot pulp was transferred to a ball-mill container with

1% SDC (550 mL) and ball-milled for 16 h at 4°. Light microscopic examination revealed ∿ 95% rupture of cells. The suspension was then centrifuged at 20,000 g for 0.5 h at 4°. The residue was washed twice with distilled water (2 x 200 mL), recovered by centrifugation, and extracted twice with phenol-acetic acid-water (2:1:1,w/v/v, 2 x 500 mL) for periods of 0.5 h after short treatments in a blender to ensure uniform suspensions. The insoluble residue was then blended with DMSO-water (9:1, v/v, 350 mL) and the suspension was sonicated for 30 min whilst maintaining the temperature at ∿ 20°. The suspension was stirred at room temperature for 16 h, the insoluble residue was separated by centrifugation, and the procedure was repeated. The final insoluble residue was washed five times with water (5 x 100 mL), at which stage a starch-iodine test was negative, and the residue (10.8 g) in aqueous suspension was isolated by freeze-drying.

Extraction of Polysaccharides from Carrot CWM. CWM (25 g) was extracted three times by stirring in water (1 L) for 2 h at 80°. After each extraction the suspension was centrifuged and the combined supernatant liquids were freeze-dried to give polysaccharide fraction A (0.5 g).

Residue A was extracted three times with aqueous 1% ammonium oxalate (1 L) for 2 h at 80° with maintenance of pH at 5. The final suspension was removed by centrifugation to give residue B (17.2 g), the combined supernatant liquids were dialyzed against distilled water (with frequent changes) for 2 days, and polysaccharide fraction B (4.4 g) was isolated by freeze-drying.

Residue B (13.5 g) suspended in water (1.35 L) was delignified by treatment with acetic acid (1.7 mL) and sodium chlorite (4.1 g) at 70° in an inert atmosphere, and the suspension was stirred for 15 min. The residue was separated by centrifugation, the procedure was repeated twice, the delignified CWM (residue C) was separated after washing twice with water, and the combined centrifugates and washings were dialyzed against distilled water and freeze-dried to give polysaccharide fraction C (0.9 g).

Residue C (11.5 g) was treated at 4° by stirring with dilute sodium hydroxide (200 mL) at pH 12 in an inert atmosphere in order to effect de-esterification of remaining pectin with minimum base-catalyzed degradation. Sodium hydroxide was then added to obtain a total volume (1 L) of molar reagent, and the suspension was stirred for 2 h at room temperature. The insoluble residue was separated and extracted again, and the combined extracts were neutralized with acetic acid, dialyzed against distilled water, and freeze-dried to give polysaccharide fraction D (0.8 g). The residue from the foregoing extraction was further extracted in a similar manner with 4M sodium hydroxide, and the extracts were worked up to give polysaccharide fraction E (0.25 g), leaving residue E (9.3 g).

Preparation of Carrot AIR and Isolation of Polysccharide Fraction F. Carrots (900 g) were cut into small pieces and blended in 95% ethanol (1.5 L), and the suspension was heated under reflux for 2 h. The residue was then extracted continuously with fresh ethanol until all colour had disappeared affording carrot AIR (35 g). Carrot AIR (35 g) was extracted thrice by stirring vigorously in water (2 L) at room temperature to give polysaccharide fraction F (5.3 g).

Examination of Polysaccharide Fractions. Fraction A. Fraction A (425 mg) was chromatographed on a column (1.6 x 60 cm) of DEAE-Sephadex A-50 (formate form) which was eluted with water and then sequentially with 0.2M, 0.4M, 0.6M and M sodium formate. Fraction A1 (220 mg), eluted with water, gave on hydrolysis arabinose and galactose in the ratio of 5:1 with traces of glucose and mannose, but contained no uronic acid. Cetyltrimethylammonium hydroxide solution (2 mL) and M NaOH (2 mL) were added to fraction A1 (160 mg) in water (20 mL). After 20 min the flocculent white precipitate was removed by centrifugation. The precipitate was dissolved in water (20 mL) containing sufficient acetic acid for neutralization and the polysaccharide derivative was reprecipitated with M NaOH (2 mL). A solution of the precipitate in dilute acetic acid was deionized successively with Dowex 1X4 ($HCO_3^-$) and 50WX8 ($H^+$) resins, and freeze-dried to give arabinan (1) (15 mg). Hydrolysis of the polysaccharide gave arabinose (92%) and galactose (7%). A sample of arabinan was methylated and hydrolysis of the methylated polysaccharide, followed by reduction and acetylation gave the alditol acetates shown in Scheme 2.

Fraction D. Fraction D (205 mg) (carbohydrate content, 50%) was chromatographed on a column (1.6 x 50 cm) of DEAE-Sepharose CL-6B (acetate form) which was eluted with water to give fraction D1 (22 mg), with a linear gradient of 0--0.5M potassium acetate (500 mL) to give fractions D2 (12 mg) and D3 (17 mg), and 0.5M sodium hydroxide (200 mL) to give fraction D4 (33 mg) (total recovery of carbohydrate, 86%). A sample of fraction D4 was converted into the carboxyl-reduced polysaccharide (9), hydrolysis of which gave 4-O-methylglucose as an additional neutral sugar constituent.

Aqueous 7% cupric acetate (~8 mL) was added dropwise to fraction D (200 mg) in water (60 mL), the precipitate was removed by centrifugation, the supernatant liquid was concentrated (to 25 mL) and poured into ethanol (100 mL). The resulting precipitate was washed with ethanolic 1% hydrogen chloride to remove copper ions and then with ethanol to remove acid. The residue in water was chromatographed on a column (1 x 25 cm) of microcrystalline cellulose which was eluted successively with water, 7M urea, and

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0.5M sodium hydroxide to give fractions D5 (9 mg), and D7 (24 mg). Fraction D7 was further chromatographed on DEAE-Sepharose CL-6B to give fractions D7a and D7b eluted with water and M potassium acetate. The polysaccharide sub-fractions were analyzed for sugar composition (Table IV) and selected samples were subjected to methylation analysis (Table V).

Fraction F. The polysaccharide had $[\alpha]_D$ + 169° (c 0.16) and the methoxyl content (7.6%) corresponded to a degree of esterification of 59%. The crude pectin (210 mg) was chromatographed on a column (1.6 x 40 cm) of DEAE-Sephacel (acetate form) which was eluted with 0.2M and 0.4M potassium acetate buffered at pH 5 to give fractions F1 (22 mg) [Found: rhamnose, 3; arabinose, 13; galactose, 18; uronic acid, 32%] and F2 (135 mg) [Found: rhamnose, 3; arabinose, 3; galactose, 6; uronic acid, 84%].

A sample of fraction F2 was methylated (14, 15). Methanolysis of the methylated polysaccharides followed by acetylation gave a mixture of methyl glycoside acetates amongst which derivatives of 2,3-di-O-methylgalacturonic acid predominated (glc on column b). A further sample of the methylated polysaccharide was hydrolyzed and the derived partially methylated alditol acetates (from neutral sugar constituents only) were examined by glc-ms (Table III). A sample of fraction F2 was converted into the carboxyl-reduced polysaccharide by the procedure of Taylor and Conrad (9). Methylation analysis of the carboxyl-reduced polysaccharide was performed in the usual way (Table III).

Preparation of Apple CWM and AIR and Extraction of Pectin Fractions. Apples ('Granny Smith' variety) were cored and peeled and the resulting pulp (2 kg batches) was stored in ethanol suspension to minimize enzymic oxidation. Separate batches of pulp were processed, as described for carrot preparations, to furnish apple CWM (32.5 g) and apple AIR (39.8 g) for which analytical data are recorded in Table VI. Polysaccharide fractions B (4 g) and C (2.5 g) were extracted from CWM with water at 80° and aqueous 1% ammonium oxalate at 80°. Likewise polysaccharide fractions A (2.1 g), B (5.2 g), and C (4.5 g) were isolated from AIR by extraction with water at room temperature, 80°, and with aqueous 1% ammonium oxalate respectively.

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# Legumes and Their Soluble Fiber: Effect on Cholesterol-Rich Lipoproteins

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Water-soluble plant fibers and soluble-fiber rich foods have distinct hypocholesterolemic effects in man and experimental animals. We evaluated the hypocholesterolemic effects of dried beans, rich in soluble fiber, for ten hypercholesterolemic men on a metabolic ward. They were fed a control diet for seven days followed by a bean-supplemented diet for 21 days. The bean-supplemented diet provided 115 g/d (dry weight) of dried beans but otherwise resembled the control diet. Control and bean-supplemented diets had similar energy, carbohydrate, protein, fat and cholesterol contents but the bean-supplemented diet had more soluble and total plant fiber. Average serum total cholesterol concentrations were 19 per cent lower on bean-supplemented than on control diets. Bean supplementation lowered low-density lipoprotein cholesterol more than high-density lipoprotein cholesterol concentrations resulting in an increase in the high-density to low-density ratios. Soluble-fiber rich foods may have an important role in the long-term management of patients with hypercholesterolemia.

High-fiber foods or food products have valuable health promoting effects. Intake of selected high-fiber items can improve diabetic control (1), lower blood lipid concentrations (2,3) and perhaps reduce the risk of heart attack. Plant fibers, defined as the polysaccharide and lignin components of plant foods which are not digested in the human small intestine, affect the physiology and influence the metabolism of various nutrients. For example, certain plant fibers have substantial hypocholesterolemic effects. Selected water-soluble fibers such as pectin, guar and oat gum have unique cholesterol-lowering properties. We will review the effects of selected plant fibers or fiber-rich foods on serum cholesterol concentrations. We also

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will provide a preliminary report on the metabolic effects of bean-supplemented diets for hypercholesterolemic men. Current information indicates that fiber-rich foods have notable therapeutic potential for treating certain metabolic conditions and may prevent the development of certain diseases. However, since the available information is largely descriptive in nature, we have little understanding of the mechanisms underlying the important metabolic effects of plant fibers. Further work is required to delineate the mechanisms for plant fibers and to determine the practical role of fiber-rich foods in the management or prevention of certain human disorders.

Hypocholesterolemic Effects of Plant Fibers-Rat Studies. In Table I we compare the cholesterol-lowering effects of selected fibers used in experimental studies with rats. Since many different experimental designs were used, this collation provides only general guidelines to the effects of different fiber sources.

TABLE I. EFFECTS OF PLANT FIBER SUPPLEMENTS ON SERUM CHOLESTEROL CONCENTRATIONS OF RATS.[1]

| Ingredient | No. of Studies | Cholesterol % of control |
|---|---|---|
| Cellulose | 9 | 96 |
| Wheat bran | 6 | 100 |
| Oat Bran | 2 | 81 |
| Oat gum | 1 | 59 |
| Pectin | 13 | 80 |
| Guar | 3 | 81 |
| Bengal gram | 2 | 72 |

1. Data from Table I (4) except for oat bran (7) and oat gum (8).

Laboratories have consistently reported that neither purified cellulose nor wheat bran have significant cholesterol-lowering effects (4). From these observations we conclude that the water-insoluble types of polysaccharides have minimal cholesterol-lowering effects. In sharp contrast, purified water-soluble fibers such as pectin and guar have distinct hypocholesterolemic effects. The cholesterol-lowering properties of water-soluble fibers are shared by fibers from quite diverse sources. Pectins are cell wall constituent of many plants and usually are extracted from citrus products. Oat gum is a gum extracted from oat bran while guar is a storage polysaccharide extracted from the Indian cluster bean.

In addition, these three soluble fibers range in chemical structures ranging from the beta glucan of oat gum and the galactamannan of guar to galactouronic acid, the major component of pectins. Preliminary studies suggest that the physiologic properties of fibers can be correlated with their cholesterol-lowering properties since the viscosity and the

extent of methoxylation of pectin influences the hypocholesterolemic effect (5).

Hypocholesterolemic Effects of Plant Fibers-Human Studies. Most of the purified fibers and fiber-rich foods that have been tested in animals also have been used for human studies (Table II). These human studies yield similar conclusions.

TABLE II. EFFECT OF PLANT FIBER SUPPLEMENTS ON SERUM CHOLESTEROL CONCENTRATIONS OF HUMANS[1]

| Source of Fiber | No. of Studies | Plant Fiber, g/d | Serum Cholesterol % of control |
|---|---|---|---|
| Cellulose | 3 | 16 | 100 |
| Wheat bran | 14 | 17 | 101 |
| Whole oats | 1 | 15 | 89 |
| Oat bran | 2 | 27 | 83 |
| Pectin | 10 | 25 | 87 |
| Guar | 3 | 24 | 84 |
| Bengal gram | 1 | Unknown | 78 |
| Beans | 1 | 30 | 81 |

1. Data from Table II (4) except for oat bran (3) and beans (current study).

Although the experimental designs varied, certain generalizations seem apparent. Cellulose and wheat bran do not have discernible cholesterol-lowering properties while oat products and legumes which are rich in water-soluble fibers and the purified soluble pectins have distinct hypocholesterolemic effects. The purified pectin and guar preparations have practical limitations because these materials develop a gummy, glue-like material when hydrated and must be ingested rapidly to avoid nausea and vomiting. Guar, however, has been successfully incorporated into a crispbread preparation which is reasonably palatable (6). Oat bran can be eaten as a hot cereal or incorporated into a tasty muffin. As we will describe later, moderate quantities of beans can be served without difficulties for most individuals.

## METHODS

Overview of Study. We designed this study to determine if supplementing the diet of hypercholesterolemic men with 115 g (dry weight) of beans/day was associated with significant changes in blood lipid concentrations. On a metabolic ward we fed a control diet for seven days to obtain baseline measurements and then fed a bean-supplemented diet for 21 days. For each patient the control and bean diets provided identical quantities of carbohydrate, protein, fat and cholesterol. Thus

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these two diets were designed to provide identical intakes of energy and nutrients and differed only in the amounts of soluble and insoluble plant fiber.

Patient Selection. We identified men whose serum total cholesterol concentration had exceeded 260 mg/dl on at least two previous measurements. We did not include patients with secondary causes of hypercholesterolemia (such as hypothyroidism or renal disease) or with serum triglyceride concentrations exceeding 500 mg/dl. None had received lipid-lowering drugs in the last six months. Thus we invited patients with type IIa or IIb hyperlipoproteinemia to participate in this study. We obtained informed consent from each patient in accordance with the Human Investigations Subcommittee of the University of Kentucky. After admission to the metabolic ward, patients were randomly allocated to the bean-diet study or the oat-bran study. All patients enrolled completed the study. To avoid bias in selection, the principal investigator did not learn of the diet allocation until after admission to the hospital. We will summarize the response of ten consecutive patients to the bean diet since the response to the oat bran diet will be reported elsewhere.

Diets. The control and bean diets were composed of commonly available foods (Table III) and were computed to provide identical amounts of energy, carbohydrate, protein, fat and cholesterol (Table IV).

The bean diet provided 115 g of dried beans/day served in soups

TABLE III. FOODS SERVED ON REPRESENTATIVE DAYS

| CONTROL DIET | BEAN DIET |
|---|---|
| Milk 480 g | Milk 400 g |
| Eggs 38 g | Eggs 38 g |
| Butter 8 g | Butter 20 g |
| | Margarine 8 g |
| Ham 95 g | Turkey 48 g |
| Ground beef 100 g | Ground beef 48 g |
| White bread 85 g | White bread 28 g |
| Wheat flake cereal 30 g | Graham crackers 17 g |
| Sweet potatoes 110 g | Potatoes 200 g |
| Macaroni 92 g | Cabbage 105 g |
| Green beans 100 g | Kale 90 g |
| Brussel Sprouts 100 g | Tomatoes 110 g |
| Peaches 100 g | Peaches 112 g |
| Apricots 130 g | Orange 220 g |
| Apple juice 100 g | Orange juice 110 g |
| | Navy beans 50 g (dry) |
| | Pinto beans 65 g (dry) |

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and as cooked beans. To incorporate beans into the diet we had to reduce the intake of beef and pork from approximately 200 to 100 g/day and increase the intake of butter and margarine to replace the fat content of these meats. The average control diet provided approximately 20 grams of total plant fiber and 5 grams of soluble fiber/day whereas the average bean diet provided approximately 50 grams of total and 20 grams of soluble fiber/day. Beans provided approximately 30 grams of total and 13 grams of soluble fiber/day. The nutrient and fiber content of each diet was measured, the actual food consumption was measured and the energy, nutrient and fiber intake was computed as previously described (1-3).

TABLE IV. COMPOSITION OF FOOD SERVED TO REPRESENTATIVE PATIENT

| | Control | Bean Diet |
|---|---|---|
| Kcalorie | 1715 | 1711 |
| Carbohydrate, total (g) | 182.1 | 183.4 |
| Simple | 91.1 | 91.3 |
| Complex | 90.8 | 91.9 |
| Protein (g) | 85.2 | 85.8 |
| Fat, total (g) | 70.3 | 70.5 |
| Saturated | 33.2 | 30.6 |
| Monounsaturated | 28.7 | 26.6 |
| Polyunsaturated | 6.6 | 10.6 |
| Cholesterol (mg) | 447.4 | 449.0 |
| Plant fiber, total (g) | 19.8 | 49.1 |
| Soluble | 4.5 | 19.4 |
| Insoluble | 15.3 | 29.7 |

Measurements. We weighed patients daily. Each morning we collected blood after a 10-hr fast and measured the serum total cholesterol, triglycerides and glucose. On the last three days of each diet we measured serum high density lipoprotein (HDL) cholesterol concentrations and calculated low density lipoprotein (LDL) cholesterol values. We collected all stools and measured frequency, wet and dry weights. Selected stool samples will be analyzed later for neutral steroids, cholesterol and bile acids. We used the paired T-test for statistical comparisons.

## RESULTS

Serum Cholesterol Concentrations. Bean-supplemented diets effectively lowered serum cholesterol concentrations by an average of 19%. Since the low density lipoproteins (LDL) are considered to be atherogenic and the high density lipoproteins (HDL) may protect against the development of atherosclerosis, we were interested in the effects of bean diets on these

lipoproteins. Average serum total cholesterol concentrations decreased from 299 mg/dl on the control diet to 242 mg/dl on the bean diet (Table V). Most of this reduction was due to the reduction in LDL cholesterol concentrations which decreased in each individual; average values dropped from 219 to 169 mg/dl, a 23% reduction. Average serum HDl cholesterol concentrations also decreased on the bean diet although the 15% reduction was smaller than observed for LDL cholesterol concentrations. The ratio of HDL:LDL cholesterol concentrations, considered to be an indicator of the atherogenic potential of serum, changed favorably with a slight, but insignificant, increase form 0.15 on the control to 0.17 on the bean diet.

TABLE V. RESPONSE OF FASTING SERUM CHOLESTEROL, TRIGLYCERIDES AND PLASMA GLUCOSE CONCENTRATIONS TO BEAN DIET. VALUES FOR 10 MEN

| | Control Diet | Bean Diet | % Change |
|---|---|---|---|
| Serum cholesterol, mg/dl | | | |
| Total | 299±14 | 242±12 | -19 |
| LDL | 219±15 | 169±11 | -23 |
| HDL | 33±3 | 28±2 | -15 |
| Serum triglyceride, mg/dl | 238±31 | 221±38 | -7 |
| Plasma glucose, mg/dl | 98±4 | 95±3 | -3 |

Four patients had type IIa hyperlipoproteinemia characterized by an elevation of serum cholesterol concentrations with normal serum triglyceride concentrations; this represents a selective elevation of the LDL. These patients with hypercholesterolemia alone responded better to the bean diet than did six other patients. These six patients had type IIb hyperlipoproteinemia characterized by an elevation of both serum cholesterol and triglyceride concentrations reflecting abnormally high LDL and very low density lipoproteins (VLDL) concentrations. Thus the four patients with type IIa hyperlipoproteinemia had a 24% reduction in serum total cholesterol concentration with values decreasing from 314 to 239 mg/dl; their LDL cholesterol concentrations decreased by 27%. The six patients with type IIb hyperlipoproteinemia had a 16% decrease in serum total and a 19% decrease in LDL cholesterol concentrations. We will need to study more patients to determine if these different responses are consistent and have important therapeutic implications.

Serum Triglyceride Concentrations. The bean diet did not have a significant effect on serum triglyceride concentrations. While average serum triglyceride concentrations decreased by 7%, values decreased in seven patients and increased in three patients. This confirms our earlier experience with oat bran

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(3) and indicates that diets which are selectively enriched with soluble plant fibers are not effective in lowering serum triglyceride concentrations. High fiber diets which provide a mixture of insoluble and soluble fibers from a variety of different foods, however, are effective in lowering serum triglyceride concentrations (2).

Serum Glucose Concentrations. Bean diets did not have a significant influence of fasting serum glucose concentrations; values decreased in seven men and increased in three men. However, all three men who had average serum glucose concentrations above 100 mg/dl (suggesting a diathesis to diabetes) on the control diet, showed a reduction with the bean diet. Previous studies (4) have indicated that soluble fiber supplements have a greater impact on postprandial plasma glucose concentrations than on fasting glucose concentrations. In these studies we did not measure postprandial glucose concentrations or do glucose tolerance tests; these studies may have demonstrated an effect of bean-supplemented diets on glucose metabolism.

Body Weights. Over a four week period, average weights decreased from 76.4 ± 4.2 kg (mean ± SEM) on control diets to 75.3 ± 4.4 kg on the bean diet. This reduction in body weight is largely atrributable to the fact that most of these patients were overweight and wanted to lose weight in the hospital. Although we strongly encouraged patients to eat all of the food that was served, most did not consistently eat everything that was served. Thus their caloric intake was lower than anticipated. Careful analysis of their intake indicates that there was a proportional decrease in energy, nutrients and fiber without selective changes in any one item. Changes in body weight, however, were not correlated with changes in serum cholesterol concentrations. Using a tertile analysis, we observed for three patients who lost the most weight that average reductions in serum cholesterol concentrations were identical to average reductions for three patients who lost the least amount of weight.

Other Effects. We carefully monitored these inpatients daily for side effects related to the increase in total plant fiber intake from an average of 20 to 50 grams/day. Only two patients reported gastrointestinal side effects with bean diets. Both noted abdominal distention and discomfort during the first ten days on the bean diet. These side effects were tolerable and neither patients requested that we modify or discontinue the bean diets; the diet was well tolerated by one patient during the last week of observation and was reasonably well tolerated by the other patient during the last week. With direct questioning we learned that all patients has more gas production with the bean diet. They had more flatulence and

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more eructations (belching). None of these patients developed diarrhea or other side effects.

## DISCUSSION

Our current studies confirm previous reports (4) that supplementing the diet with soluble fibers or fiber-rich foods significantly lowers serum cholesterol concentrations in man. We documented that supplementing the diet with 115 g of dried beans lowered serum total cholesterol concentrations by an average of 19%. Bean supplements were reasonably well tolerated and appear to offer promise as a practical measure for the long-term management of hypercholesterolemia. While bean-supplemented diets were as effective as oat-bran supplemented diets in lowering serum cholesterol concentrations, oat bran was better tolerated by our patients. Nevertheless, because beans are widely available and are a commonly used food, they offer promise as an adjunct in the long-term management of patients with hypercholesterolemia. Based on reported side effects associated with guar and pectin supplements and on discussions with investigators who have used these purified fibers, we feel that bean supplements are better tolerated and associated with fewer side effects than are these purified fibers.

Earlier we reported (3) that supplementing the diet with 100 grams of oat bran/day selectively lowered LDL cholesterol concentrations and did not alter HDL cholesterol concentrations of hypercholesterolemic men. In this study, we compared the effects of oat bran with that of beans using the same protocol; hypercholesterolemic patients were randomly allocated to either bean or oat-bran supplemented diets. We have not completed our analysis of the oat-bran branch of the study. However, preliminary observations suggests that beans and oat bran have similar effects on LDL and HDL cholesterol concentration over a 21-day period of study. Our current studies do indicate that bean supplemented diets favorably influence the ratio of HDL to LDL cholesterol concentrations and thus reduce the theoretical diathesis to atherosclerosis. Currently we are examining the long-term effects of diets supplemented with either beans or oat bran to assess this question in a more practical fashion.

The lack of an effect of bean-supplemented diets on serum triglyceride concentrations is consistent with our previous experience and that of others (4). While a mixture of soluble and insoluble fibers from a wide variety of foods has a significant triglyceride-lowering effect, (2) we have not identified specific types of fibers or fiber-rich foods which are effective in lowering serum triglyceride concentrations in humans or experimental animals. Nevertheless, we have documented that weight-maintaining, high-fiber diets dramatically lower serum triglyceride concentrations of hyperetriglyceridemic patients. When we treated ten patients

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with serum triglyceride concentrations exceeding 1000 mg/dl (six-fold higher than normal) with high-fiber diets, we observed an 80% reduction in serum triglyceride concentrations while these patients were hospitalized. With longer term follow-up we have noted that high-fiber diets are accompanied by an even further reduction in serum triglyceride concentrations which now average only 10% of values initially observed (Anderson, J.W. unpublished observations). Further studies are required to determine the type of fibers or fiber-rich foods which act to lower serum triglyceride concentrations. The mechanisms responsible for the reduction in serum triglyceride concentrations associated with high-fiber diets have not been determined.

The influence of high fiber diets on glucose metabolism has been reviewed in detail elsewhere (4). Our studies of these patients with a diabetic diathesis suggest that bean-supplemented diets improve glucose metabolism but we did not rigorously examine this question in this study. In a previous study where we fed similar amounts of soluble fiber from an oat bran source, we observed a significant improvement in oral glucose tolerance tests and a significant reduction in average insulin concentrations in response to oral glucose administration (unpublished observations, see Ref 10). Other workers have documented that supplementing the diet with soluble fibers improves the disposal of glucose after meals.

The mechanisms responsible for the cholesterol-lowering effects of soluble fibers are reviewed elsewhere (4). Our studies and those of others suggest that two mechanisms may play a major role in the cholesterol-lowering effects of soluble-fiber supplemented diets. First, these diets are accompanied by significant increases in fecal bile acid excretion (3). This may divert cholesterol which is synthesized in the liver from cholesterol-containing lipoprotein particles into the synthesis of bile acids. Furthermore, we have documented that biliary bile acid excretion is significantly higher in oat bran treated rats than in control rats (11). Second, short chain fatty acid metabolites resulting from the fermentation of plant fiber in the colon may influence cholesterol synthesis. Soluble fibers are almost completely hydrolyzed to short chain fatty acids such as acetate, butyrate and propionate in addition to other products in the right side of the colon. Our preliminary studies indicate that these short chain fatty acids in physiologic concentration are able to significantly inhibit hepatic cholesterol synthesis utilizing rat hepatocytes preparations (12). These studies suggest that plant fibers not only deplete the body of bile acids, but they also act to attenuate hepatic cholesterol synthesis. Further studies are required to evaluate the physiologic importance of these observations and to delineate other mechanisms by which plant fibers lower serum cholesterol concentrations.

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## CONCLUDING COMMENTS

Fiber-rich foods offer many health-promoting benefits for humans. High fiber diets lower insulin requirements of diabetic individuals, lower serum cholesterol concentrations of hypercholesterolemic individuals, dramatically lower fasting serum triglyceride concentrations of patients with severe hypertriglyceridemia, offer therapeutic advantages for obese patients because of their high satiety value (9), and alleviate symptoms of hypoglycemia of patients with reactive hypoglycemia (13). High-fiber or fiber-supplemented diets have the potential to lower the risk for atherosclerosis or even reverse atherosclerosis in humans and reduce the risk for certain types of cancer.

The medicinal value or therapeutic potential of high-fiber food supplements has not been examined carefully. Studies of the cholesterol-lowering properties of high-fiber foods have provided the strongest evidence that selected foods may have potential therapeutic effects. Additional studies in diabetic patients have documented the therapeutic effects of fiber supplements. Our studies with oat bran indicate that high fiber foods can effectively and safely lower serum cholesterol concentrations of hypercholesterolemic men. Our current studies document that the intake of 115 g of dried beans daily lowers average serum cholesterol concentrations by 19%. These results clearly demonstrate the need to examine the effectiveness, palatability, practicality and safety of other high-fiber or fiber-supplemented food items for a variety of metabolic derangements in humans.

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# Purified Psyllium Seed Fiber, Human Gastrointestinal Tract Function, and Nutritional Status of Humans

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Because of its high hemicellulose content, purified psyllium seed fiber has excellent hydrophilic qualities which account for its effectiveness as a bulking-type laxative. In spite of this long usage, purified psyllium seed fiber has not been popularly suggested as a source of dietary fiber in the supplementation of food products. In a series of studies conducted in this laboratory, purified psyllium seed fiber was added as a fiber supplement to bread and to peanut butter. Products were fed to human subjects as part of a mixed food diet in which the psyllium seed provided the major source of dietary fiber. Different studies were designed to compare effects of psyllium seed fiber with other purified and food fiber sources. Results indicated that while positive effects associated with increased feeding of fiber tended to be greater with psyllium than with other test fibers employed, the negative effects associated with decreased protein and mineral utilization also tended to be greater.

Recent concern for increasing dietary fiber intake of Americans has generated interest in a multitude of plant products which theoretically might be of use as fiber supplements to foods destined for consumption by humans. Often suggestions for the use of these products seem to be promted on the basis of economical criteria rather than by using desired human physiological response as a justification. For example, many currently-used or projected, fiber-rich ingredients are by-products from established industries; hence, are readily available at relatively low cost. Since all forms of dietary fiber do not elicit similar physiological effects in the human, this approach may lead to false expectations on the part of both the food processor and the consumer when the fiber enriched product is actually produced, sold and used.

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Ground psyllium seed husks have been fed to humans for generations as a pharmaceutical to increase stool bulk and to achieve and/or maintain gastrointestinal tract motility. Some of the many bulk laxative products sold in the United States which are based on psyllium seed fiber for their functions effects include the following: Mucilose Flakes, Effersyllium, Mutamucil, Konsyl, L.A. Formula, Syllact, and Syllamalt (1). Sometimes these products are mixed with sweetening agents or flavorings to increase palatability. Warnings to drink the products suspended in water or with water are also included to avoid intestinal blockage problems. While it may not be part of the popularly accepted image of dietary fiber, the desired physiological effect of fiber from laxatives or less directly from food resources is the same.

Psyllium is a member of the genus plantage. Psyllium or more correctly, Plantago psyllium, is also referred to as fleawort. It has been cultivated for many years in Spain and France expressively for its mucilaginous seed coatings for use as a laxative. Its laxative properties are associated with its high content of various hemicelluloses. Hemicelluloses in comparison to the other major fiber classes are notable for their hydrophilic and, hence, bulking properties.

At the University of Nebraska, Department of Human Nutrition and Food Service Management, a commercial psyllium seed fiber laxative product, Mucilose Flakes (Winthrop Laboratories, Sterling Drug Co.) has been used for more than 30 years as a fiber source for human subjects maintained on synthetic and semi-synthetic research diets. The usual carrying agents used in these studies were starch breads and starch cookies. Several years ago when this laboratory first became interested in effects of dietary fiber on gastrointestinal tract function and on nutrient utilization, it appeared rational to continue to use the same fiber source in the initial studies and as comparative fiber to other fiber sources in later studies. The purpose of this paper will be to review some of the results of these studies (2-20). In order to simplify presentation, results of several studies involving a total of 85 adult subjects have been grouped together. Not all subjects received all experimental treatments; however, the same basic control diet was used in all studies. This basal diet was based on ordinary foods and included milk, corn flakes, rice, noodles, ground beef, tunafish, peanut butter, peaches, pears, jelly and sugar. Fiber supplementation levels were moderately high ranging from 14 to 20 g/subject/day. Bread or peanut butter were used as the carrying agent. Data from all studies were pooled. Statistical analyses (Biometrics Laboratory, University of Nebraska) included Analysis of Variance and Least Square Means test.

## Effect on Gastrointestinal Tract Function and Fecal Bulk

Psyllium fiber is composed of several hemicelluloses, which are complex polymers of pentosans, hexosans and galactans. Hemicelluloses are noted for their hydrophilic properties which make them excellent bulking agents. Many studies have been conducted seeking to define relationships among fiber intake, fecal bulk, and fecal transit time times. Results of these studies have been confusing in that different fibers seem to elicit different degrees of response (21-25). Furthermore, individual subject response often is tremendously variable. For example, increased dietary fiber has been found to be useful in relieving symptoms of both diarrhea by decreasing stool bulk and increasing fecal time as well as constipation by increasing stool bulk and decreasing fecal time. Therefore, mean values sometimes involve individual values changing not only to different degrees but actually in opposite directions depending upon the subjects involved.

In the current project, fecal transit times were measured by calculating time required for colored glass bead markers to pass through the gastrointestinal tract (solid phase markers). Fecal transit times of subjects were not significantly affected due to the feeding of dietary fiber in comparison to the fecal transit times when no fiber supplements were given with the laboratory controlled diet. However, numerically, the feeding of psyllium resulted in the shortest fecal transit time. All subjects in this study were assumed to have normal gastrointestinal tract function so it is perhaps not surprising that fecal transit times were not significantly affected as a result of feeding the fiber supplements (Table I).

Feces of subjects were divided into period lots by use of fecal dye markers. Fecal wet weights were significantly affected as a result of feeding the fiber supplements (Table I). When psyllium was the fiber supplement used, fecal wet weights were double those when no fiber supplement was given. These values were numerically higher than those obtained when other test fiber sources were used. Fecal excretion on a dry weight basis were also increased when psyllium was used as a fiber source in comparison to when no fiber supplements were employed; however, these values were not as high as those obtained when such fiber supplements as cellulose or corn bran were employed suggesting that water bulking was the primary cause of increased fecal wet weight when psyllium was fed.

## Effect on Proximal Composition of Feces

Feeding of psyllium fiber resulted in a significant increase in percentage of water contained in feces during this project in comparison to values obtained when the laboratory controlled diet was fed without fiber supplementation or when other sources

Table I

Effect of Psyllium and Other Fiber Sources on Various Parameters of Gastrointestinal Tract Activity, Fece Composition, and Blood Lipid Patterns of Humans

| Parameter | Fiber source | | | | | | |
|---|---|---|---|---|---|---|---|
| | Psyllium | Cellulose | Pectin | Wheat Bran | Rice Bran | Corn Bran | None |
| Fecal transit time (hr) | 36.1 | 39.1 | 39.7 | 40.8 | 41.6 | 45.2 | 45.3 |
| Fecal weight-wet (g/day) | 154.9[c] | 114.1[bc] | 96.6[b] | 105.5[b] | 99.2[b] | 96.0[b] | 69.8[a] |
| Fecal weight-dry (g/day) | 30.5[b] | 34.8[b] | 25.2[ab] | 29.0[ab] | 30.4[b] | 37.2[b] | 20.3[a] |
| Fecal water (%) | 80.3[c] | 69.5[b] | 73.9[b] | 72.5[b] | 69.2[b] | 61.2[a] | 70.9[b] |
| Fecal fat (%) | 2.03[c] | 1.62[b] | 1.42[ab] | 1.97[c] | 2.04[c] | 1.74[b] | 1.30[a] |
| Fecal protein[1,2] (g/day) | 18.8[c] | 15.7[b] | 15.9[b] | 18.7[c] | 17.8[bc] | 20.0[c] | 10.7[a] |
| Fecal cellulose (g/day) | 4.0[ab] | 12.6[c] | 2.8[a] | 3.4[a] | 3.3[a] | 4.7[b] | 2.2[a] |
| Fecal hemicellulose (g/day) | 7.5[b] | 3.8[a] | 3.1[a] | 9.0[bc] | 5.4[a] | 11.5[c] | 2.5[a] |
| Fecal lignin (g/day) | 0.86[ab] | 0.95[b] | 0.68[a] | 0.69[a] | 1.02[b] | 0.98[b] | 0.60[a] |
| Fecal copper[3] (mg/day) | 5.04[b] | 4.07[a] | 3.91[a] | 5.81[b] | 5.55[b] | 5.07[b] | 3.78[a] |
| Fecal zinc[4] (mg/day) | 13.32[b] | 10.26[a] | 9.63[a] | 12.13[b] | 11.13[ab] | 10.03[a] | 9.64[a] |
| Fecal magnesium[5] (mg/day) | 379[b] | 264[a] | 278[a] | 450[c] | 375[b] | 403[bc] | 252[a] |
| Fecal calcium[6] (mg/day) | 899[b] | 873[b] | 752[a] | 910[b] | 827[ab] | 799[a] | 796[a] |
| Blood serum cholesterol (mg/dl) | 158[a] | 168[b] | 172[b] | 173[b] | 172[b] | 179[b] | 172[b] |
| Blood serum triglyceride (mg/dl) | 91[a] | 105[b] | 105[b] | 102[b] | 122[c] | 117[c] | 103[b] |

Table I (cont)

| Parameter | Fiber source | | | | | | |
|---|---|---|---|---|---|---|---|
| | Psyllium | Cellulose | Pectin | Wheat Bran | Rice Bran | Corn Bran | None |
| Blood serum phospholipid (mg/dl) | 210[a] | 209[a] | 202[a] | 195[a] | 189[a] | 205[a] | 220[a] |

[1]Protein calculated by multiplying nitrogen x 6.25. Since not all fecal nitrogen is from protein, these values are probably over-estimations.

[2]Protein intake was 65 g/subject/day during all periods except when bran supplements were used. Amount of additional protein/subject/day during these periods were as follows: wheat bran, 2.01; corn bran, 2.93; and rice bran, 2.62.

[3]Copper intake was 4 mg during all periods except when bran supplements were used. Amounts of additional copper/subject/day during these periods were as follows: wheat bran, 0.053; corn bran, 0.007; and rice bran, 0.008.

[4]Zinc intake was 10 mg/subject/day during all periods except when bran supplements were used. Amounts of additional zinc/subject/day during these periods were as follows: wheat bran, 0.270; corn bran, 0.066; and rice bran, 0.213.

[5]Magnesium intake was 1450 mg/subject/day during all periods except when bran supplements were used. Amounts of additional magnesium during these periods were as follows: wheat bran, 21.28; corn bran, 30.32; and rice bran, 42.13.

[6]Calcium intake was 1450 mg/subject/day during all periods except when bran supplements were used. Amounts of additional calcium/subject/day during these periods were as follows: wheat bran, 25.32; corn bran, 20.15; and rice bran, 12.12.

Values with different letter superscripts are significantly different from one another ($P < 0.05$).

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of fiber were fed. In spite of the high water content, stools were well-formed during periods when the psyllium fiber was used. This attests to the water holding capacity of this fiber source.

Fecal fat loss (Goldfisch method) was also increased when psyllium fiber was fed in comparison to losses when no fiber supplements were employed (Table I). However, these losses were no greater than when several of the other test fiber supplements were used. Loss of fat in feces may be considered either an advantage or a disadvantage depending upon whether problems of obesity and atherosclerosis are being considered or whether malabsorption syndromes are the topic in question.

Fecal nitrogen losses (Kjeldahl method) were significantly increased as a result of feeding any of the test fiber sources in this project (Table I). Psyllium feeding resulted in higher fecal nitrogen losses than did the feeding of the other two "purified" fiber sources, cellulose and pectin, but no higher losses than the feeding of the three test brans. Nitrogen values have been converted to protein (N x 6.25) in this project. Since all fecal nitrogen is not protein nitrogen, these values are undoubtedly falsely high but can be used for comparative purposes.

## Effect on Fecal Fiber Composition

Since psyllium fiber is composed of several hemicelluloses, it might be supposed that feces produced when high levels of psyllium are fed would reflect this high content of hemicelluloses. Similarly, it might be expected that feces produced when other sources of dietary fiber are fed would reflect the fiber composition of the fiber source. A complexing factor to this simplistic approach is that the feeding of dietary fiber may result in changes in lower gut microflora and that microflora residue also will influence fecal fiber composition. Furthermore, about half of the hemicellulose is known to undergo degradation in the lower gut. In addition, chemical methodology for classification of fiber classes is rather inexact when applied to the clinical laboratory situation. Methods of Goering and Van Soest were used in the present study.

In the current project, the cellulose fraction in feces was increased only when the cellulose supplement was given. Numerically, the feeding of psyllium resulted in some increase in this fraction but not at the statistically significant level. Hemicellulose content of feces was significantly increased as a result of feeding psyllium; however, these values were not as high as those achieved when wheat bran or corn bran was fed. Corn and wheat bran contain considerable amounts of hemicellulose but not at the high levels found in psyllium. Furthermore, the water-holding capacity of these two fiber sources were not as high as those for psyllium. Hence, it would appear that fecal residual fiber is perhaps not a good index of functionality of

the fiber. Fecal lignin levels (Table I) were increased as a result of fiber feeding but these were not statistically different from one another.

## Effect on Mineral Utilization

Considerable investigation has been reported on the effects of increasing dietary fiber on utilization of various minerals. Fiber supplementation may dilute the concentration of minerals by increasing fecal bulk, may lessen the absorption by decreasing fecal transit time, may encourage absorption of minerals upon fiber residues or trap minerals within residues, and/or may complex with phytate and other inhibitors of mineral utilization. However, results of studies investigating utilization of different minerals under conditions of additions of different levels and sources of fiber have in no way been conclusive (26-29).

In the present project, fecal copper losses were significantly increased when psyllium fiber was used as a fiber source in comparison to levels when no fiber supplements were given (Table I). These losses were significantly greater than when the cellulose and pectin supplements were given but not greater than when the bran supplements were used. Fecal zinc loss was also significantly increased when psyllium supplements were used as well as losses of calcium and magnesium. It is important to remember, however, that increased fecal mineral losses are of nutritional significance only if the subjects have marginal or inadequate intakes of the minerals in question. When diets supply adequate amounts of these nutrients, this negative effect is of no physiological importance.

## Effect on Blood Serum Lipid Patterns

One of the proposed advantages of inclusion of dietary fiber in foods and eating patterns is the decrease in blood serum lipids which may occur since increased circulating blood serum lipids (lipidemias) have been identified as risk factors for development of atherosclerosis and coronary heart disease. It is thought that not only will dietary fiber inhibit the absorption of dietary fat by dilution of fat in the chyme and/or through absorption of fat in the fiber itself but will also inhibit the absorption of bile salts and thus lower the absorption of endogenous cholesterol. While survey studies indicate that vegetarians and other groups customarily consuming high fiber diets have low levels of circulating blood lipids and a lower incidence of atherosclerosis and coronary heart disease than do people consuming typical American or westernized diets, it is difficult to separate the variables of fiber from those of total dietary fat, dietary cholesterol, unsaturated to saturated fatty acid ratio, and sucrose intake, all of which are known to affect circulating blood lipids (30-34). In laboratory controlled studies with

humans, these effects of dietary fiber on circulating blood lipids have not been consistently demonstrated (35-40).

In the present series of studies, blood serum lipids were determined by a commercial laboratory (Stat Laboratory, Wichita, Kansas). Blood cholesterol levels of subjects were lower when subjects received the experimental diets than when subjects consumed their self-selected diets. The mean cholesterol level of subjects while on the self-selected diets was 193 mg/dl and while receiving the controlled diet alone or with psyllium, cellulose, pectin, wheat bran, rice bran or corn bran were 172, 158, 168, 172, 173 and 179 mg/dl, respectively. The general lowering of blood cholesterol levels due to feeding of the experimental diet was probably due to its low fat (30% of energy) and low cholesterol (less than 100 mg/day) levels. Psyllium fiber feeding resulted in significantly lower blood serum cholesterol levels than did the feeding of other test dietary fibers or than the feeding of the basal diet without fiber supplementation.

Blood serum triglyceride levels were also depressed in the current project in comparison to normal mean values of subjects on self-selected diets (133 mg/dl) as a result of feeding the laboratory controlled diet whether or not fiber supplements were included. However, as shown on Table I, psyllium feeding resulted in a statistically significant further decrease in blood serum triglyceride levels in comparison to the other test sources of fiber or when no test fiber was given. Blood serum phospholipid levels were also measured; however, no statistically significant changes in this serum lipid fraction were observed either as a result of feeding the experimental control diet or as a result of fiber supplementation of this diet.

## Conclusion

Fiber derived from psyllium seed has a long history of use as a pharmaceutical to produce laxation. It's high bulking properties are due to its high content of hemicellulose. When incorporated into food products and fed to humans, it produces the desirable effects of increased stool bulk, decreased fecal transit time, and decreased blood serum cholesterol and triglyceride levels. However, concurrently psyllium fiber supplementation results in some depression in absorption of selected minerals, fat and protein. This was also true of some but not all of the other fiber sources tested.

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# Locust Bean Gum in Food Products Fed to Familial Hypercholesterolemic Families and a Type $II_B$ Patient

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Familial hypercholesterolemia (FHC) is associated with severe premature coronary artery disease and death and is resistant to conventional dietary treatment. Locust bean gum (LBG) incorporated into food products was fed to adults and children with FHC. Twenty-eight patients, 18 adults and 10 children were divided into two groups (Group A and Group B), and using a cross-over design were fed identical food products with and without LBG (8-30 gm/d) as outpatients in four-week periods. Three additional adults with different lipid abnormalities were studied using the same protocol. Diet records, food product consumption, lipids, and lipoproteins were analyzed every two weeks. Total cholesterol (C) was lowered 11% (275 to 246 mg%) in Group A at four weeks ($p < .001$) and 17% (255 to 215 mg%) in Group B at eight weeks of LBG diet ($p < .001$). Low density lipoprotein cholesterol (LDL) fell 10% at four weeks in Group A ($p < .001$) and 17% at eight weeks in Group B ($p < .001$). HDL/LDL ratios increased in both groups. Two patients with FHC, one with a partial ileal bypass and one taking lipid-lowering medications, experienced no further lowering of serum lipids or lipoproteins. A patient with elevations in cholesterol and triglycerides (Type $II_B$) had a 22% drop in both. LBG food acceptance was good. There were no significant side effects of the diet. LBG was more effective than some pharmacological agents in lowering lipids, and in outpatients over a two-month period appears to be one of the most effective dietary treatments of FHC. LBG in food products appears to be a safe, effective means of lowering serum lipids in normal and hypercholesterolemic adults and children with FHC.

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FHC patients are often resistant to standard dietary management of their hyperlipidemia. Pharmacological treatment in children who will require long-term management involves problems with safety, expense, effectiveness, and may not be acceptable to the child or the parents. For these reasons, drugs are seldom used in the management of hyperlidemic children.[1] Atherosclerosis has is origins in children and potentially may be moderated by lowering serum cholesterol and LDL cholesterol. The prevention of atherosclerosis should begin in children with FHC at an early age, safe and effective treatment is needed, and dietary fiber is of potential use in these patients.

The introduction of fiber into the field of lipid metabolism stems from epidemiological data which compares the incidence of diseases common in the Western world with native African populations.[2-4] Specifically, hyperlipidemia and FHC are associated with a striking incidence of coronary artery disease in the U.S.[5-8]

It is often difficult to manage hyperlipidemia in FHC with standard dietary management. Specific fibers in appropriate amounts exert a pronounced hypolipidemic effect in man and animals.[9] Pectin and Guar have been especially effective in lowering cholesterol in normal and hyperlipidemic patients.[10-11] However, not all fibers are effective in lowering lipids. No two sources of dietary fiber have the same effect on lipid metabolism.[12] Some dietary fibers, although effective as hypolipidemic agents, are not palatable or are difficult to incorporate into food because of increased viscosity or other chemical or taste properties.

LBG comes from the endosperm of the locust bean,[13] which the first-century Greeks used as a laxative and as a paste to bind mummies.[14] It is a white, odorless powder without a distinctive taste and is not to be confused with carob, which is a chocolate substitute which comes from the locust bean husk.

LBG has previously only been used in trace amounts in food products and has not been evaluated in treating hyperlipidemia. It is a hydrophylic compound that in an unhydrated form could create a bolus-like obstruction of the bowel. Bowel obstruction has been reported with the use of a similar hydrophylic compound,[15] although Kahn has recently reported feeding subjects 9 gm/day of Guar in capsules which were not hydrated without side effects.[16]

Study Design and Population: Thirty-two patients were selected from the Hennepin County Medical Center's Hyperlipidemia Clinic on the basis of their willingness to cooperate and make frequent clinic visits. One patient, a four-year-old child, withdrew from the study before the food products were fed because of fear of blood drawing. The FHC patients were defined by published criteria which include: elevation of total cholesterol and LDL cholesterol, normal levels of VLDL cholesterol and triglycerides, one parent and one child, and at least one first-degree relative, with FHC. The families also often had individuals by family history with premature cardiovascular disease. Twenty-one adults (aged 22-53) and 10 children (aged 10-18) completed

the study. Three of the adults were analyzed separately because of the different lipid abnormaliites.

Twenty-eight patients were divided to attempt to balance FHC and N, adults and children, males and females, and baseline lipid levels into two groups, A and B. The study was a modified cross-over design (see Table 1). This allowed a simultaneous comparison of two nearly identical diets with and without LBG, and also provided information after two months of LBG food products in Group B. At Week 16, subjects returned for lipid measurements while back on the normal baseline diet for 4 weeks, .

Normals were included with their hyperlipidemic spouses and siblings because entire families followed the diet and thus compliance was improved. Also, there is speculation that normals might respond differently from FHC patients to dietary management and the normals served as an additional control. Of interest, Group A had more FHC patients (11 vs 7) and Group B had no FHC females (Table 2).

The total cholesterol, VLDL-C, and triglycerides were similar in both groups. Group A had higher LDL-C and lower HDL-C levels, reflecting perhaps the greater number of FHC patients in Group A (Table 2). It is interesting that Group A, which had 8 women versus 5 in Group B, had lower HDL-C levels. This may be explained by the absence of FHC females in Group B (Table 3).

Three patients with different lipid abnormalities were studied simultaneously using the same protocol. One patient was a 53-year-old man with FHC who had a partial ileal bypass two years prior to the study. The second patient was a 49-year-old Type $II_B$ man with elevations of both his cholesterol and triglycerides. A third patient, a 51-year-old man with FHC, was on cholestyramine, nicotinic acid, and atromid during the entire study.

Identical-appearing food products were fed with or without locust bean gum. The food products were bread, crackers, cookies, and a potato product. The food products contained 8-10% LBG by dry weight. Each portion, i.e. slice of bread, one cookie, two crackers, six

TABLE 1

DIET STUDY DESIGN

| FEEDING PERIOD | | I | II | III | |
|---|---|---|---|---|---|
| WEEK NUMBER | -2,0 | 2,4 | 6,8 | 10,12 | 16 |
| GROUP A | BL | E | C | E | BL |
| GROUP B | BL | C | E | E | BL |

BL - BASELINE DIET
C - CONTROL WITHOUT LBG
E - EXPERIMENT WITH LBG

In Unconventional Sources of Dietary Fiber; Furda, I.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1983.

TABLE 2

SEX, LIPID TYPE, AND AGE

| | | GROUP A (ECE) | | GROUP B (CEE) | |
|---|---|---|---|---|---|
| | | YOUNG | ADULT | YOUNG | ADULT |
| FHC | MALE | 2 | 3 | 3 | 4 |
| | FEMALE | 1 | 5 | 0 | 0 |
| | TOTAL | 3 | 8 | 3 | 4 |
| NORMAL | | | | | |
| | MALE | 2 | 0 | 1 | 0 |
| | FEMALE | 1 | 1 | 1 | 4 |
| | TOTAL | 3 | 1 | 2 | 4 |

ECE EXPERIMENTAL, CONTROL, EXPERIMENTAL DIET
CEE CONTROL, EXPERIMENTAL, EXPERIMENTAL DIET

TABLE 3

PATIENT LIPID BASELINE VALUES - MG%, (SD)

| | GROUP A (ECE) | GROUP B (CEE) |
|---|---|---|
| CHOLESTEROL | 265 (82) | 255 (83) |
| LDL CHOLESTEROL | 200 (82) | 177 (87) |
| HDL CHOLESTEROL | 46 (11) | 55 (18) |
| VLDL CHOLESTEROL | 20 (12) | 24 (17) |
| TRIGLYCERIDE | 89 (57) | 101 (57) |

potato pieces, contained between 1.7 and 2.0 gm of LBG. Food products with and without LBG were analyzed in duplicate. The products with LBG contained higher amounts of total and soluble dietary fiber (using the revised Furda Method).[17] The amount of insoluble dietary fiber was the same for products with and without LBG. There were no significant differences in carbohydrate, protein, and fat content between LBG and non-LBG containing products. Fat analysis was performed for palmitic, oleic, linoleic, linolenic, stearic, arachidic, and arachidonic acid.[18-19] No significant differences were found between products with and without LBG (Table 4).

The families were seen at two-week intervals to collect three-day diet histories and two-week daily dietary records for the food products. The study families had been followed in the lipid clinic and given extensive dietary instruction over a period of six months to seven years before the study was initiated. The above data was recorded on nine consecutive visits at two-week intervals. The first two and the last visits were obtained while the patients were on their normal baseline diets. The feeding periods were four weeks in

In Unconventional Sources of Dietary Fiber; Furda, I.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1983.

TABLE 4

PRODUCT ANALYSIS

| | BREAD | | COOKIES | | CRACKERS | | POTATO | |
|---|---|---|---|---|---|---|---|---|
| | LBG | C | LBG | C | LBG | C | LBG | C |
| PROTEIN | 10.6 | 12.6 | 4.9 | 5.4 | 12.5 | 13.0 | 2.6 | 2.5 |
| CARBOHYDRATE | 49 | 55 | 62 | 64 | 67 | 69 | 23 | 21 |
| MOISTURE | 36 | 29 | 8 | 4.7 | 4.7 | 3.1 | 67 | 65 |
| FAT | 2.4 | 2.7 | 21 | 23.7 | 11 | 11.2 | 4.5 | 9 |
| LINOLEIC | 37 | 37 | 13.9 | 14.3 | 55 | 55 | 58 | 57.5 |
| OLEIC | 36 | 35 | 59 | 59 | 29 | 28 | 25.7 | 26.2 |
| PALMITIC | 17 | 16 | 13.5 | 13.5 | 11 | 11.2 | 11.6 | 11.3 |
| T.D.F. | 20 | 11.5 | 16 | 4.2 | 24 | 17 | 25 | 12 |
| I.D.F. | 9 | 8 | 3.5 | 1.6 | 11 | 11 | 12 | 9 |
| S.D.F. | 11 | 3.5 | 12.5 | 2.6 | 13 | 6 | 13 | 3 |

T.D.F. = TOTAL DIETARY FIBER
I.D.F. = INSOLUBLE DIETARY FIBER
S.D.F. = SOLUBLE DIETARY FIBER
LINOLENIC, STEARIC, AND ARACHIDIC WERE IN LESSER AMOUNTS (0-10%)
RALTEC ANALYSIS
C = CONTROL DIET
UNITS VARY DEPENDING UPON TYPE OF ASSAY

In Unconventional Sources of Dietary Fiber; Furda, I.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1983.

duration. Total calories, cholesterol, saturated fat, unsaturated fat, protein, carbohydrate, crude fiber, vitamin and mineral intake were coded using the USDA Handbook 456[20] and scored by the USDA Handbook 8[21] on a Digital Equipment Corporation computer PDP 11/34. Using the Keys, Anderson, Grande equation, the dietary intake of saturated and polyunsaturated fat and cholesterol was used to estimate the change in serum cholesterol due to non-LBG changes in the diet.[22] This was termed the dietary effect.

The food products were distributed to the families at their two-week clinic visits. The families were instructed not to change their diets during the study period. Exchanges were arranged within food preferences so that protein, carbohydrate, and fat consumption changes would be minimized. Because the food products primarily contained carbohydrates, carbohydrate-caloric exchanges were planned individually to minimize changes from the patients' baseline diets.

LBG in food products was prescribed to the subjects on the basis of body size. Adult males ate 25-35 gm/day, adult females age 10-25 gm/day, and children ate 10-20 gm/day of LBG in the food products. Most subjects ate selections from all four of the food products.

Laboratory and Exercise Evaluation: Laboratory evaluations performed at each two-week visit included plasma total cholesterol, LDL-C, HDL-C, VLDL-C, and plasma total triglycerides.[23] The lipoprotein determinations, LDL-C, HDL-C, and VLDL-C were not performed at Week 16. White blood cell counts, calcium, and serum glutamic oxaloacetic-transaminase (SGOT) were done before and after feeding LBG products. Weight was recorded at each visit.

Glycosylated hemoglobin determinations were performed on stored frozen hemolysates from eight Group B subjects at the end of the four-week control period and again at the end of the eight-week LBG feeding period. All eight subjects demonstrated a decrease in total cholesterol and LDL cholesterol in response to LBG. None of the subjects were diabetic or had symptoms or a past medical history of carbohydrate intolerance.

Glycosylated hemoglobin was determined by modification of a colorimetric assay which measures stable ketoamine-linked sugar.[24] Mild acid hydrolysis of the sugar linked to hemoglobin yields 5-hydroxymethyfurfural which reacts with thiobarbituric acid to form a colored product. The procedure was performed on globin extracts to prevent colorimetric interference by heme. Chromatographically purified $HbA_{aC}$ was used as the standard for the method. Glycosylated hemoglobin was expressed as relative precentage of the total hemoglobin.

Exercise forms were filled out at the end of the control and experimental periods for 65 different types of exercise activity. This data was recorded for each month of the study and included the number of times the activity was performed and the duration per occasion.[25] The study was performed during the months of February,

March, and April, when outdoor activities remained similar. Subjects were asked to maintain their normal physical activity. No apparent change of exercise as determined by these forms was observed during the study, and thus no correlation could be made between exercise and lipid changes.

The study was approved by the Human Volunteer Committee at the Hennepin County Medical Center to include adults and children.

Statistical Methodology: The relationships between LBG intake and lipid changes were analyzed by regression analysis which allowed for adjustment for various covariates. The within-person correlation coefficients between LBG and specific lipid determinations were calculated. The means of these correlation coefficients are approximately normally distributed, so they were tested by the t-test, using the empiric standard error. To ensure that other dietary changes did not explain lipid changes, the correlations between lipid changes and the dietary effect were similary tested. The regressions were carried out for all persons together and for Group A and B separatedly. Conditional F-tests were applied to see if Group A and B differed in the lipid (or lipid-fraction) response to LBG. The significance of a covariate was tested by the t-test on the regression coefficient.

All statistical analysis was carried out on the PDP 11/70 computer at the Laboratory of Physiological Hygiene using a Fortran program for the correlation analysis and the Biomedical Data Processing statistical package program BMDP1R for the regression analyses.[26]

## Results

Thirty-one patients completed the study. The lipid and lipoprotein levels were averaged for Group A and B for each of three periods and the baseline levels and are presented with standard deviations in Table 5. Three patients were evaluated separately because of the different nature of their lipid disorder and surgical or medical treatment.

Total Cholesterol: Comparing the control diet (Period II) to the experimental diet (Period III), Group A decreased their total cholesterol from 276 to 246 mg%, an 11% decrease ($p<.001$). The cholesterol and triglyceride values returned to baseline levels at Week 16, four weeks after resuming the normal baseline diet. Group A did not significantly decrease their cholesterol levels during the first experimental feeding period (Period I) when compared to baseline values; this could not be explained on the basis of LBG consumed, exercise, weight change, or any dietary variable evaluated.

Group B, comparing the control diet (Period I) to the experimental diet (Period II and III), decreased their total cholesterol from 255 to 215 mg%, a decrease of 16% ($p<.001$) (Table 5).

In Unconventional Sources of Dietary Fiber; Furda, I.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1983.

TABLE 5

LBG, CHOLESTEROL, LIPOPROTEIN CHOLESTEROL, TRIGLYCERIDE LEVELS BY GROUP FOR EACH TWO-WEEK PERIOD (MG/DL) (S.D.)

| WEEK | DIET | CHOL | LDL-C | HDL-C | VLDL-C | TG | LBG |
|---|---|---|---|---|---|---|---|
| **GROUP A** | | | | | | | |
| -2 | BL | 263.5(78) | 199.5(78) | 45.9(12.6) | 18.1(12.9) | 80.5(49.7) | 0 |
| 0 | | 266.3(88) | 200.7(88) | 45.3(10.7) | 21.3(12.4) | 99.2(69.0) | 0 |
| PERIOD I | E | | | | | | |
| 2 | | 257.5(87) | 195.9(87) | 45.5(9.9) | 16.3(9.4) | 94.3(60.8) | 15.4(6.4) |
| 4 | | 261.7(89) | 202.6(89) | 43.7(10.3) | 15.3(9.0) | 91.5(61.1) | 15.1(4.6) |
| PERIOD II | C | | | | | | |
| 6 | | 277.9(96) | 217.3(94) | 48.1(10.6) | 12.9(11.3) | 88.6(58.2) | 0 |
| 8 | | 272.7(93) | 209.1(89) | 45.1(10.1) | 18.9(13.8) | 100.8(72.4) | 0 |
| PERIOD III | E | | | | | | |
| 10 | | 253.3(88) | 191.4(85) | 43.6(11.1) | 15.4(11.9) | 95.9(85.1) | 12.2(4.3) |
| 12 | | 246.3(86) | 191.7(86) | 42.9(10.2) | 11.7(12.2) | 82.9(57.9) | 11.5(6.3) |
| 16 | BL | 266.9(92) | ---- | ---- | ---- | 84.6(58.0) | 0 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| GROUP B | | | | | | | |
| -2 | BL | 251.8(86) | 174.9(87) | 53.5(16.4) | 23.8(17.1) | 107.0(56.4) | 0 |
| 0 | | 257.2(18) | 177.6(89) | 55.1(20.1) | 23.7(18.3) | 108.4(61.0) | 0 |
| PERIOD I | C | | | | | | |
| 2 | | 262.5(82) | 183.4(93) | 55.2(18.1) | 23.8(19.1) | 116.8(64.5) | 0 |
| 4 | | 249.2(84) | 176.8(90) | 52.0(16.3) | 20.4(19.2) | 109.3(54.8) | 0 |
| PERIOD II | E | | | | | | |
| 6 | | 229.5(73) | 160.6(83) | 51.7(18.4) | 17.2(12.7) | 93.2(44.9) | 14.4(5.5) |
| 8 | | 221.2(66) | 151.7(75) | 52.6(15.9) | 17.5(11.5) | 80.9(40.9) | 13.4(7.3) |
| PERIOD III | E | | | | | | |
| 10 | | 219.2(61) | 151.3(69) | 47.6(16.8) | 20.2(15.2) | 102.9(49.5) | 12.0(5.4) |
| 12 | | 214.7(67) | 150.0(70) | 48.5(13.8) | 16.2(6.3) | 92.5(27.2) | 11.9(7.3) |
| 16 | BL | 251.6(82) | ---- | ---- | ---- | 102.8(58.0) | 0 |

E - FOOD PRODUCTS WITH LBG
C - FOOD PRODUCTS WITHOUT LBG
BL - BASELINE DIET WITHOUT FOOD PRODUCTS

LDL Cholesterol: Comparing control to experimental feeding periods, Group A LDL-C was lowered 10% from 211 to 191 mg% ($p < .001$). Comparing Period I experimental diet to baseline diet, there was no significant change in LDL-C in Group A (Table 5).

Group B lowered their LDL-C 17% from 181 mg% during control diet (Period I) to 141 mg% on the experimental diet (Period II and III) ($p < .001$) (Table 5).

HDL Cholesterol: In Group A, comparing control to experimental diet periods, HDL-C levels fell 7%. In Group B, HDL-C levels fell 10%. The HDL/LDL ratio in Group A increased 4% (N.S.), while in Group B the ratio increased 12% from .29 to .33 ($p < .001$.) (Table 5).

VLDL Cholesterol: The association between VLDL-C changes and LBG was just significant. Both groups demonstrated an erratic response in VLDL-C levels to LBG, but by the end of the feeding experiment (Week 12), both groups were over 30% lower in VLDL-C than at baseline (Table 5).

Triglycerides: All subjects had normal triglyceride levels before, during, and after the feeding studies. And there was no change during LBG feeding of significance (Table 5).

Males vs Females, FHC vs Normal, and Children vs Adults: All subgroups demonstarted a lowering of both total cholesterol and LDL-C. There was no difference in the cholesterol or LDL-C response to LBG between males and females of either group. FHC patients consistently had a greater lowering of cholesterol and LDL-C than normals in both groups ($p < .05$). Cholesterol and LDL-C in adults were consistently lowered more than in children (Table 6). This was not significant when adjusted for weight.

Patient with Elevations in Triglycerides and Cholesterol: The patient with Type $II_B$ hyperlipidemia had a 22% and 18% decrease in cholesterol and 24% and 22% decrease in triglycerides at 4 and 8 weeks during the experimental LBG feeding periods. VLDL cholesterol fell 24% at 4 and 8 weeks.

Partial Ileal Bypass and LBG: The patient with the partial ileal bypass was unusual in that he had consistently elevated HDL levels. This patient had no significant lipid lowering on the experimental LBG diet. It is interesting that the HDL cholesterol increased on the LBG diet and fell during the control diet.

LBG Plus Pharmacological Treatment: To help understand the mechanism of action, we also fed the LBG food products to a 52-year-old man with FHC who was on three lipid lowering medications during

TABLE 6

LIPID RESULTS BY PERIOD, SEX, LIPID TYPE, AND AGE - MG%

| GROUP | | BASELINE A | BASELINE B | PERIOD 1 A | PERIOD 1 B | PERIOD 2 A | PERIOD 2 B | PERIOD 3 A | PERIOD 3 B |
|---|---|---|---|---|---|---|---|---|---|
| | | | | E | C | C | E | E | E |
| CHOL | M | 264 | 296 | 265 | 296 | 281 | 258 | 256 | 253 |
| | F | 266 | 188 | 255 | 184 | 270 | 173 | 244 | 159 |
| | FHC | 304 | 313 | 300 | 316 | 320 | 272 | 289 | 261 |
| | N | 156 | 185 | 147 | 184 | 150 | 170 | 141 | 164 |
| | Y | 233 | 252 | 232 | 250 | 234 | 230 | 216 | 227 |
| | A | 286 | 256 | 278 | 259 | 303 | 222 | 272 | 210 |
| LDL | M | 200 | 226 | 208 | 231 | 222 | 199 | 200 | 193 |
| | F | 200 | 97 | 191 | 99 | 206 | 88 | 185 | 83 |
| | FHC | 237 | 244 | 238 | 252 | 256 | 214 | 230 | 203 |
| | N | 96 | 97 | 90 | 96 | 94 | 88 | 85 | 90 |
| | Y | 173 | 180 | 178 | 186 | 179 | 164 | 167 | 163 |
| | A | 217 | 174 | 214 | 176 | 236 | 151 | 208 | 143 |
| HDL | M | 36 | 44 | 37 | 44 | 38 | 42 | 35 | 40 |
| | F | 54 | 70 | 51 | 69 | 54 | 68 | 50 | 61 |
| | FHC | 45 | 41 | 44 | 41 | 46 | 39 | 42 | 38 |
| | N | 48 | 70 | 48 | 67 | 48 | 67 | 47 | 60 |
| | Y | 42 | 54 | 42 | 52 | 41 | 53 | 40 | 50 |
| | A | 48 | 55 | 48 | 55 | 50 | 52 | 46 | 47 |

Y = CHILDREN M = MALE E = EXPERIMENTAL DIET
A = ADULT F = FEMALE C = CONTROL DIET

In Unconventional Sources of Dietary Fiber; Furda, I.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1983.

the study, cholestyramine (24 gm/day), nicotinic acid (4 gm/day), and chlofibrate (2 gm/day). His usual cholesterol levels were in the 400's mg% range when not on medication and were in the low 200's mg% range when on the medications. There was no change in serum lipids on the medications with the addition of LBG. In evaluating these two patients, it would seem that the bile acid loss by ileal bypass or cholestyramine may have been maximized, thus there was no additional effect in bile acid binding and loss with the LBG.

Glycosylated Hemoglobin: The incorporation of LBG into a controlled diet for eight weeks did not change glycosylated hemoglobin levels.

Dietary Data: The amount of LBG was analyzed for each group and subgroup - males versus females, adults versus children, and FHC versus normals - for each of the three periods. Amounts of LBG were calculated by body weight of the subjects and by kilocalories. No significant differences were seen between groups or subgroups. Group A children consumed less LBG than adults in Period I; this difference persisted after adjustment for kilocalories, but not after adjustment for body weight (Table 7).

Diet analysis of both groups at the two baseline clinic visits revealed children ate 161 (+/- .46) mg/day of cholesterol and P/S ratio of .58 (.14). Adults in our study ate 242 (145) mg/day of cholesterol and the P/S ratio was .56 (.20).

The intake of protein, carbohydrate, total fat, saturated fat, monounsaturated fat, and polyunsaturated fat did not change significantly during the study (Table 8). Separate analysis of these dietary factors did not correlate with the changes seen in the serum lipids.

Total calories for both groups averaged 1700 at baseline, increased slightly and then decreased to 70 less than baseline during Period III. The subjects were 2.0 pounds above baseline weight at Period I, and 1.8 pounds above baseline at Period III. Non-LBG fiber intake was analyzed from the dietary records and did not differ or change over the study from group to group. There were no significant difference between or within groups at baseline or during any of the three feeding periods for total calories, non-LBG fiber, or weight change (Table 9).

There were preferences for the individual food products which remained quite constant over the study. The bread and cookies were the food products consumed more often. The potato product was the least used.

Side Effects of LBG Intake: No serious side effects were reported during the feeding of either the control or experimental food products. Subjects reported increased flatulence while on the LBG-containing products. Flatulence decreased and at about one to weeks the amount

TABLE 7

LBG MG/DAY

| | PERIOD 1 A | PERIOD 1 B | PERIOD 2 A | PERIOD 2 B | PERIOD 3 A | PERIOD 3 B |
|---|---|---|---|---|---|---|
| AMOUNT LBG | | | | | | |
| M | 14.5 (6.3) | 0 | 0 | 15.6 (6.1) | 13.0 (6.6) | 13.7 (5.5) |
| F | 16.1 (3.2) | 0 | 0 | 11.2 (5.8) | 10.8 (3.4) | 9.1 (4.3) |
| Y | 12.4 (2.1)* | 0 | 0 | 13.5 (1.5) | 9.2 (2.6) | 10.6 (2.2) |
| A | 17.3 (5.1) | 0 | 0 | 14.2 (8.0) | 13.6 (5.7) | 12.8 (6.8) |
| FHC | 15.3 (5.2) | 0 | 0 | 16.0 (6.5) | 12.2 (5.8) | 13.6 (6.0) |
| N | 15.3 (4.0) | 0 | 0 | 11.6 (5.3) | 10.9 (2.9) | 10.0 (4.5) |
| LBG/WEIGHT | | | | | | |
| M | 12.3 (4.2) | 0 | 0 | 12.5 (5.3) | 10.6 (3.7) | 10.8 (3.9) |
| F | 11.7 (2.6) | 0 | 0 | 9.7 (4.6) | 7.9 (2.7) | 8.0 (4.0) |
| Y | 12.5 (3.7) | 0 | 0 | 14.7 (4.5)* | 9.4 (3.8) | 11.5 (3.7) |
| A | 11.5 (3.3) | 0 | 0 | 9.4 (4.4) | 9.0 (3.3) | 8.6 (4.0) |
| FHC | 11.2 (3.2) | 0 | 0 | 12.2 (5.7) | 8.7 (3.2) | 10.1 (3.6) |
| N | 14.0 (3.4) | 0 | 0 | 10.5 (4.5) | 10.5 (4.1) | 9.3 (4.8) |
| LBG/KCALS | | | | | | |
| M | 6.1 (2.7) | 0 | 0 | 7.2 (2.1) | 5.7 (2.0) | 6.3 (1.5) |
| F | 8.3 (2.3) | 0 | 0 | 7.3 (3.3) | 7.4 (2.1) | 5.6 (1.7) |
| Y | 5.2 (0.7)* | 0 | 0 | 6.6 (1.3) | 4.8 (1.5)* | 5.1 (0.6) |
| A | 8.7 (2.6) | 0 | 0 | 7.7 (3.0) | 7.8 (1.6) | 6.6 (1.8) |
| FHC | 7.6 (2.9) | 0 | 0 | 7.5 (2.1) | 7.0 (2.3) | 6.4 (1.7) |
| N | 6.5 (1.9) | 0 | 0 | 7.0 (3.1) | 5.7 (1.6) | 5.6 (1.5) |

*SIGNIFICANT DIFFERENCE (<.05)

In Unconventional Sources of Dietary Fiber; Furda, I.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1983.

TABLE 8

DIET ANALYSIS BY GROUP AND PERIOD (S.D.)

| | | PROT | CHO | FAT | SAFA | MUFA | PUFA |
|---|---|---|---|---|---|---|---|
| BL | A | 16.0(2.9) | 56.2(6.5) | 29.1(4.0) | 9.0(1.3) | 12.0(1.5) | 4.7(1.5) |
| -2,0 | B | 15.9(1.2) | 51.2(12.3) | 32.4(9.9) | 10.4(4.5) | 13.4(3.9) | 5.7(1.5) |
| PERIOD 1 | A | 13.8(2.5) | 55.7(5.6) | 31.8(4.0) | 8.9(1.6) | 13.6(1.6) | 6.1(1.7) |
| +2, +4 | B | 15.1(2.4) | 43.3(4.9) | 30.8(2.8) | 9.0(1.2) | 12.0(2.6) | 5.8(1.0) |
| PERIOD 2 | A | 14.6(2.6) | 55.0(7.5) | 32.8(4.8) | 9.1(1.7) | 13.5(1.4) | 6.2(2.1) |
| +6,+8 | B | 16.9(3.8) | 53.9(9.1) | 28.1(4.1) | 8.8(1.9) | 11.5(3.3) | 5.1(1.3) |
| PERIOD 3 | A | 15.1(2.1) | 54.4(7.0) | 31.7(4.4) | 8.4(1.4) | 13.4(1.8) | 6.3(1.6) |
| +10,+12 | B | 16.3(2.6) | 50.6(7.8) | 34.2(4.3) | 10.3(2.4) | 13.8(3.6) | 5.5(1.1) |

SAFA - SATURATED FATTY ACIDS
MUFA - MONOUNSATURATED FATTY ACIDS
PUFA - POLYUNSATURATED FATTY ACIDS

TABLE 9

CALORIES NON-LBG FIBER AND WEIGHT CHANGE

(S.D.)

| | | CALORIES | FIBER (GM/DAY) | WEIGHT (LBS) |
|---|---|---|---|---|
| BL | A | 1845 (686) | 5.0 (2.0) | 130.6 (35) |
| | B | 2075 (430) | 4.3 (1.3) | 126.7 (41) |
| PERIOD 1 | A | 2248 (610) | 2.3 (2.4) | 133.0 (36) |
| | B | 2215 (351) | 2.7 (0.6) | 128.3 (41) |
| PERIOD 2 | A | 1964 (704) | 3.1 (0.9) | 133.0 (36) |
| | B | 1903 (506) | 2.4 (1.0) | 128.5 (41) |
| PERIOD 3 | A | 1878 (706) | 3.3 (3.5) | 133.0 (36) |
| | B | 1945 (533) | 2.3 (1.4) | 127.6 (40) |

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reported was usually not a problem. No one had diarrhea, but stool size increased. One patient had significant constipation which remitted after discontinuing the diet temporarily. After three days this person was able to resume the diet without further problem. There was no change observed in the WBC counts, serum calcium, or SGOT determinations.

## Discussion

LBG food products successfully lowered serum cholesterol and LDL-C. In Group A (comparing control to experimental diet feeding periods), serum cholesterol decreased 11% and LDL-C decreased 10%. In Group B, serum cholesterol decreased by 13% at 4 weeks and 17% at 8 weeks of LBG feeding, and LDL cholesterol decreased 17% at 4 and 8 weeks. HDL/LDL ratios increased in both groups, but not significantly in Group A. Group A cholesterol and LDL-C at 4 weeks of LBG feeding did not change from the baseline diet. Group A cholesterol and lipoprotein values increased while on the control diet, suggesting that there was a suppresion effect on cholesterol levels during LBG feeding.

Evaluation by Subgroup: Group A and B patients were evaluated by subgroup for their response to LBG over the three feeding periods. Both groups and subgroups experienced a decrease in their total cholesterol, LDL-C, and to a lesser extent HDL-C comparing control to LBG feeding periods. Both males and females decreased their total cholesterol, LDL-C, and HDL-C. Both FHC and normal lipid patients, children and adults, decreased their lipid levels. Contrary to Tuomilehto's report,[27] the FHC females in our study decreased their serum cholesterol on LBG by 10% from 319 to 288 mg% ($p < .001$).

Control of Dietary Variables: It is necessary to analyze and compare as many of the dietary variables as possible[28]; the food products with and without LBG contained similar amounts of carbohydrate, protein, and fat. Diet analysis at baseline and at two-week intervals did not reveal significant differences for dietary intake between groups during the control and experimental feeding periods. There were no significant differences during the various feeding periods or between groups for total fat, saturated fatty acids, monounsaturated fatty acids, polyunsaturated fatty acids, total calories, non LBG fiber, or weight change (Tables 8 & 9). In fact, both groups, in face of serum cholesterol and LDL-C lowering, gained a slight amount of weight, which often is associated with a rise in serum lipids.

Analysis of our data revealed that Group A responded to LBG only after being fed the control diet; no change was observed in lipids in Period I when compared to the baseline diet, which did not contain the same food products. Group B responded after they had been fed

control food products. These observations highlight the necessity of controlling as many dietary variables as possible.

Dietary Cholesterol and P/S Ratio: The low dietary cholesterol and modest P/S ratio in our patients provided a reasonable but not maximal conventional dietary approach to controlling hypercholesterolemia. The mean intake in children of 161 mg/day and P/S of .58 compares to USDA figures of 370 mg/day of cholesterol and P/S of .36 for children. Adults consumed 242 mg/day and P/S of .56 as compared to national norms for adults of 451 mg/day of cholesterol and P/S ratio of .22.[28-29] The changes in cholesterol, LDL-C, HDL-C, and VLDL-C attributed to the LBG food products were in addition to the presumed effect of the baseline diet which was not altered significantly during the study. It is not known what the effect of LBG food products would be on a similar group whose baseline diet included average cholesterol and P/S intake.

Glycosylated Hemoglobin: Kiehm[31] and Anderson[32] found that when diabetic patients on small doses of insulin were treated with high-carbohydrate, high-fiber diets, insulin requirements were reduced. Jenkins demonstrated that Guar gum lessened glycosuria in diabetics.[33] Miranda found bread with high content of cellulose flattened the glucose tolerance curve.[34] Jenkins found that viscosity was important in flattening the glucose tolerance curve in his work with Guar and tragacanth. There was a slowing of absorption of xylose from the small intestine.[35]

None of the subjects that we report have carbohydrate intolerance and to our knowledge glycosylated hemoglobin has not been evaluated in normoglycemic patients on fiber diets.

LBG did not influence carbohydrate metabolism sufficiently to alter glycosylated hemoglobin levels despite lowering blood lipids.[36] More sensitive techniques of protein glycosylation have recently become available[37] and glycosylation of short-lived proteins might be useful in retrospective evaluation of possible recent hypoglycemic episodes.

Potential Role of LBG in Treating Hypercholesterolemia: Standard dietary treatment of hypercholesterolemia by decreasing saturated fat, increasing polyunsaturated fat, and decreasing animal protein and cholesterol intake remain the mainstay of therapy. Free living, non-institutionalized patients, for reasons of compliance, individual response, or dietary preference, may not successfully lower their serum lipids or the result may be suboptimal. Thus, the significance of the lipid lowering observed in this outpatient study may be greater than similar results with institutionalized patients where compliance is greater. Some individuals in the study, despite comparable LBG intake, did not lower their lipids, perhaps just as feeding monkeys

dietary cholesterol indicates that there are diet responders and diet non-responders.[38] Adults in an outpatient setting, after initiation of standard dietary treatment, usually do not respond by more than a 5-10% lowering of serum cholesterol. Of a group of FHC children evaluated at age 2, only 40% could maintain normal serum cholesterol levels despite near maximal dietary treatment.[39] Prevention of heart disease and/or regression of atherosclerotic lesions may require a decrease of serum lipids to near normal levels. If the dietary response is suboptimal, pharmacological management of hyperlipidemia usually is necessary. In children, pharmacological agents are often not accepted because of their potential for side effects, expense, and the long duration with which they must be consumed.[40] Specific dietary fibers, such as LBG, may enhance current treatment regimens, if they can tastefully be incorporated into foods.

Physiology of LBG: LBG was used in the present study because of its similarity to Guar gum, which in food products has proved to be an effective means of lowering serum lipids. Guar gum is similar to LBG in molecular weight, galactomannan content, moisture, protein, and ash. Although both gums are viscous, LBG is less viscous than Guar. A 1% addition of Guar gum to distilled water develops a viscosity of 6000 CPS as compared to LBG, which develops a viscosity of 3000 CPS measured on a Brookfield RVF spindle No. 3 at 20 rpm at 25$^{o}$ C. Guar has one D-Galacto pyranose for every two D-Manno pyranose units, while LBG has one D-Galacto pyranose for unit for every four D-Manno pyranose units.[41-42] Because LBG is less viscous than Guar, its incorporation into food products is less difficult. The lower viscosity of LBG food products may decrease the gumminess or stickiness, and thus improve palatability. In small doses (6.7 gm/day), LBG increased stool weight by 10%[43]. Guar in comparable doses to the present study increased stool weight by 20%.[44]

Fiber Analysis: The revised Furda method of fiber analysis used on our food products determines soluble and insoluble fractions as well as the total fiber.[17,44] The lipid lowering effect of fiber appears to be in the soluble portion of the fiber because the amount of insoluble fiber was the same for food products with and without LBG. This study does not, however, isolate a lipid-specific portion of the fiber, nor can one determine whether the lipid effect is restricted to the soluble or the insoluble fiber fraction.

Mechanism of Action: Dietary fibers such as LBG are thought to lower serum lipids by binding bile acids and increasing bile acid excretion. Micellular formation which is essential for cholesterol absorption is interefered with. LBG fiber may act as a water-holding and cation-exchange agent, increasing total fecal bile acid and neutral sterol output because of the increased water-holding capacity. Fiber

in the large intestine may act as a physiological chromatography column, whose gel filtration and acid ion-exchange properties decrease bile acid absorption and hence lower serum lipids.[45]

## Conclusions

LBG food products provided a safe, effective means to lower serum lipids in hypercholesterolemic and normal adults and children over a three-month feeding study. Serum cholesterol was lowered 11-16%, LDL-C was lowered 10-17%, and HDL/LDL ratios increased (4-12%). The LBG food products were consumed without significant side effects and were well accepted.

It is appealing to develop safe, effective, bio-engineered foods which can be consumed by asymptomatic children and adults over a long period of time. Utilizing standard hypolipidemic dietary therapy, fiber-enriched foods can be used either as a primary treatment or as an adjunct to pharmacological management and prevention of premature atherosclerosis can be approached in the young and asymptomatic. It appears possible that bio-engineered food products can displace the pills and powders which often serve as negative reinforcements to prolonged treatment programs.

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# Pectins and Guar Gum: Effect on Plasma Lipoproteins and Tissue Lipoprotein Lipase Activity in Rats

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Various dietary fiber supplementations were given to rats fed semisynthetic diets with 20% casein and 20% peanut oil for 3-6 months. When added to cholesterol free diets, 5% guar gum slightly decreased total plasma and HDL cholesterol, whereas 5% low or high methoxylated pectin had no significant effect or a slightly elevating effect. When 0.5% cholesterol was included in the diets, 5% pectin decreased total cholesterol and increased HDL cholesterol, thus increasing substantially the relative HDL concentration. Wheat bran increased total and HDL cholesterol, and there seemed to be a dose-response relationship. Differences in plasma triglycerides were generally insignificant. Lipoprotein lipase activity was slightly elevated in heart tissue after feeding wheat bran and slightly lowered in adipose tissue after feeding high methoxylated pectin.

Dietary fiber might be a factor protecting against atherosclerotic disease (1). This hypothesis is based on epidemiological data linking a large number of "Western" diseases with a low intake of dietary fiber (2). It has got experimental support, however in that certain types of dietary fiber might alter plasma lipoprotein concentrations.

The relationship between the plasma lipoprotein profile and clinical appearance of atherosclerotic heart disease is now well established. During the latest decade increased interest has focused on the differential effect of various lipoprotein classes; specifically the protective function of high-density lipoproteins

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(HDL) against atherosclerosis has been firmly established (3), whereas increased levels of low-density lipoproteins (LDL) are regarded as a major risk factor for cardiovascular disease.

Effects of dietary fiber on plasma lipids have been most pronounced and most consistent in studies using purified, viscous types of fiber, such as pectin and guar gum. The decrease in total and LDL cholesterol when feeding these fibers is accompanied by an increased fecal bile-acid excretion. Increased bile-acid loss with increased de novo synthesis from cholesterol is usually considered the main mechanism by which dietary fiber may alter plasma cholesterol. However, decreased absorption of dietary cholesterol or altered cholesterol and lipoprotein metabolism by other mechanisms may also be important (for review see for instance 4).

Wheat bran binds bile-acids in vitro (5) but in vivo effects on plasma cholesterol have been almost uniformly negative (6). Some studies on small groups of humans (7,8), however, indicate that bran might increase HDL cholesterol, although unchanged (9) or even decreased (10) levels have also been reported.

Colon cancer is the form of cancer most strongly linked to dietary factors, especially fat and fiber. It is suggested that the bulking effect of dietary fiber would dilute carcinogens and diminish the time during which the colonic mucosa is exposed to carcinogens (11). Fibers such as pectin and guar gum, however, increase the flow of bile salts to the large bowel and have poor bulking effect as they are readily degraded by the intestinal microflora. Since bile salts and/or their microbial degradation products are cocarcinogens, this complicates the dietary fiber/colon cancer hypothesis.

Experiments with chemically induced colon cancer in the rat have partially, but not consistently, supported the dietary fiber hypothesis (12). Thus, Bauer et al. (13,14) recently reported that both high- and low-methoxylated pectin (6.5 or 5% in a diet containing 20% casein, 20% fat and no other dietary fiber) given during induction of colon cancer with subcutaneous 1.2-dimethylhydrazine, enhanced the yield of tumors. Guar gum, 5%, also tended to increase the tumor yield, although not significantly compared with controls on a virtually fiber-free diet. Others(15) however reported a protective effect of 15% pectin.

With this background, the present investigation was undertaken. The effect on plasma total and HDL cholesterol, and triglycerides of two kinds of pectin with different degrees of methoxylation, and of guar gum was studied in 3-6 months feeding experiments with rats. Wheat bran and oat bran were also studied for comparison. Pectin and wheat bran was fed both with and without dietary cholesterol.

To throw some light upon mechanisms by which dietary fiber could influence plasma lipoproteins, heart and adipose tissue lipoprotein lipase activities were measured after feeding some of the dietary fiber containing diets. This key enzyme in lipoprotein metabolism is one of the most important factors determining plasma triglyceride and plasma HDL concentrations (16).

## Materials and Methods

Animals. Male SPF Sprague-Dawley rats (Anticimex, Stockholm, Sweden), weighing approximately 135 g at the start of the experiment, were used. They were kept individually in steel-wire cages with free access to water and were adapted to a 12 h light and dark shift. The room was air-conditioned, maintained at 23° and 5o-60% relative humidity. The different diets were fed *ad lib.* in metal containers designed to give minimal losses.

Diets. A basal diet with 20% casein, 20% fat, wheat starch as main carbohydrate source and proper amounts of vitamins and minerals was prepared as described earlier (17). The various dietary fiber containing diets were prepared by substituting dietary fiber preparations for wheat starch.

Analysis of dietary fiber. Total dietary fiber including both water-soluble and water-insoluble components was analysed with an enzymic method as described by Asp et al. (18). The dietary fiber was characterized by gas-liquid chromatographic assay of monosaccharides after acid-hydrolysis and gravimetric determination of acid insoluble lignin. Uronic acids were assayed with a decarboxylation method. These analyses were performed as described by Theander and Åman (19).

Analysis of plasma lipids. HDL was separated by selective precipitation of very-low-density lipoprotein (VLDL) and low-density lipoprotein (LDL) by dextran sulphate and manganese chloride (20), and HDL cholesterol was measured by cholesterol determination of the clear supernatant fraction. Cholesterol and triglycerides were analysed by enzymic procedures (21,22).

Experiment 1. Diets containing 5% high-methoxylated (HM) pectin, 5% low-methoxylated (LM) pectin, 5% guar gum or 20% wheat bran (corresponding to about 10% bran fiber) were fed for 6 months to rats (15 in each group) and compared with the basal diet. After 6, 12 and 26 weeks, 5 rats in each group were killed after 16 hours fasting by carbon dioxide narcosis. Blood was withdrawn by cardiac puncture (17).

Experiment 2. In this experiment the basal diet, a 5% HM-pectin diet and a 10% wheat bran diet (corresponding to 5% bran fiber) were fed to groups of 7 rats both with and without 0.5% dietary cholesterol. For comparison, 10% wheat bran and 10% oat bran diets were also studied (without cholesterol).

Plasma lipids were analysed as in Experiment 1. Lipoprotein lipase activities in adipose tissue and heart muscle was assayed with tri ($^3$H) oleoylglycerol emulsion as substrate (23). Activities are expressed as nmol of fatty acid released per minute per mg protein.

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Statistical evaluation. Student's t-test (two-tailed) was used for weight of rats and food intake. Differences in plasma lipids, lipoproteins and lipoprotein lipase activities were evaluated with Wilcoxon's test (two-tailed).

## Results and Discussion

Experiment 1. The dietary fiber content and composition of the various dietary fiber preparations used have been reported earlier (17). The HM and LM pectin preparations had 74 and 37% respectively of their carboxyl groups methoxylated. The uronic acid content was 80 and 86% respectively (dry weight basis). The content of neutral sugars, mainly galactose, was 6 and 4% respectively. The guar gum preparation contained 89% neutral sugars (dry weight basis), mainly mannose and galactose. The wheat bran contained 52% dietary fiber (dry weight basis) with a typical composition dominated by arabinoxylan and cellulose. The acid insoluble lignin content was 5%.

Figure 1 shows the growth curves in the first experiment. The guar gum group (G) had slightly slower weight development than the other groups. When the weight increase was calculated per unit energy consumed, the guar gum group had a significantly lower value, in spite of an 8% increase in the food intake. Fecal losses could not explain this lower weight development since fecal dry weight increased only 0.3 g/day and most of this increase can be expected to represent undegraded guar gum. It is interesting in this context to recall an investigation (24) showing uncoupling of oxidative phosphorylation in liver and heart mitochondria of rats given small doses of gum arabic, gum tragacanth or modified celluloses.

Total plasma cholesterol (Figure 2) was significantly lowered by guar gum after 12 weeks but the pectins did not significantly alter the level. Wheat bran increased total cholesterol at all the time points studied (17).

HDL cholesterol (Figure 3) was also lowered by guar gum but increased by wheat bran. The relative HDL concentration (percent of total cholesterol) was close to 0.7 in all cases.

Plasma triglycerides decreased with age but were similar in all groups. On gel electrophoresis, no chylomicrons were demonstrable, indicating that plasma triglyceride concentrations essentially corresponded to VLDL concentrations.

Experiment 2. As in the first experiment, weight development and food intake was similar in the different gorups.

Figure 4 shows total plasma cholesterol after 3 months on the different diets. In the groups not fed dietary cholesterol pectin gave a slight but significant plasma cholesterol increase. As in Experiment 1, wheat bran also increased the total cholesterol and there seemed to be a dose-response relationship.

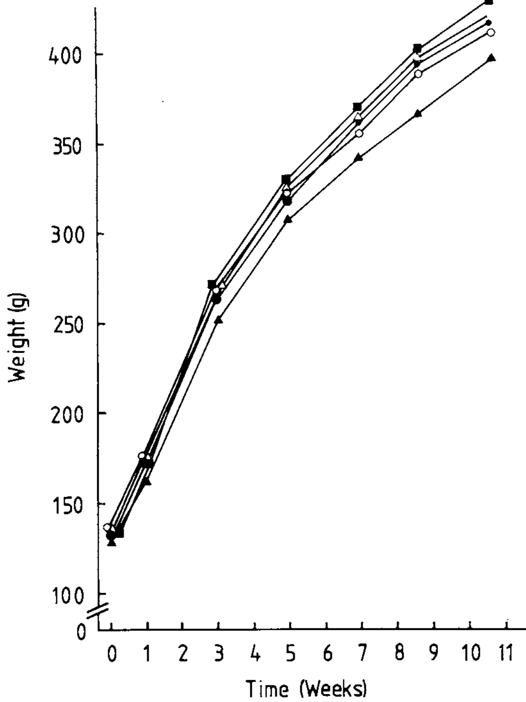


*Figure 1. Growth curves of rats fed various dietary fiber containing diets. Key: ●, HM pectin, 5%; △, LM pectin, 5%; ▲, guar gum, 5%; ■, wheat bran, 20%, corresponding to 10% bran fiber; and ○, basal diet.*

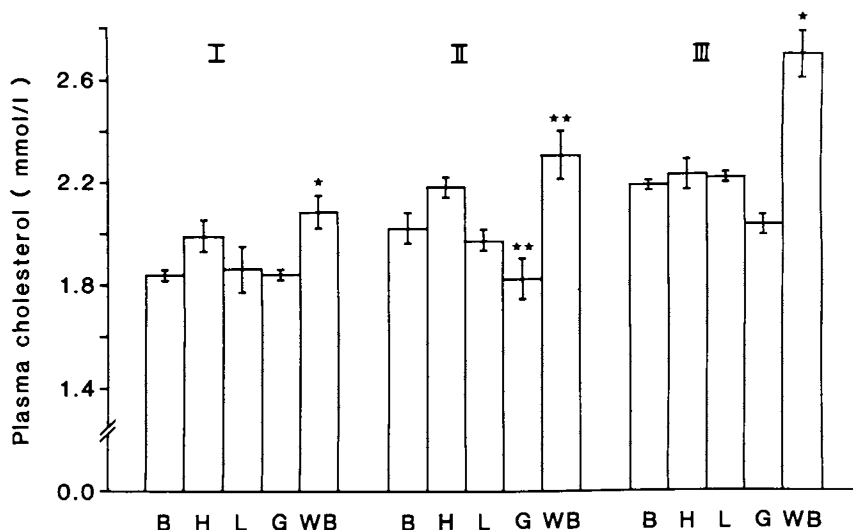


*Figure 2. Total plasma cholesterol after 6 (I), 12 (II), and 26 (III) weeks in rats given diets containing different dietary fibers. Key: Group B, basal diet; Group H, high-methoxyl pectin diet; Group L, low-methoxyl pectin diet; Group G, guar gum diet; Group WB, wheat bran diet; *, P < 0.05; and **, P < 0.01. Mean values are given with their standard errors represented by vertical bars. (Reproduced with permission from Ref. 17. Copyright 1981,* British Journal of Nutrition.)

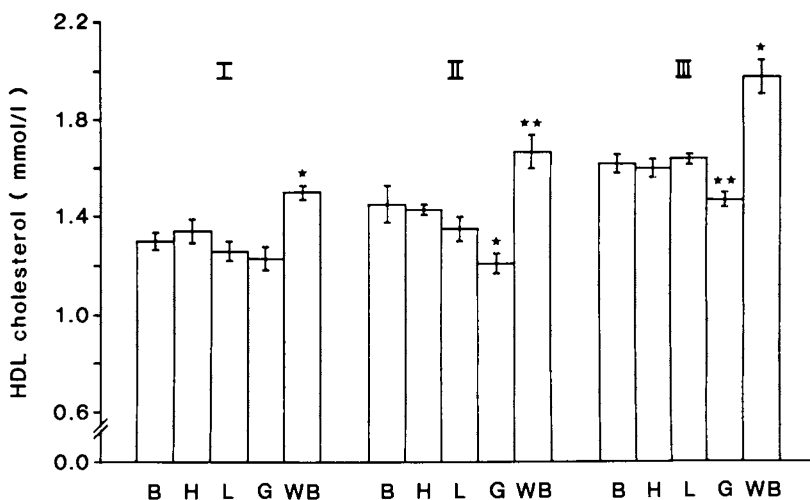


*Figure 3. High-density-lipoprotein cholesterol after 6 (I), 12 (II) and 26 (III) weeks in rats given diets containing different dietary fibers. Key is the same as in Figure 2. Mean values are given with their standard errors represented by vertical bars. (Reproduced with permission from Ref. 17. Copyright 1981,* British Journal of Nutrition.)

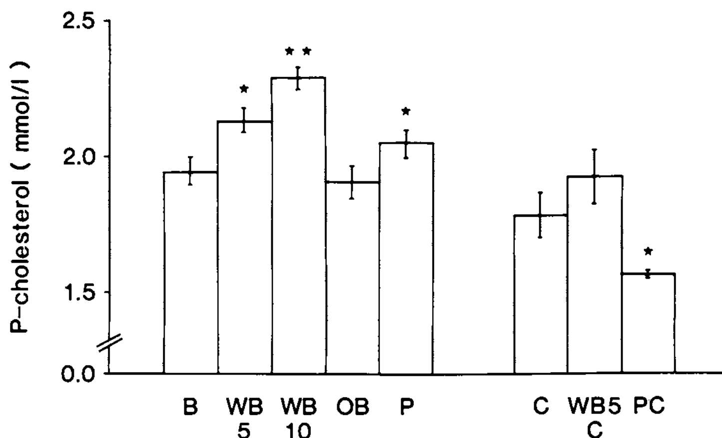


*Figure 4. Total plasma cholesterol after 3 months in rats given diets containing different dietary fibers, without cholesterol (left) and with 0.5% cholesterol (right).* *Key: Group B, basal diet; group WB5 and WB10, wheat bran diets with 5 or 10% wheat bran fiber; Group OB, oat bran diet with 10% fiber; Group P, pectin diet with 5% high-methoxylated pectin; Group C, basal diet with 0.5% cholesterol; Group WB5C, wheat bran diet (5% fiber) with 0.5% cholesterol; Group PC, pectin diet, 5%, with 0.5% cholesterol; *, $P < 0.05$; and **, $P < 0.01$. Mean values are given with their standard errors indicated by vertical bars.*

When 0.5% cholesterol was added to the diets, total cholesterol levels decreased slightly whereas HDL cholesterol concentrations fell by about 30%. When added to the cholesterol containing diet, 5% pectin significantly decreased the total plasma cholesterol and increased the HDL cholesterol level (Figure 5). Wheat bran increased HDL cholesterol both with and without dietary cholesterol.

These changes demonstrate that the relative HDL concentration i.e. HDL cholesterol as percent of total cholesterol, was strongly decreased by dietary cholesterol despite the slight reduction of the total plasma cholesterol levels (Figure 4, 5). Despite its lack of effect in rats fed without cholesterol, pectin could partly reverse the negative effect of dietary cholesterol on the Total/HDL cholesterol ratio. Wheat bran did not significantly change this ratio since it led to similar elevations in both total and HDL cholesterol.

Oat bran did not change plasma cholesterol levels. Others (25) have shown that oat bran decreases total plasma cholesterol and increases HDL in cholesterol fed rats. The effect seems to be related to soluble oat gums. The lack of effect of oat bran in our experiment might be due to the fact that we studied this fiber only in cholesterol-free diets. Soluble gums may also have been lost in preparing this fiber enriched oat bran (81% dietary fiber).

Plasma triglycerides were not altered by pectin but significantly lowered in the wheat bran group with 5% bran fiber and in the oat bran group.

Lipoprotein lipase activity in heart tissue was not altered by pectin but slightly increased in the wheat bran group. In adipose tissue the activity was significantly lowered in the group fed pectin.

## General Discussion

The evaluation of effects of dietary constituents on plasma lipid and lipoproteins requires selective measurements of individual lipoprotein classes. For example, the addition of cholesterol to the diet in the rat does not increase total plasma cholesterol levels; in the present study a slight reduction in plasma cholesterol was in fact seen. However, the HDL cholesterol fraction was markedly reduced, and the VLDL and/or LDL cholesterol level must therefore have been increased. Extrapolation from data in humans would indicate that this shift in lipoprotein profile is disadvantageous from the atherogenic point of view. The increase in VLDL/LDL cholesterol may readily be explained by the dietary load of cholesterol which is transported to peripheral tissues by these lipoprotein particles (16). The reduction in HDL which are considered to be involved in cholesterol transport from peripheral tissues to the liver, is however an interesting but so far unexplained phenomenon.

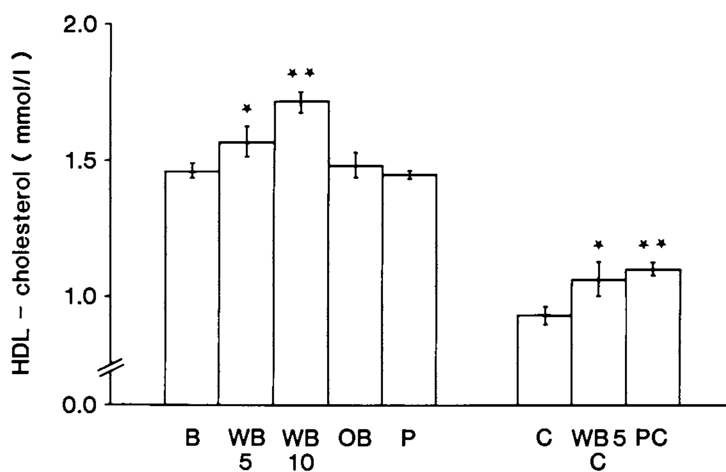


*Figure 5. High-density lipoprotein cholesterol after 3 months in rats given diets containing different dietary fibers, without cholesterol (left) or with 0.5% cholesterol (right). Key is the same as in Figure 4.*

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The effects of dietary fiber on plasma lipoproteins are in some cases, strongly affected by the presence of cholesterol in the diet. This study confirms that dietary cholesterol is essential for the plasma cholesterol lowering effect of pectin. In fact no such effect was obtained in the absence of cholesterol in our high protein/high fat diets, and the effects of pectin might be interpreted as a partial reversal of the disadvantageous effects of dietary cholesterol on the plasma lipoprotein profile.

Interference with the reabsorption of bile salts leading to increased fecal excretion and *de novo* synthesis from cholesterol is generally regarded to be the main mechanism by which pectin and other types of viscous fiber affect plasma lipid concentrations. Some studies indicate, however, that the presence of dietary cholesterol might be important (*26,27*). This is strongly supported by our data. The mechanism *might* be related to disturbed micellar formation affecting intestinal cholesterol absorption. The highly significant favourable effect of pectin (in the cholesterol containing diets) on the HDL/total cholesterol ratio may be related to altered endogenous cholesterol synthesis due to decreased absorption of dietary cholesterol.

Several mechanisms are involved in the regulation of plasma HDL levels. Besides the rate of production of HDL in the liver and intestine and the rate of elimination in peripheral tissues, the pattern of intravascular metabolism is also important in the setting of HDL concentrations. LPL catalyzes the rate-limiting step in the lipolytic degradation of triglyceride-rich lipoproteins; since this reaction is also associated with a transfer of amphipathic surface components (e.g. cholesterol, phospholipids and apoproteins) to the HDL particles, a high LPL activity will generally be associated with high plasma levels of HDL (*16*).

The increase in HDL and total cholesterol in the group fed bran did not seem related to dietary cholesterol. The increase in heart tissue lipoprotein lipase activity indicates an increased peripheral elimination of triglycerides in muscular tissue which is consistent with an elevation in HDL cholesterol. This increase, however, is small compared with changes in lipoprotein lipase activity induced by for instance ethanol (*28*). It is therefore more probable that the effect of wheat bran on HDL cholesterol is also related to altered rate or site of lipid absorption in the intestine.

## Acknowledgments

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# Aminopolysaccharides—Their Potential as Dietary Fiber

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Aminopolysaccharides could serve as a new source of dietary fiber having special physiological and *in vitro* functional properties. The fact that they are of animal and microbial origin and currently not approved for use in foods may be reasons why they have not been considered within the context of dietary fiber. Chitin and chitosan, two major readily available aminopolysaccharides are described in terms of their functional properties in foods, and in their possible role as potential pharmaceutical or food additives. Special attention is paid to their physiological effects in rats. The interaction of chitin and chitosan with lipids and cholesterol, and their effect on lipid absorption suggest that some aminopolysaccharides show strong binding activity towards specific lipids. This activity seems to be a function of the density of positive charge in the aminopolysaccharide molecule where electrostatic forces between lipids and aminopolysaccharides play a greater role than hydrophobic interactions. Since aminopolysaccharides are nondigestible and some reduce lipid absorption in rats, they could be considered as ingredients having "negative" calorie value. The hypocholesterolemic activity of chitosan and its side effects are compared with currently available hypocholesterolemic agents such as cholestyramine.

The widely accepted definition of dietary fiber as proposed by Trowell (1) refers to "plant polysaccharides and lignin which are resistant to hydrolysis by digestive enzymes of man" as the sole constituents of dietary fiber. Being limited to the plant polymers only, this definition does not include undigestible polysaccharides from other sources such

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as those of animal origin, or those which are prepared synthetically. There are several food-approved cellulose derivatives and synthetic gums, and recently polydextrose which "are resistant to hydrolysis by digestive enzymes of man" and therefore, they should be considered dietary fiber as well. Animal aminopolysaccharides which are part of the traditional diet of the Eskimos offer another example (2).

Godding (2) spelled out the limitations of Trowell's definition and proposed a modified definition of so called "edible fiber", which consists of "polysaccharides, related polymers and lignin, which are resistant to hydrolysis by the digestive enzymes of man". It seems logical that all non-digestible edible polymers, regardless of their origin, should be considered dietary or edible fiber because of two common denominators - their polymeric nature and their resistance to hydrolysis by the digestive enzymes of man.

## General Properties

The common natural aminopolysaccharides include chitin, chitosan, keratan sulphate, hyaluronic acid, chondroitin and dermatan sulphates, heparin and blood group substances. While chitin and chitosan contain hydroxyl, amino and acetyl groups, the others contain also carboxyl and sulphate groups. In this paper, attention is paid only to chitin and chitosan. Chitin is a cellulose like polymer which is present in fungal cell walls and exoskeletons of arthropods such as insects, crabs, shrimps, lobsters and others. Chemically, chitin and chitosan are polyglucosamines which are differentiated only by the extent of acetylation of amino groups as shown in Figure 1. Although there is no clear distinction between chitin and chitosan, it is generally accepted that chitin is extensively acetylated, while chitosan is virtually deacetylated. The typical chitins have usually 70-95% degree of acetylation (DA) which corresponds to 15-20.7% acetyl content while chitosans commonly have 15-25% DA corresponding to 3.2-5.3% acetyl content (3). The degree of acetylation is probably the most important parameter in these polysaccharides and it greatly determines their physiological and *in vitro* functional properties.

Another important parameter is the molecular weight (MW) and its associated viscosity. The average MW of some chitins can exceed $10^6$. Chitosan, since it is prepared from chitin by alkaline deacetylation has a lower number average MW ranging usually between $1x10^5$ - $3x10^5$. Chitosan displays a wide range of viscosities in diluted acid media which depend mainly on its MW (3). The relative viscosity of high viscosity chitosans is comparable with the viscosity of guar or tragacanth gums. The viscosity of chitosan likely plays an

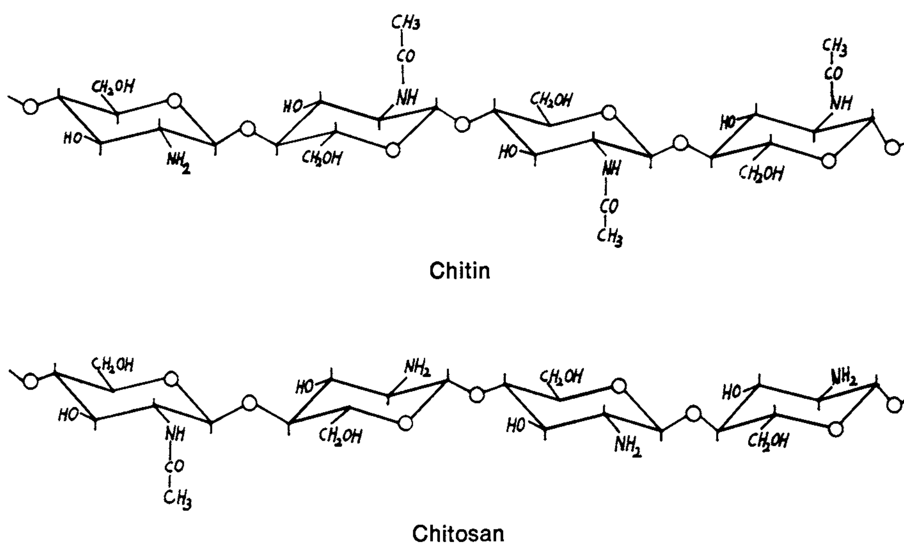


*Figure 1. Chemical structure of chitin and chitosan.*

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important role in its physiological properties later discussed. Only chemically treated (acid hydrolyzed) chitin forms viscous solutions (4).

While chitin is insoluble in common solvents (is soluble in concentrated mineral and formic acids and some special solvents), chitosan is soluble in diluted mineral and organic acids (3). Chitosan is not soluble at pH $> 6.0$ and it functions only in acid systems, which is relevant to its food applicability. Due to the high density of the positive charges chitosan behaves in aqueous acid solutions as a polycationic molecule. This behavior is not typical for chitin because of its high degree of acetylation. Chitosan in the polycationic form binds various anions forming salts or complexes with them. It can be considered a weak anion exchange resin. Chitosan is a strong base toward hydrochloric acid, since the primary amino groups with $pK_a$ 6.3, easily form quaternary nitrogen salts at low pH. At high pH, however, the amino groups of chitosan are weak bases and therefore do not interract with anions and do not dissociate neutral salts.

## Potential Applications in Foods

Chitin and chitosan have been recommended as potential feed and food additives because of their useful functional properties. Though officially not approved for use in foods in the U.S., some aminopolysaccharides are part of the traditional diet of Eskimos as well as being present in different Oriental foods such as tempeh, sufu and even aged beef (5). In the nonpurified form, chitin is present in oyster shell powder which is used in animal feed as a source of calcium. Glucosamine, the monomeric unit of chitosan is used extensively in oral pharmaceutical preparations.

Microcrystalline chitin produced by controlled acid hydrolysis may be suitable for use as food thickener and stabilizer (4). The viscosity and emulsion stability of microcrystalline chitin is 10 to 20 times higher than that of crystalline cellulose making it suitable for applications in mayonnaise, peanut butter and other emulsion-type foods (6). Addition of microcrystalline chitin to white and protein fortified breads has been shown to increase specific loaf volume (7).

Chitosan due to its high viscosity in systems of pH $< 5.5$ can function as thickener, stabilizer or dispersing agent (6). Other applications include gel and film formation (8,9), encapsulation and inclusion in packaging materials (10). Bough (11) recommended chitosan as a coagulating agent for food processing wastes with its subsequent utilization as animal feed. Recently, Rha and Sanchez (12) prepared and described chitosan globules which simulate the cellular

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texture of cooked rice and may have other specific texture applications in foods.

## Physiological Activity

Chitin and chitosan are believed to be of low toxicity. According to Arai (13), the $LD_{50}$ of chitosan in laboratory mice is 16g/kg of body weight. This is similar to that of salt or sugar. In an eight weeks feeding study (14), it has been suggested that chitosan is safe in rats up to 10% in the diet. At 15% level, enlargement of liver and kidneys with a few other changes have been observed. While chitin is believed to be virtually inert in the gastrointestinal tract of mammals, chitosan has been recently associated with strong hypocholesterolemic activity in rats.

Sugano *et al* (15), compared chitosan, cholestyramine, cellulose and different brown algae at 5% level in rat diets containing cholesterol. Besides showing lowest weight gain and highest fecal weight, rats fed chitosan also had the lowest plasma cholesterol and showed its greatest excretion. The excretion of bile acids was only slightly enhanced by chitosan as compared to cholestyramine. The results of twenty days feeding experiment are shown in Table I. Of particular interest was the observation that chitosan caused repression of microbial transformation of cholesterol to coprostanol. Cholestryamine, on the other hand, was more effective in reducing liver cholesterol.

Kobayashi *et al* (16), compared the effectiveness of chitosan with konjac flour and two hypocholesterolemic drugs. Their experiment included one week feeding of cholesterol-containing diet to rats with 4% level of test materials. They observed that chitosan caused the greatest depression of serum cholesterol level and was superior to konjac flour used at the same level and to moristerol and benikol used at 0.5% and 0.8% levels respectively. While konjac flour and benikol caused severe diarrhea, chitosan did not affect the feces.

Nagyvary *et al.* (17), studied high viscosity (4,000 c.p.) chitosan in relation with cellulose, citrus pectin (9% $CH_3O$) and Al salt of pectin at 4% level in rat diet containing cholesterol. The feeding period lasted four weeks. Their observations are summarized in Table II. The level of serum cholesterol in chitosan fed group was approximately 44% lower than in cellulose fed group, 22% lower than in pectin group and about 16% lower than in Al-Pectin fed group. The liver cholesterol was about 60% lower in chitosan-fed group than in the three other groups. There was also a pronounced hypolipidemic effect of chitosan on serum and liver as compared to the other fibers. What seems to be noticeable is the fact that both cholesterol and triglycerides were lowered even more dramatically in liver than in serum.

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Table I.

PLASMA AND LIVER LIPID CONCENTRATIONS AND FECAL OUTPUT OF NEUTRAL STEROLS*

| DIETARY REGIMENS 5% | PLASMA LIPIDS (MG/DL) | | LIVER LIPIDS (MG/G) | |
|---|---|---|---|---|
| | CHOLESTEROL | TRIGLYCERIDES | CHOLESTEROL | TRIGLYCERIDES |
| CELLULOSE | $162 \pm 11^{a}$ | $110 \pm 6^{a}$ | $33.3 \pm 2.6^{a}$ | $42.2 \pm 1.7^{a}$ |
| CHOLESTYRAMINE | $107 \pm 7^{b}$ | $146 \pm 13^{a}$ | $5.8 \pm 0.7^{b}$ | $24.3 \pm 1.8^{b}$ |
| CHITOSAN | $94 \pm 12^{b}$ | $123 \pm 9^{a}$ | $11.0 \pm 1.7^{c}$ | $30.3 \pm 2.5^{b}$ |
| UMITORANOO ALGAE | $143 \pm 16^{a}$ | $125 \pm 7^{a}$ | $36.0 \pm 1.9^{a}$ | $49.5 \pm 3.0^{a}$ |

| DIETARY REGIMENS | CHOLESTEROL EXCRETED (MG/DAY) | COMPOSITION OF FECAL STEROLS (% OF TOTAL) | | |
|---|---|---|---|---|
| | | COPROSTANOL | CHOLESTEROL | PHYTOSTEROLS |
| CELLULOSE | $45.3 \pm 2.2^{a}$ | $28.6 \pm 3.2^{a}$ | $68.4 \pm 3.5^{a}$ | $3.0 \pm 0.8^{ac}$ |
| CHOLESTYRAMINE | $50.7 \pm 4.1^{a}$ | $39.0 \pm 3.1^{b}$ | $54.4 \pm 2.7^{b}$ | $6.6 \pm 1.4^{b}$ |
| CHITOSAN | $68.7 \pm 2.4^{b}$ | $18.3 \pm 3.0^{c}$ | $79.2 \pm 3.3^{c}$ | $2.5 \pm 0.7^{c}$ |
| UMITORANOO ALGAE | $44.5 \pm 5.3^{a}$ | $45.1 \pm 2.0^{bd}$ | $49.9 \pm 2.4^{bd}$ | $5.0 \pm 0.8^{abd}$ |

a,b,c,d: Values represent the mean ± SEM of 6 rats per group. Values within same column not sharing a common superscript letter are significant at $p < 0.01 - 0.05$
*Sugano *et al.* (15)

Table II.

EFFECT OF FIBER ADDITIVES ON CHOLESTEROL AND TRIGLYCERIDE LEVELS*

| ADDITIVE (4%) | CHOLESTEROL LEVELS | | TRYGLYCERIDE LEVELS | |
|---|---|---|---|---|
| | SERUM MG/DL | LIVER MG/G | SERUM MG/DL | LIVER MG/G |
| CELLULOSE | 244±35 | 44±11 | 563±72 | 172±33 |
| PECTIN | 175±13[a] | 42±7 | 404±78[a] | 156±17 |
| Al-PECTIN[f] | 164±10[a] | 36±6[c] | 382±58[a] | 138±12[b] |
| CHITOSAN | 137±9[a,d] | 15±4[a,d] | 303±33[a,e] | 87±20[a,d] |

[a] p<0.01 vs Cellulose; [b] p<0.05 vs Cellulose;
[c] p<0.01 vs Cellulose; [d] p<0.01 vs Al-Pectin;
[e] p<0.02 vs Al-Pectin; [f] Al content of this diet was 0.2%.

*Nagyvary et al. (17)

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Recently Sugano *et al* (18) published a more comprehensive paper on this subject. They observed that on feeding a high cholesterol (0.5%) diet to male rats for three weeks, addition of 2-5% chitosan resulted in a significant reduction of plasma cholesterol, e.g., 25-30%, without influencing either food intake or growth. Concentrations of liver cholesterol and triglycerides were also decreased significantly. Sugano's results are shown in Table III. Plasma cholesterol lowering by chitosan was similar to that of cholestyramine while the liver cholesterol lowering was less extensive, but still showed an approximate 50% reduction. Chitosan used at 10% level in the diet showed additional reduction in plasma cholesterol which was accompanied by growth reduction. The growth reduction was also observed with fine particles of chitosan at 2% level in the diet. Chitosan increased significantly the excretion of neutral sterols, but not acidic sterols. Chitin, which was also used in this study, did not show any physiological activity.

Of particular importance was the long term (81 days) feeding study of a diet containing no cholesterol and a very low (0.5%) level of chitosan, which resulted in the occurence of more cholesterol as high density lipoproteins (HDL) and less as very low density lipoproteins (VLDL) as compared to the basal diet (18).

Unpublished experiments (19) using rats with a cannulated left thoracic lymphatic chanel (20) indicated that during acute conditions, there was a massive reduction in absorption of cholesterol as well as oleic acid in the presence of chitosan. In this experiment, rats were given by gastric intubation, tritiated cholesterol, $C^{14}$ oleic acid and sodium taurocholate to which either chitosan or cholestyramine were added. The lymph was collected for twenty-four hours. The results shown in Table IV indicate that chitosan inhibits the absorption of cholesterol and oleic acid at least as effectively as cholestyramine. In chronic studies (19), when rats were fed diet containing 1% and 5% levels of chitosan and cholestyramine respectively for 4 weeks, the same inhibition of absorption of cholesterol and oleic acid between chitosan and cholestyramine was observed.

Recently, the hypocholesterolemic effect of chitosan in Guinea pigs has been reported (21).

## Possible Mechanism of Hypocholesterolemic and Hypolipidemic Activity of Chitosan

The mechanism of hypocholesterolemic and hypolipidemic action is not universal and it frequently varies from one compound to another. However, compounds having chemical and other similarities may also display a similar mechanism of action.

Table III.

EFFECTS OF CHITOSAN AND CHITIN AND RELATED COMPOUNDS ON PLASMA AND LIVER LIPIDS AND FECAL EXCRETION OF STEROLS[a],*

| EXPERIMENT[b] | DIETARY MANIPULATIONS | PLASMA | | LIVER | | STEROID EXCRETION | |
|---|---|---|---|---|---|---|---|
| | | CHOLESTEROL | TRIGLYCERIDE | CHOLESTEROL | TRIGLYCERIDE | NEUTRAL | ACIDIC |
| | % | MG/DL | | MG/G | | MG/DAY | |
| 1 | CONTROL | 127±5[d] | 104±10[d] | 35±0.9[d] | 53.7±6.2[d] | 49.6±3.7[c] | 13.1±1.1[c] |
| | CHITOSAN, 5 | 93±5[e] | 110±12[d] | 18.3±2.9[e] | 31.9±3.3[e] | 67.7±2.4[d] | 13.6±0.4[c] |
| | CHITOSAN, 10 | 79±6[e] | 106±10[d] | 11.1±2.0[f] | 26.8±3.0[e,f] | 73.6±3.4[d] | 13.9±0.8[c] |
| | CHOLESTYRAMINE,5 | 86±11[e] | 114±9[d] | 4.9±0.4[f] | 19.3±1.5[f] | 45.2±5.4[c] | 30.3±1.0[d] |
| 3 | CONTROL | 114±4[e] | 125±11[d] | 41.0±2.8[e] | 53.1±5[e] | 50.1±5[d] | 17.0±2.7[d] |
| | CHITIN, 2 | 106±3[e] | 132±12[d] | 37.8±3.9[e] | 52.5±4.1[e] | 50.6±4.3[d] | 16.6±1.3[d] |
| | CHITOSAN, 2 | 76±4[d] | 137±9[d] | 11.3±3.0[f] | 33.7±4.9[d] | 74.7±4.6[c] | 17.2±1.2[d] |

[a]Mean ± SEM. [b]The age of animals at the start of experiment was: 52 days (experiment 1), 37 days (experiment 3). Feeding period: 20 days. All diets contained 0.5% cholesterol.
[d-f,c-e]Values in the same column in each experiment not sharing common superscript letters are significantly different at $p<0.05$ to 0.01.

*Sugano et al. (18).

Table IV.

CHOLESTEROL AND OLEIC ACID ABSORPTION IN RATS WITH CANNULATED THORACIC LYMPHATIC CHANEL[a]

| MEASUREMENT (NO. OF RATS) | CONTROL (3) | 50 MG CHOLESTYRAMINE (5) | 50 MG CHITOSAN (7) |
|---|---|---|---|
| CHOLESTEROL | | | |
| % ABSORPTION | 59.5 ±3.8 | 31.7 ±5.6 | 29.4 ±9.4 |
| MG ABSORBED | 29.8 ±1.9 | 15.9 ±4.7 | 14.7 ±7.9 |
| % INHIBITION | ---- | 46.7 ±9.4 | 50.6 ±15.8 |
| OLEIC ACID | | | |
| % ABSORPTION | 69.1 ±2.2 | 46.9 ±5.2 | 41.4 ±12.6 |
| MG ABSORBED | 194.9 ±6.2 | 131.3 ±14.6 | 115.1 ±35.3 |
| % INHIBITION | ---- | 32.1 ±7.6 | 40.9 ±18.1 |

[a]Vahouny et al. (19), unpublished results.

Among the typical food ingredients which have been shown to be hypocholesterolemic is the group of specific natural polysaccharides. This includes food gums such as guar gum, locust bean gum, oat B-glucan, konjac mannan, some pectins and most recently chitosan. In spite of their different chemical structure, these substances have a few common denominators of which some may be responsible for their hypocholesterolemic and hypolipidemic action. These properties include nondigestibility in the upper GI tract, high viscosity, polymeric nature and high water binding *in vitro* and low water binding in the lower GI tract. Their nondigestibility and high viscosity in the upper GI tract are believed by some researchers to be primarily responsible for their hypocholesterolemic action. The high viscosity of undigestible polymers likely reduces diffusion and it may result in coating of the inner walls of the gut and thus prevent or delay the absorption of nutriens.

Chitosan, while possessing most of these properties, has an additional highly relevant characteristic resulting from its chemical structure. It is the ability to bind different anions, i.e., bile acids or free fatty acids at low pH by ionic bonds (18,22). The *in vitro* binding of taurocholate by chitosan was observed (18) and was comparable in magnitude to that of cholestyramine. *In vivo* however, the excretion of acidic sterols after feeding chitosan was the same as the control and it was approximately 2x lower than that corresponding to cholestyramine (18). This could be possibly explained by the fact that chitosan as a weak basic anion exchange resin, effectively binds anions such as bile acids only in low pH environment. At pH > 6.0, the ion exchange capacity is greatly reduced, the binding is not strong enough and bile acids can be released and reabsorbed in the lower GI tract. Cholestyramine, colestipol and DEAE Sephadex on the other hand are strong basic anion exchangers from which the bile acids are difficult to be released. This hypothesis is supported by the fact that feeding the cholestyramine resulted in reduction of liver cholesterol significantly more than feeding of chitosan (18). The unique ability of chitosan to reduce serum cholesterol without increasing the fecal acidic sterols may have a great significance since the increased binding and excretion of bile acids caused by some fibers and drugs, specifically by cholestyramine, has been implicated in the possible enhancement of cancer of the colon (23,24). Cassidy *et al* (25) have observed that a six week ingestion of diets containing 2% levels of cholestyramine, colestipol or DEAE Sephadex, or 15% levels of alfalfa or pectin resulted in considerable changes in ultrastructural topography of rat jejunum and colon. The authors hypothesized that the observed accumulation of lipids in the small intestinal mucosa suggested an interference with lipid transport from the cell (25).

Chitosan appears to have a stronger affinity toward lipids than cholestyramine (26). According to Nagyvary (26), the maximum binding capacity of chitosan is about 12 mg lipids/mg of chitosan. His *in vitro* experiments indicate that chitosan binds lipid droplets *in toto* unlike cholestyramine which is more selective for bile salts. Vahouny *et al.* (27) have observed however, that cholestyramine has an extremely high binding capacity toward individual micellar components *in vitro* including bile salts, lecithin, cholesterol, fatty acids and monolein.

It is noteworthy to mention that unabsorbable antibiotics such as neomycin, tetracyclines and others are known to possess hypocholesterolemic properties and they can induce steatorrhea (28,29). Although not polymeric, these substances are polycationics having several free amino groups, mainly primary, but also secondary and tertiary. Eyssen *et al* (30) studied the cholesterol lowering effect of basic antibiotics in chicks fed cholesterol containing diet. They concluded that the hypocholesterolemic activity may not be caused by antibiotic properties of these drugs, but rather by the polybasic nature of these molecules. One of the reasons which supported this conclusion was that N-methylated neomycin which is a basic neomycin derivative without antibiotic properties, was also an effective cholesterol lowering agent. The effectiveness of tested basic antibiotics depended on number of basic amino groups in their molecules since neither N-acetylated neomycin nor streptomycin with only three basic amino groups were effective. A condensation product of two molecules of streptomycin, bearing seven basic groups, however, was hypocholesterolemic. The authors suggested that the ability of polybasic antibiotics to form insoluble salts with bile acids in the gut and their increased excretion is likely responsible for serum cholesterol lowering effect. There seems to be an analogy between the non-effective chitin and N-acetylated neomycin versus highly effective chitosan and neomycin in ability to reduce the serum cholesterol. Chitosan, however, does not seem to possess a variety of intestinal functions such as antibiotic and other properties specific for neomycin.

The available data indicate that the hypocholesterolemic and hypolipidemic activity of chitosan is probably due to disruption and/or inhibition of micelle formation. At pH 6.0-6.5 chitosan begins to precipitate and as the linear chains of the polysaccharide start to aggregate, they can entrap the whole micelles. The entrapped cholesterol, fatty acids and monoglycerides thus escape absorption. Such "polar entrapment," shown in Figure 2, can occur in the duodenum. Another mode of action could be the "disintegration" of mixed micelles, which can start before the precipitation of chitosan, and in which the free fatty acids and bile acids are selec-

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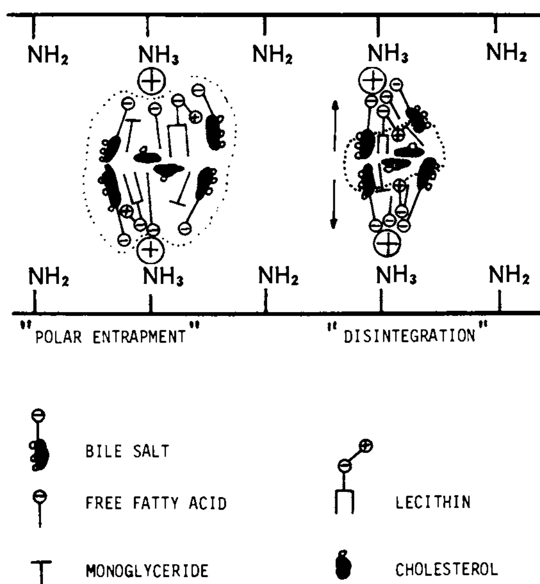


*Figure 2. Possible mode of interaction of chitosan with mixed micelles.*

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tively removed from mixed micelles by forming electrostatic bonds with the protonized amino groups of chitosan. Yet another possibility may exist in which the mixed micelles are not formed at all, since the protonized amino groups can immediately bind negatively charged free fatty acids and bile acids prior the micelle formation can take place.

An alternative possible mechanism of hypolipidemic and hypocholesterolemic action of chitosan could be the inhibition of intestinal lipases. A limited work on inhibition of pancreatic lipase by neomycin in humans (31) and in vitro (32), and by cellulose and xylan (32) also in vitro have been reported. No work on the possible inhibition of intestinal lipases by chitosan has been published yet.

Though chitosan may have some similarity with cholestyramine and other hypocholesterolemic/hypolipidemic drugs having character of basic anion exchangers, there seem to be several significant differences in the mechanism of their action as well as in their side effects. The latest results indicate that chitosan is equally effective as cholestyramine in reducing the absorption of dietary cholesterol and lipids however, it does not cause the structural deviations in the intestine of rats when used at 1-5% levels (19). The more detailed comparison of hypocholesterolemic resins, specifically cholestyramine with chitosan is shown in Tables V and VI.

Due to these unique properties, the relative safety and availability, some amino-polysaccharides will likely find, sooner or later, specific applications either in processed foods, special dietary or medical foods or in pharmaceutical preparations.

Table V.

COMPARISON OF HYPOCHOLESTEROLEMIC RESINS WITH CHITOSAN

| AGENT | FUNCTIONAL GROUPS | AVERAGE BILE ACID BINDING CAPACITY % | INTESTINAL VILLI OR COLONIC RIDGES WITH STRUCTURAL DEVIATIONS % | | EFFECT ON BILE ACIDS EXCRETION |
|---|---|---|---|---|---|
| | | | JEJUNUM | COLON | |
| CHOLESTYRAMINE | QUATERNARY AMINES | 80-100[b] | 64.2[a] | 39.5[a] | INCREASE ($\leq$x 10) |
| COLESTIPOL | SECONDARY AND TERTIARY AMINES | 50-60[b] | 35.9[a] | 55.0[a] | INCREASE (x 2-5) |
| DEAE SEPHADEX | TERTIARY AND QUATERNARY AMINES | 30-40[b] | 13.0[a] | 40.6[a] | INCREASE (x 3) |
| CHITOSAN | PRIMARY AMINES | PROBABLY LOW | VERY SMALL[c] | NO EFFECT[c] | NO EFFECT |

[a] 2% agent in rat diet; [b] *in vitro* binding at pH 7.0; Cassidy *et al.* (25)

[c] 1% and 5% agent in rat diet; Vahouny *et al.* (19)

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Table VI.

COMPARISON OF *IN VITRO* AND *IN VIVO* PROPERTIES OF CHITOSAN AND CHOLESTYRAMINE

| | CHITOSAN | CHOLESTYRAMINE |
|---|---|---|
| ANION EXCHANGER | WEAK | STRONG |
| VISCOSITY | HIGH | NONE |
| SOLUBILITY | AT pH < 6.0 | INSOLUBLE |
| LIPID BINDING | HIGH | MODERATE |
| BILE ACIDS BINDING | ? | VERY HIGH |
| SERUM CHOLESTEROL LOWERING | HIGHLY EFFECTIVE | HIGHLY EFFECTIVE |
| LIVER CHOLESTEROL LOWERING | HIGHLY EFFECTIVE | EXTREMELY EFFECTIVE |
| SERUM TRIGLYCERIDES LOWERING | NOT CLEAR | SLIGHT INCREASE |
| LIVER TRIGLYCERIDES LOWERING | EFFECTIVE | HIGHLY EFFECTIVE |
| HDL,LDL | INCR. CHOL.IN HDL | LOWERS LDL |
| LIPID AND CHOLESTEROL LYMPHATIC ABSORTPION | HIGHLY INHIBITED | HIGHLY INHIBITED |
| NEUTRAL FECAL STEROLS | HIGH | LOW |
| ACIDIC FECAL STEROLS | LOW | HIGH |
| FECAL CHOLESTEROL/ COPROSTANOL | HIGH | LOW |
| CONSTIPATION, DIARRHEA | NONE | CONSTIPATION |
| FECAL VOLUME | LARGE | SMALL |
| GUT MORPHOLOGY | NEGLIGIBLE EFFECT | ADVERSE EFFECT |

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9

# Catabolism of Mucopolysaccharides, Plant Gums, and Maillard Products by Human Colonic *Bacteroides*

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The mixture of complex carbohydrates which enters the human colon includes not only plant cell wall polysaccharides but also a variety of other types of carbohydrate such as mucopolysaccharides (from dietary meat or host secretions), plant gums which are added to foods as emulsifiers, and Maillard reaction products. Some strains of colonic Bacteroides which can degrade plant cell wall polysaccharides can also degrade one or more of these other types of carbohydrates. Bacterial strategies for degrading these substances differ. For example, the enzymes responsible for catabolism of mucopolysaccharides by Bacteroides are cell-associated rather than extracellular and are not associated with the bacterial membranes. By contrast, the catabolism of guar gum, a viscous plant galactomannan, involves both an extracellular enzyme and at least one membrane-bound enzyme. Breakdown of polygalacturonic acid involves a membrane-bound enzyme. A feature which is common to all three of these polysaccharide-degrading systems is that the enzymes are inducible, that is they are produced only when the appropriate substrate is encountered by the bacteria. The Maillard product, fructosylglycine, is not utilized by strains of Bacteroides which can ferment fructose and fructosyl compounds such as sucrose, presumably because of difficulties in hydrolyzing the C-N bond which binds fructose to glycine. Another Maillard product, isomaltol-galactoside, which contains a glycosidic bond, can be degraded and the galactose moeity utilized. Utilization of this compound, which is an analogue of lactose, appears to occur via the lactose system. The enzyme which degrades

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isomaltolgalactoside, unlike the polysaccharidases which are produced by the same organism, is constitutive rather then inducible.

Nutritional studies in humans have demonstrated quite clearly that some components of dietary fiber are degraded extensively during passage through the intestinal tract (1). Degradation of dietary fiber appears to occur in the colon where component polysaccharides are fermented by saccharolytic bacteria which reside there. Although this fermentation occurs in the colon, it may contribute to human nutrition since a significant percentage of the bacterial fermentation products can be absorbed through the colonic mucosa (1, 2). Because of the potential contribution of dietary fiber components to host nutrition and also because of an increased awareness of the role of the bacterial flora of the colon in human disease, considerable attention has been devoted in recent years to the possible effects of dietary fiber on the colonic flora.

The focus on bacterial fermentation of components of dietary fiber from conventional sources such as bran should not prevent us from considering other types of carbohydrate which may also be fermented by colon bacteria. For example, the host itself produces a variety of polysaccharides which reach the colon. These include mucins from the secretions which lubricate the intestinal tract and mucopolysaccharides from slonghed epithelial cells. It is difficult to obtain precise information concerning how much of this material is produced daily, but the amount could easily be comparable to the amount of polysaccharide which is ingested in the diet. Some of these host-produced polysaccharides, particularly the mucopolysaccharides, are readily utilizable by some of the same bacterial species which ferment plant polysaccharides (3).

Another potential source of carbohydrate for colon bacteria consists of polysaccharides which are added to foods as emulsifiers (e.g. guar gum, alginate) or which are used as stool softeners (e.g. psyllium). Plant polysaccharides such as guar gum are also being evaluated for use in the treatment of diabetes because they retard glucose absorption (4, 5). The amount of polysaccharide from these sources will vary from person to person but could be appreciable in some cases.

Maillard products, associated with the browning of foods, could also provide some carbohydrate for intestinal bacteria. At present, this possibility is merely speculative. Many possible Maillard products exist (6, 7). The Maillard products which are most likely to be utilizable by bacteria are the early products, such as glycosylamines and aryl-glycosides, which resemble fermentable disaccharides. Some Maillard products such as catechols or substituted pyridines could actually be toxic to

colon bacteria. No estimates are yet available as to the amounts of these substances which actually reach the colon. Moreover, the number and variety of Maillard products which can be formed in foods makes it difficult to decide which compounds to investigate. Some Maillard products such as aryl-glycosides could be cleaved by enzymes in the host's small intestine, although this might not be the case for individuals with digestive enzyme deficiencies such as lactose intolerance. Moreover, some recent nutritional studies have shown that varying amounts of compounds such as sucrose or starch, which are generally considered to be completely digestible, can escape digestion in the small intestine and thus reach the colon (8, 9). Accordingly, the possibility that colon bacteria are exposed to Maillard products should be considered.

## Polysaccharide Utilization by Colon Bacteria

From these examples, it can be seen that studies of the effect of diet on the intestinal flora must take into account the availability of a variety of polysaccharides. Information about *in vitro* fermentation of different polysaccharides by colon bacteria can help us to make predictions about what sorts of carbohydrate may be utilizable *in vivo*, but this information raises still other questions. To see this, consider some of the results of surveys of polysaccharide fermentation by numerically predominant species of colon bacteria (3, 10). Some of these results are summarized in Table 1. One thing that is immediately apparent from Table 1 is that the most versatile fermenters of polysaccharides are *Bacteroides* species. Moreover, many of these species can ferment several types of polysaccharides. This raises the question of which type of polysaccharide would be utilized preferentially *in vivo* if these bacteria were to be confronted by several utilizable polysaccharides at the same time. Not only do individual strains or species exhibit the ability to ferment a variety of polysaccharides, but it is also the case that each polysaccharide can be fermented by more than one species. Do all of these species actually participate in degrading a particular polysaccharide *in vivo* or does one species predominate due to a more efficient utilization system? In short, results of surveys of polysaccharide fermentation allow us to predict that many types of carbohydrate (including dietary fiber components) will be fermented in the colon, but these results do not provide answers to other questions such as which organisms actually degrade a polysaccharide *in vivo* or whether a particular dietary carbohydrate (e.g. an emulsifier such as guar gum) might upset the normal patterns of polysaccharide catabolism in the colon if it were present in high enough concentrations in the diet.

Table 1. Species of intestinal bacteria which can degrade various indigestible polysaccharides (3, 10)

| Polysaccharide | Species of colonic bacteria which can degrade it |
|---|---|
| Chondroitin sulfate (mucopolysaccharide) | Bacteroides thetaiotaomicron<br>Bacteroides ovatus<br>Bacteroides '3452A'*<br>Bacteroides fragilis subsp.a<br>Bacteroides uniformis |
| Guar gum, locust bean gum (galactomannan) | Bacteroides ovatus<br>Bacteroides uniformis<br>Ruminococcus albus |
| Xylan | Bacteroides ovatus<br>Bacteroides fragilis subsp.a<br>Bacteroides eggerthii<br>Bacteroides vulgatus<br>Bifidobacterium adolescentis<br>Bifidobacterium infantis |
| Pectin, polygalacturonic acid | Bacteroides thetaiotaomicron<br>Bacteroides ovatus<br>Bacteroides '3452A'*<br>Bacteroides fragilis subsp.a<br>Bacteroides vulgatus<br>Eubacterium eligens |
| Arabinogalactan | Bacteroides thetaiotaomicron<br>Bacteroides ovatus<br>Bacteroides '3452A'*<br>Bacteroides uniformis<br>Bacteroides vulgatus<br>Bacteroides 'T4-1'* |

* Unnamed DNA homology groups (11).

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Our approach to these and similar question has been to investigate the mechanisms by which pure cultures of colon bacteria utilize individual polysaccharides *in vitro*, with a view to determining what factors affect the organism's decision to utilize a particular type of carbohydrate. This approach is based on the assumption that information about the specific features and limitations of polysaccharide-catabolizing systems in colon bacteria will permit us either to make predictions about the extent to which catabolism of a particular class of polysaccharides can occur *in vivo* or to develop specific methods for detecting metabolic states in bacteria *in vivo*.

## Strategies for Polysaccharide Metabolism

To illustrate some characteristrics of polysaccharide utilization systems of *Bacteroides* species, we will compare the systems involved in utilization of a host-produced mucopolysaccharide (chondroitin sulfate), a plant galactomannan (guar gum) and polygalactaronic acid. *Bacteroides thetaiotaomicron* ferments not only chondroitin sulfate but also structurally similar mucopolysaccharides such as hyaluronic acid and heparin. The degradative enzymes are inducible, i.e. they are only produced in appreciable amounts only when the organism is exposed to the appropriate substrate (12). The rate at which the enzymes are produced following exposure to the polysaccharide and the amount of enzyme produced are both proportional to the concentration of polysaccharide in the medium. Thus the higher the concentration of a polysaccharide the more likely it is to be utilized. Induction of enzyme activity is fairly specific. Growth on chondroitin sulfate is accompanied by production of enzymes which attack chondroitin sulfate and those mucopolysaccharides which closely resemble chondroitin sulfate (e.g. hyaluronic acid). Enzymes for degrading heparin, a mucopolysaccharide which contains a different glycosidic linkage, are not induced by chondroitin sulfate. Bacteria which are growing on heparin produce enzymes which attack heparin but not chondroitin sulfate or hyaluronic acid (K. Voss and A. Salyers, unpublished results).

None of the enzymes which are involved in the breakdown of chondroitin sulfate by *B. thetaiotaomicron* are extracellular. In fact, the first degradative enzyme, chondroitin lyase, appears to be located in the periplasmic space between the inner and outer membranes of these gram negative organisms (13). Chondroitin lyase breaks chondroitin sulfate into disaccharides and these disaccharides are further degraded by enzymes which are located in the cytoplasm. None of these enzymes is membrane-bound. However there are probably some membrane proteins involved, e.g. receptors on the cell surface which bring the chondroitin sulfate into contact with the lyase and

proteins in the cytoplasmic membrane which transport the disaccharides into the cytoplasm.

The chondroitin sulfate-degrading systems of two species of colon bacteria which are closely related to B. thetaiotaomicron, Bacteroides ovatus and Bacteroides '3452A'(an unnamed DNA homology group of Bacteroides), appear to be similar to the chondroitin sulfate-degrading system of B. thetaiotaomicron in that the degradative enzymes are inducible and are cell-associated rather than extracellular (A. Salyers and M. O'Brien, unpublished data). Moreover, none of these enzymes in B. ovatus or B. '3452A' are membrane-bound. It is interesting to note that DNA homology data indicates that B. ovatus and B. '3452A' have 30-45% cross homology with B. thetaiotaomicron (11). We are currently investigating the possibility that some of this shared genetic material contains the genes for chondroitin sulfate utilization, i.e. that these three species have identical systems for degrading chondroitin sulfate.

Guar gum, a galactomannan which is used as an emulsifier in processed foods, is utilized by a different sort of system. When B. ovatus is growing on guar gum, there is an enzymatic activity in the extracellular fluid which decreases the viscosity of guar gum (14). This enzyme reduces the molecular weight of guar gum from $10^6$ to $4 \times 10^5$, but does not produce small carbohydrate fragments. In addition to this enzyme, there is also cell-associated galactomannanase activity, most of which stays with the cell membranes even after treatment with 0.5M NaCl. This indicates that the enzyme is an integral membrane protein. The product of this galactomannanase appears to be a trisaccharide (15). Thus the galactomannan-degrading system differs from the chondroitin sulfate-degrading system in the location of the initial degradative enzymes. However, these two systems resemble each other in that the degradative enzymes are inducible.

B. thetaiotaomicron does not ferment guar gum. However it does ferment another viscous polysaccharide, polygalacturonic acid. Recent investigations of polygalacturonic acid breakdown by B. thetaiotaomicron have shown that this organism produces an inducible membrane-bound polygalacturonase (J. Leedle and A. Salyers, unpublished results). No extracellular enzyme was detected.

Thus colonic Bacteriodes appear to have different strategies for degrading different polysaccharides. It may be the case that in general the type of mechanisms used for degrading a particular polysaccharide depends on the type of polysaccharide being utilized rather than on the species of the organism.

## Utilization of Maillard Products

At present there is little information concerning what types of Maillard products reach the colon. The lignin-like

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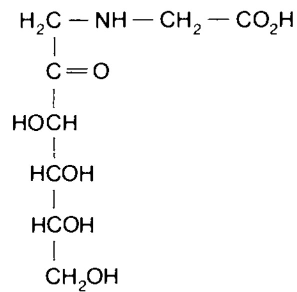
polymers which are the end products of the Maillard reaction reach the colon but are not degraded by colon bacteria (Van Soest, private communication). It is not known whether earlier products in the Maillard reaction (e.g. aryl-glycosides, glycosylamines) are digested in the small intestine. However, we can determine whether these Maillard products could be utilized by saccharolytic bacteria if they were to reach the colon.

We have investigated the utilization of two Maillard compounds, fructosyl-glycine and isomaltol-galactoside, by *Bacteroides* species. Fructosyl-glycine is formed during condensation of glucose with glycine (7, 16). Isomaltol-galactoside is formed as a consequence of the interaction of an amine with lactose. The resulting reactions culminate in dehydration of the glucose moeity to produce isomaltol (17). Isomaltol and isomaltol-galactoside are found in baked breads and contribute to the characteristic odor and flavor of these foods (18).

The structures of fructosyl-glycine and isomaltol-galactoside are shown in Figure 1. Fructosyl-glycine could be considered a structural analogue of sucrose or of other fructosyl compounds such as inulin. We tested two species of colonic *Bacteroides* (*B. ovatus* and *B. thetaiotaomicron*) for the ability to grow in medium which contained fructosyl-glycine as the sole carbon source. Both of these species ferment sucrose and *B. ovatus* also ferments inulin, but neither could utilize the fructose in fructosyl-glycine. Moreover, crude extracts from sonically disrupted *B. ovatus* or *B. thetaiotaomicron* (grown on fructose, sucrose or inulin) did not degrade fructosyl-glycine. The inability of these *Bacteroides* species to utilize fructoryl-glycine as a carbon source does not rule out the possiblity that other intestinal becteria might be able to utilize it.

Although fructosyl-glycine was not utilized as a carbon source by *B. ovatus*, it did appear to inhibit growth of *B. ovatus* on other carbohydrates (Figure 2). This inhibitory effect occurred with glucose as well as with fructose and fructosyl compounds. Note that this effect was not very pronounced and that it required high concentrations of fructosyl-glycine. Thus it is not likely to be important in the colon where concentrations of compounds such as fructosyl-glycine would be expected to be much lower. Fructosyl-glycine did not inhibit the growth of *B. thetaiotaomicron* on fructose, glucose or sucrose. So the effect of fructosylglycine on growth may be limited to certain species.

Investigations, such as those described above, of the effect of compounds like fructosyl-glycine on growth rate of bacteria have a major drawback. The compound being tested must be incubated for 10-20h at 37°C in growth medium. Although fructosyl-glycine is relatively stable and there was no visible darkening of the medium during the incubation period, we were able to detect by thin layer chomatography traces of compounds



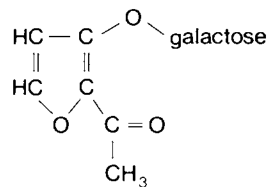




*Figure 1. Structures of fructosyl–glycine and isomaltol galactoside.*

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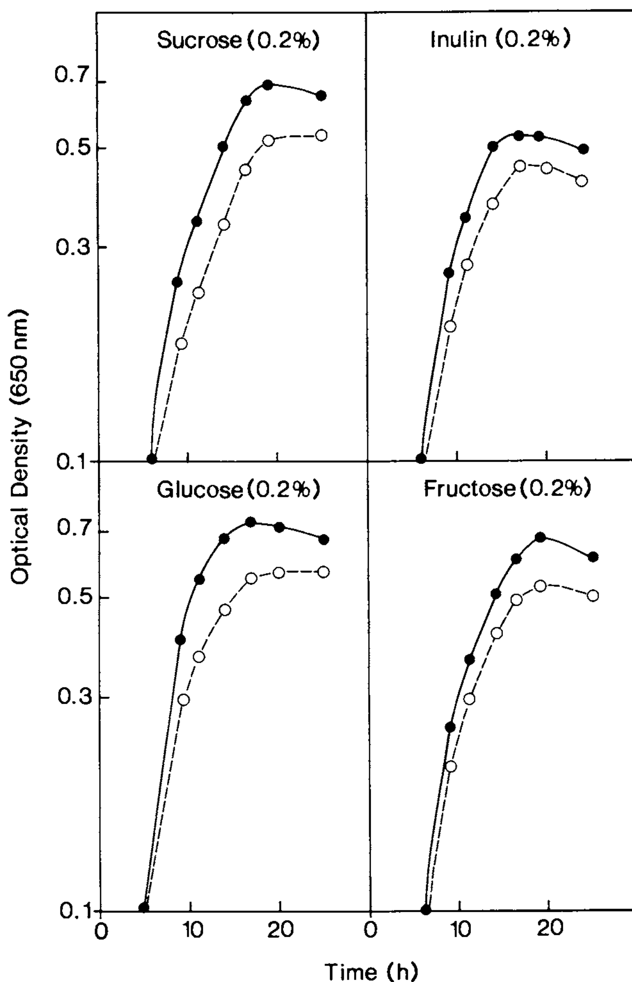


*Figure 2. Effect of fructosyl–glycine on the growth of* B. ovatus *in a medium that contained glucose, fructose, sucrose, or inulin as the sole source of carbohydrate. The basal medium has been described previously (*12*).*

*The carbohydrate and fructosyl–glycine were filter-sterilized and added to the basal medium after autoclaving. The initial concentration of both the carbohydrate and fructosyl–glycine was 2 mg/mL. Cultures grew more slowly in the medium that contained fructosyl–glycine plus the fermentable sugar (○) than in the medium that contained only the fermentable sugar (●).*

other than fructosyl-glycine in uninoculated medium which had been incubated for 12h at 37°C. This raises the question of whether fructosyl-glycine itself or a further reaction product was in fact responsible for the effect on *B. ovatus*.

We tested the effect of fructosyl-glycine on uptake of glucose, fructose and sucrose by *B. ovatus*. We found no significant effect of fructosyl-glycine on uptake of any of these compounds. However, the uptake of [$^3$H]sucrose was so slow that we could have failed to detect reductions in the rate of uptake which were on the order of 20% or less. We also tested the effect of fructosyl-glycine on sucrase activity in crude extracts from disrupted bacteria. Fructosyl-glycine did inhibit sucrase activity. Further investigations along these lines are needed to establish whether fructosyl-glycine has any other effects on bacterial metabolism which might explain why it inhibits growth.

The results of our investigations of the effects of another Maillard product, isomaltol-galactoside, can be summarized as follows (B. Schmetter and A. Salyers, unpublished data): *B. thetaiotaomicron* utilized the galactose in isomaltol-galactoside. During utilization, isomaltol accumulated in the extracellular fluid. Isomaltol was not utilized, but at high enough concentrations (4-5 mg/ml) it inhibited growth. Thus isomaltol is toxic to *Bacteriodes*, but only at concentrations much higher than those which are likely to be found *in vivo*. Isomaltol did not affect uptake of simple sugars on amino acids. However, it stopped bacterial synthesis of protein and DNA. Since [$^3$H]-labeled isomaltol was not taken up by the bacteria in detectable quantities, it is not clear how isomaltol exerts this effect. One possibility is that it binds to membranes.

Isomaltol-galactoside appears to be utilized via the lactose system of *B. thetaiotaomicron*. Isomaltolgalactoside inhibited uptake of [$^{14}$C]-lactose. Moreover mutants which were deficient in lactose uptake did not utilize isomaltol-galactoside. Isomaltol-galactoside was hydrolyzed almost as rapidly by bacterial enzymes as lactose. Whether or not isomaltol-galactoside itself is actually present in significant quantities in the colon, it is interesting to note that lactose analogues of this sort are readily utilized by a lactose-fermenting organism. Other Maillard aryl-glycosides may also be utilized by bacteria which normally ferment structurally similar saccharides.

The enzyme activity which was associated with the breakdown of isomaltol-galactoside was constitutive. In this respect, the catabolic system for isomaltol-galactoside differs from the catabolic systems for degrading poysaccharides. It also differs from the lactose system in *E. coli* which is an inducible system. The fact that isomaltol-galactoside breakdown can occur without

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prior adaptation of the bacteria indicates that these substances would be rapidly utilized *in vivo*.

## Conclusions

Human colonic bacteria are quite versatile with respect to their ability to ferment polysaccharides. Moreover, individual species do not seem to have a consistent strategy for degrading polysaccharides. In some cases, cell-associated periplasmic enzymes are used. In other cases the enzymes are extracellular or even membrane-bound. It may turn out to be the case that the catabolic strategy is specific for the type polysaccharide rather than for the species, i.e. different species may utilize a particular type of polysaccharide in the same way.

Because of the variety of polysaccharides which can be fermented by some *Bacteroides* species, it is difficult to predict with certainty which polysaccharides in the complex mixture of dietary and host-produced carbohydrates that enter the colon will be degraded most rapidly and most extensively. Further information about how these organisms make choices between different polysaccharides *in vitro* may help to clarify this issue. However, nutritionists who are interested in catabolism of dietary fiber components *in vivo* should be aware that the bacteria may prefer other sources of carbohydrate, such as mucopolysaccharides from host secretions or even Maillard products, to the dietary polysaccharide under study, and that this preference may influence catabolism of a particular polysaccharide in ways which we cannot at present predict. Effects of this sort may be responsible for some of the individual-to-individual variation which is encountered in nutritional studies of dietary fiber utilization.

## Acknowledgments

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# 10

# Some in Vitro and in Vivo Properties of Dietary Fibers from Noncereal Sources

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Non-cereal dietary fiber sources are compared and evaluated for cation exchange, water holding capacity and *in vitro* fermentability by gut microflora. Fiber sources studied include Psyllium gum, alfalfa, purified wood cellulose, pectin, cabbage, synthetic Maillard product, soybean hulls and propol. Sources are compared to the standard AACC wheat bran. Fermentability of the substrates alfalfa, wood cellulose and wheat bran show the largest unfermentable residue and the greatest differences between inocula sources (rumen inocula greater than human fecal inocula in all cases). Water-soluble sources (pectins) are completely fermentable, the exception of Psyllium gum which contains about 44% of an unfermentable residue. Cabbage pectin is unusual in having considerable cold water (50%) solubility. Cation exchange binding measured with copper are the highest for pectin, cabbage, bran, Maillard product = alfalfa and soybean hulls descending in that order. Wood cellulose, propol and Psyllium gum have essentially no metal binding capacity. Dietary fibers of high prophylactic value appear to have substantial metal binding capacity and are divisible into two classes based on fermentability. Human feeding studies indicate that the fiber sources containing unfermentable residues will contribute to fecal bulk and, therefore, transit and passage, while the more fermentable have their major effects upon gut microflora. These two aspects are complementary relative to the effectiveness of dietary fiber.

The identification of dietary fibers as an overlooked factor in human health and disease has promoted a great deal of interest

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in the character and quality of food fiber sources. While considerable attention has been given to cereal brans, other sources of dietary fiber exist that offer a variety of qualities. These include vegetables, gums, wood products and synthetic polymers.

Dietary studies with humans and other animal models have emphasized the varied nature of responses to different fiber sources. Transit and passage depend on the bulk of colonic contents which in turn appears to depend upon lignified unfermentable residues as found in wheat bran. The particle size of bran is an important factor influencing its bulking effect, coarser residues having more effect (1). In general unlignified sources such as vegetables and pectin have less effect upon transit and influence gut microflora more because of their high fermentability. These residues being less rigid structurally may have greater swelling and hydration characteristics (2, 3).

Hypotheses to account for the effects of dietary fiber have emphasized the bulking and binding capacities, in the argument that faster transit reduces exposure to hazardous substances, while the binding effect might account for the specific binding of bile salt and other organic substances and the reported negative effects upon mineral availability (4). A less recognized factor is that of the gut microflora which if stimulated may incorporate ammonia, carbon skeletons and minerals into the cellular residue (5, 6). This incorporation into cellular mass would provide an alternative unavailable sink for these substances.

The purpose of this investigation is to present data on the variety of dietary fiber sources and characterize them relative to the physico-chemical characteristics supporting metal ion binding, hydration and fermentability.

## Fiber Sources

The following dietary fiber sources were studied: Psyllium gum, propol (a glucomannan gum), commercial pectin, solka floc (a wood cellulose), cabbage, soybean hulls, alfalfa, Maillard product and standard AACC wheat bran. The Psyllium and propol gum were donated by the Searle Company, Chicago, IL; other sources were purchased with the exception of the Maillard product which was prepared according to the procedure of Olson *et al.* (7) from sucrose and glycine. The insoluble fibers from wheat bran, alfalfa, and soybean hulls were prepared by amylase digestion and neutral detergent extraction. The cabbage was converted to a coarse powder by a threefold extraction with 95% ethanol and passing the alcohol slurry through a meat grinder. This treatment resulted in a 30-fold concentration of the dietary fiber over that in raw cabbage.

Water Holding Capacity by Filtration. The samples were added to crucibles that had been weighed at room temperature and hot (100° C). Deionized distilled $H_2O$ was added to the sample with backpressure for 2 hrs, then filtered under full vacuum for 1 minute; finally the crucible was wiped to remove excess water and weighed. The hydrated sample was dried at 100° C overnight and weighed. Grams of water per dry gram of sample was determined.

Water Holding Capacity by Osmotic Suction. Water was removed from hydrated samples by creating an osmotic suction using a modification of the Robertson and Eastwood method (8). Dialysis tubing (Mwt cutoff = 2000) was cut into 10 cm lengths and one end tied. These bags were soaked in a 0.1% w/v sodium azide solution overnight. Sample was placed in the bags and 5 ml of azide solution was added. The open end was tied and the filled bag rested in a 150 ml beaker within a dessicator at a relative humidity of 100% for 24 hrs. for hydration. Then 50 ml of an azide solution (0.1%) with 8.75 g of PEG (3350 Molecular weight) was placed in the beaker. This concentration of PEG will approximate a suction pressure of 270 osmol across the dialysis membrane after the sample has lost it's nonassociated water. Readings of the osmolarity were made at 6, 12, 24 and 48 hrs to determine equilibrium.

After 24 or 48 hrs, the tube was cut open. A subsample of the hydrated material was removed and weighed. A dry weight of the fiber subsample was obtained after overnight drying at 100° C. Grams of water per gram of sample was then calculated.

Measurement of Cation Exchange Capacity. Cation exchange capacity (CEC) was measured based on the high affinity of $Cu^{2+}$ for carboxylic acid groups (9, 10). Samples were weighed in 50 ml coarse Gooch crucibles, placed inside a 100 ml beaker and incubated with 50 ml 1 M copper sulfate for 2-3 hours. These crucibles were removed and washed with distilled, deionized water to remove unbound copper ions. Then the samples were washed 3 times (25 ml/wash) with 0.6 N hydrochloric acid in 70% (v/v) propanol-2. The washes were collected and brought to a known volume. Aliquots were brought to pH 8-9 with 2 N ammonium hydroxide. A copper indicator, cuprizon, was added, again brought to a known volume and the absorbance at 590 nm was measured after 30 minutes to allow for complete color reaction. Copper nitrate standards were prepared ranging from 0 to 8 PPM.

Fermentation Technique. A human batch culture technique was used to measure *in vitro* fermentability of the samples (11). The technique was developed to utilize microflora present in human fecal matter. Maintenance of an anaerobic environment during collecting, processing and inoculating was important. The use of a modified camping toilet allowed collection while maintaining

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anaerobiosis. A receiving medium was used in the collection vessel. The culture medium and determination of fermentability were the same as the rumen *in vitro* procedure (12).

## Composition of Fibers

Composition of the dietary fiber sources is shown in Table I. The gum sources generally show no insoluble fiber (as NDF) with the exception of the glucomannan source which contains about 5% of a white fibrous residue. Cabbage powder is intermediate with about a third of its dietary fiber in the form of pectin. This pectin is unusual in that it is not precipitated by quaternary detergent and also posseses substantial cold water-solubility in the absence of chelating agents. This characteristic has been mentioned by Bailey *et al.* (13). Soybean hulls contain significant amounts of galactan and other pectin related carbohydrates (14) in addition to the insoluble NDF fraction.

Table I. Composition of Dietary Fiber Sources

| Fiber Source | Dietary Fiber | NDF | HC[1] | Cell | Lignin | Nitrogen |
|---|---|---|---|---|---|---|
| | % DM | | | | | |
| Psyllium, whole seed | 87.7 | 52.6 | 23.7 | 25.4 | 3.5 | - |
| Psyllium, gum | 93.5 | ND | ND | 4.1 | 0.3 | - |
| Propol | 99.0 | 4.6 | 0 | 0 | 0 | 0 |
| Pectin | 100.0 | 0 | 0 | 0 | 0 | 0 |
| Wood Cellulose | 100.0 | 100.0 | 2.6 | 94.5 | 2.8 | 0.2 |
| Maillard polymer | ND[2] | 74.2 | 0 | 0 | 72.7 | 8.6 |
| Alfalfa NDF | 100.0 | 53.0 | 21.6 | 23.9 | 7.5 | 3.2 |
| Cabbage | 73.0 | 44.3 | 7.3 | 31.1 | 1.0 | 2.9 |
| Soy bean hulls NDF | 100.0 | 56.0 | 10.5 | 37.0 | 2.0 | 3.2 |
| Wheat bran AACC | 44.1 | 44.1 | 30.9 | 9.3 | 3.9 | 2.8 |

[1]Hemicellulose
[2]ND, Not Determined

## Fermentability and Water Holding Capacity

The dietary fiber in alfalfa and wheat bran is primarily in the forms of insoluble NDF, and these sources are significantly lignified and therefore contain substantial amounts of unfermentable carbohydrate (Table II). Soybean hulls, cabbage, wood cellulose are less lignified and about 90% fermentable. The wood source is not a pure cellulose and contains significant amounts of hemicellulose and lignin. The hemicellulose fraction is large enough to obtain a digestion balance with human subjects. Hemicellulose in Solka floc is substantially more fermentable than its cellulose (6).

Table II shows a comparison between fermentability values utilizing rumen and human inocula on the same substrates. A comparison of this nature may have inherent inequalities due to differences in inoculum source and collection procedures. These parameters have been evaluated by a number of workers (11, 17, 18). The differences in nutritional schemes between animal species have an effect on microbial populations. Fermentability estimates of a particular substrate are biologically acceptable with respect to the inoculum source.

Table II. Physicochemical and Biological Properties of Dietary Fiber Sources

| | Water Holding Capacity | | Exchange | Fermentability | |
|---|---|---|---|---|---|
| | Filtration | Osmotic suction, 0.3 osmol | meq Cu/ 100 g | Human Fecal | Rumen |
| | - -g $H_2O$/g Fiber- - | | | - - - % - - - | |
| Psyllium gum | -[1] | 3.2 | 3 | -[1] | 56 |
| Propol | -[1] | 2.5 | 0[1] | -[1] | 100 |
| Pectin | -[1] | 5.0 | 227 | 90 | 98 |
| Wood Cellulose | 1.4 | 1.0 | 5 | 23 | 94 |
| Maillard product | 2.4 | 1.7 | 37 | (0) | (0) |
| Alfalfa NDF | 5.8 | 1.8 | 36 | 46[2] | 57[2] |
| Cabbage | 20.7 | 3.0 | 92 | 91 | 91 |
| Soybean Hulls NDF | 5.9 | 1.7 | 18 | - | 89 |
| Coarse Bran, NDF | 3.5 | 1.3 | 87 | 53[2] | 71[2] |

[1]Not determined--see discussion.
[2]Used whole plant tissue.

Fermentability using human fecal inoculum ranges from a high of 91% (cabbage) to a low of 0% (Maillard product) demonstrating the sensitivity of human microflora to different fiber sources. Fermentability of cellulose by human fecal microflora (23%) is substantially less than rumen microflora (94%). The microflora in the human intestine may be more affected by fiber composition than rumen microflora. Work by Bryant (19) and Jeraci (11) leads to speculation that variation among inoculum sources in humans on a particular substrate could be greater than in other species.

The water holding capacity (WHC) of dietary fiber could have important relationships to many other fiber characteristics (15, 16). A new technique is utilized so that water soluble components of dietary fiber can be measured for WHC. This method differs significantly from previous techniques (8) in that the dialysis tubing had a molecular weight cutoff of 2000. The WHC measured by filtration is consistantly higher for the insoluble fibers. Wood cellulose shows the lowest value by both methods. The three water soluble polysaccharides are among the highest values measured by osmotic suction with pectin holding 5.0 g $H_2O$

per gram of fiber. Cabbage holds 3.0 g water per gram of fiber. This is probably due to the large amount of pectin in this fiber source. The Maillard product is unusual in that it has the smallest change in WHC between the methods.

The fermentability of these fibers, the microbial mass produced and the properties of the microbial products and fiber residues may be of greater importance in the large intestine than the characteristics of the unfermented dietary fiber.

## Cation Exchange Capacities

Cation adsorption appears to be important in the formation of cationic bridges as a mechanism for bile acid, fatty acid and mineral adsorption in the upper intestine (20). The affinity of copper for carboxylic acid groups has been employed in pectin precipitation (10, 21) and in determinations of cation exchange capacity (CEC) of dietary fiber (9). Table II contains estimates of cation exchange capacity with pectin having the greatest amount of copper adsorption per unit weight. Cabbage, with one third of its dietary fiber value deriving from pectin, similarily has a substantial exchange value (92 meq/100 g). The coarse bran, alfalfa and soybean hulls were neutral detergent fiber preparations using the amylase modification of Robertson and Van Soest (22) and were thus free of pectin and starch. Under these conditions lignin with both carboxyl and hydroxyl groups on the phenylpropane units has a predominant role in CEC. Differences among dietary fibers are also derived from the hemicellulose fraction including the glucuronic and galacturonic acid content. Sugar residues of the glucuronic acid become available for methylation, amidation or cationic complexes dependent on the degree of oxidation of the terminal hydroxyl. This fraction appears to be the primary source of copper adsorption in wheat bran. Alfalfa and soybean hulls have more moderate exchange values of 36 and 18 meq/100 g respectively. The glycine Maillard product exchange value of 37 meq/100 g is primarily a lignin-like effect. Wood cellulose and Psyllium are relatively inert polysaccharides with very little copper adsorption. Propol could not be accurately measured since it forms a gel. This prevents removal of excess copper. Addition of ytterbium with the propanol does not cause precipitation. This is supportive evidence that the exchange would be near zero.

## Summary

Data have been presented emphasizing the variability of dietary fiber sources. Water holding capacity estimates differed by technique as well as source. The most important advantage of the osmotic suction technique is that the measurement includes the water soluble dietary fiber components. This is a more realistic model of the digestive tract. The human fecal batch *in*

vitro fermentation method can be used to study fiber digestion. Variation in degree of fermentation are due to differences in fiber composition and/or inoculum source. Cation exchange capacity estimates also emphasize chemical composition differences with lignin and uronic acids being the primary adsorption sites.

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# Dietary Pectin: Effect on Metabolic Processes in Rats

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Dietary pectin affects lipid metabolism, especially that of cholesterol. One of the explanations proposed to explain an action of pectin on cholesterol metabolism is through its ability to bind bile acids and bile salts. However, pectin also has the property of forming a gel in water. This gel lowers the intestinal absorption of cholesterol and thereby decreases liver cholesterol. Recently, evidence has been obtained that the presence of pectin in a cholesterol diet increases the excretion of cholesterol esters. Results from the administration of cholesterol-4-$^{14}$C in the diet showed that the presence of pectin slows gastric emptying and results in more labeled cholesterol as well as cholesterol esters in all segments of the gut. These observations indicate that the higher levels of cholesterol esters were mainly exogenous. The functional role of pectin can be influenced by many factors such as its degree of esterification, its chain length and also the quantity and quality of other dietary ingredients. The effect of pectin on lowering of liver cholesterol in rats receiving diets containing corn oil or lard with or without cholesterol was greater with lard-pectin than with corn oil-pectin. The excretion of fecal free fatty acids was markedly greater; among them oleic acid was the highest in rats fed the pectin as compared to the pectin-free diet. Pectin also affects carbohydrate metabolism, reducing the postprandial rise in blood glucose and serum insulin. These effects of pectin could be beneficial in reducing risk factors associated with heart disease and diabetes.

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Metabolic processes affected by dietary pectin have been recognized for more than 2 decades. Effects of dietary pectin on lipid and carbohydrate metabolism are important and beneficial to our health.

Pectin lowers serum cholesterol in humans (1-6) and serum and liver cholesterol in rats (7, 8) as well as in many other species (9-13). The ingestion of cholesterol increases serum cholesterol levels (14, 15). A high level of serum cholesterol is generally considered a risk factor associated with coronary heart disease (16, 17). The cholesterol-lowering property of dietary pectin would thus be expected to reduce the incidence of heart problems. One of the early studies on the effect of dietary pectin on cholesterol metabolism in rats was carried out by Lin et al. (18). They found that more cholesterol was excreted in rats fed a cholesterol diet containing pectin than in rats fed a control diet and suggested that pectin inhibited exogenous cholesterol absorption. Later, Hyun et al. (19), by using a cannulated thoracic duct technique with rats, measured the quantitative absorption of cholesterol-4-$^{14}$C from test emulsions in the intestine and observed that the absorption of labeled cholesterol was significantly depressed by the presence of pectin in the emulsion and the lowering was due primarily to a decrease in esterified cholesterol. This finding supported the early results (18). However, the mechanism involved in the action of pectin on inhibiting cholesterol absorption is not clear at this time.

Many investigators have reported that dietary pectin increased fecal excretion of bile acids as well as neutral sterol (8, 20). The hypocholesterolemic properties of pectin may be due to its ability to bind bile acids (21-24). Leveille and Sauberlich (21), using everted intestinal sacs from the rat, reported that pectin depressed taurocholate absorption. They suggested that a possible mechanism for the hypolipemic action of pectin involved the binding of bile salts or bile acids which would result in reduced absorption of cholesterol and in turn in lower serum cholesterol levels. In addition, the binding of bile acids and an increased bile acid excretion would increase turnover of cholesterol into bile acids and provide less cholesterol to the circulation (24). Jenkins (25) postulated that gums and viscous fibers may reduce serum cholesterol by mechanisms other than simply increasing loss of bile salt.

In our laboratory, we have observed that pectin increased cholesterol ester excretion in rats fed a cholesterol-containing diet (26). An early report (27) indicated that fecal excretion of endogenous cholesterol ester could be influenced by the type of fat. Polyunsaturated fat (corn oil) as compared with saturated fat (lard) accelerated cholesterol ester, but not total cholesterol excretion (27). It was suggested that, under certain circumstances, cholesterol ester excretion was one of the major pathways of cholesterol catabolism and a process for lowering body cholesterol and might explain why corn oil lowers serum

cholesterol level. Pectin as well as corn oil is considered to be an hypocholesterolemic agent. Recently, we have carried out a study with young rats to elucidate how pectin affects cholesterol ester excretion. Cholesterol-4-$^{14}$C was used as a tracer in the diet during the last meals following 3-week feeding of unlabeled diets. The diet contained 0.5% cholesterol, 10% corn oil, either with 5% pectin or 5% cellulose (control diet). Rats were killed 4 hours after the last 2-hour meal feeding. The general status of the experimental rats is shown in Table I. The food intake in the last feeding was slightly more in the pectin group than in the cellulose group. The percent of the consumed $^{14}$C-cholesterol which passed from the stomach into the intestine was 28.6 and 34.8 in the pectin and cellulose groups, respectively (Table II), and the actual amount was 63.0 mg in the pectin group and 65.3 mg in the cellulose group. Thus, the difference of cholesterol levels in the contents of intestinal sections between diet groups was not attributed to the intake. The low percentage of cholesterol that passed into the intestine in pectin-fed rats at this period is consistent with the reports that pectin delays the gastric emptying rate (28). The contents found in the duodenum and jejunum were very small. Therefore, the samples from the same diet group were pooled. All data were from pooled samples. The ingested labeled cholesterol, both free and esterified, retained in the duodenum, jejunum, ileum, and cecum (Table II) was higher in pectin-fed than in cellulose-fed rats. Similarly, the non-labeled total cholesterol esters in these sections was also higher in the pectin-fed than in the cellulose-fed rats. The results

Table I
General Status of Experimental Rats

| | Cellulose diet | Pectin diet |
|---|---|---|
| Body weight, g | | |
| Initial | 160 ± 4 | 159 ± 3 |
| Final | 249 ± 5 | 243 ± 4 |
| Liver size, g/100 g body weight | 4.15 ± 0.10 | 4.08 ± 0.47 |
| Food intake, g (last meal) | 9.38 ± 0.55 | 10.99 ± 0.47 |
| Total labeled cholesterol intake, mg/rat | 46.9 ± 2.8 | 50.0 ± 2.3 |
| Serum cholesterol, mg/100 ml | 256 ± 14 | 235 ± 14 |
| Serum glucose, mg/100 ml | 166 ± 6 | 144 ± 9 |
| Fecal esterified cholesterol, mg/g (pooled sample) | 0.66 | 13.5 |
| Fecal neutral cholesterol (free), mg/g (pooled sample) | 32.0 | 50.0 |

Table II
Cholesterol Contents of Rat Digestive Tract Segments 4 Hours After a 2-Hour-Meal-Feeding of a Cholesterol-4-$^{14}C$ Diet Containing Either Cellulose or Pectin

| | Cellulose diet - cholesterol content,* mg | | | | Pectin diet - cholesterol content,* mg | | | |
|---|---|---|---|---|---|---|---|---|
| | Total | $^{14}C$-cholesterol | Total esterified | $^{14}C$-esterified cholesterol | Total | $^{14}C$-cholesterol | Total esterified | $^{14}C$-esterified cholesterol |
| Consumed | | 187.6 | | | | 219.9 | | |
| Stomach retained | | 122.3 (65.2%) | | | | 156.9 (71.4%) | | |
| Stomach emptying | | 65.3 (34.8%) | | | | 63.0 (28.6%) | | |
| Duodenum | 0.26 | 0.029 | 0.114 | 0.02 | 0.864 | 0.311 | 0.149 | 0.06 |
| Jejunum | 0.63 | 0.26 | 0.147 | 0.07 | 1.464 | 0.839 | 0.784 | 0.41 |
| Ileum | 11.85 | 7.88 | 1.744 | 1.18 | 25.38 | 17.700 | 7.876 | 4.108 |
| Cecum** | 49.64 | 18.05 | 1.709 | 0.49 | 75.84 | 24.366 | 5.997 | 1.573 |
| Total (from duodenum to cecum) | 62.38 | 26.219 | 3.714 | 1.76 | 103.548 | 43.216 | 14.806 | 6.151 |

* All values are of pooled groups of 4 rats. ** One rat in cellulose diet group had little cecum content, which might have passed into colon or as feces.

provided positive evidence supporting the contention that pectin lowers cholesterol absorption and that cholesterol ester is one of the fractions involved. The origin of labeled cholesterol esters in the lumen was not clear, since labeled cholesterol in the diet was in the free form. The conditions in the lumen generally favor hydrolysis of cholesterol esters and there is little, if any, intraluminal esterification (29). Sterol ester hydrolase (cholesterol esterase, EC 3.1.1.13) systems are pH-dependent. The optimal pH for hydrolysis is 6.7 and for esterification is 6.2 (30). Therefore, a possible explanation for the presence of labeled cholesterol esters in the intestinal lumen was due to a pH environment favoring synthesis. The gel-forming property of pectin might maintain a more acidic pH of the chyme as it leaves the stomach and enters the upper part of the proximal intestine, thus favoring cholesterol ester synthesis. Cholesterol esters cannot be directly absorbed by the mucosal cell (29). The source of the nonlabeled cholesterol ester in the lumen could not be determined under present experimental conditions. The present data showed that cholesterol esters were mainly in the ileal section of the small intestine. This could be due to the unabsorbed cholesterol from the upper proximal region passing down to the ileum where the absorption of cholesterol is less efficient (31, 32).

The profile of fatty acids in cholesterol esters showed a difference between the two diet groups (Table III). The high levels of palmitate and stearate esters presumably originated from the dietary fat. Since the absorption of palmitate and stearate is low, more of these fatty acids were available in the intestine for esterification. The percentage of oleate was three times greater in the cellulose-diet group than in the pectin-diet group.

Table III
The Profile of Fatty Acid Esters in the Crystallized Fecal Cholesterol Esters

| | Diet | |
|---|---|---|
| | Cellulose | Pectin |
| Fatty acids, % | | |
| Palmitate | 30 | 43 |
| Stearate | 32 | 19 |
| Transoctadecenate | 6 | 21 |
| Oleate | 23 | 7 |
| Linoleate | 8 | 4 |
| Arachidate | -- | 2 |

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It is possible that the source (endogenous versus endogenous + exogenous) of the esters may be responsible for the difference. It has been reported that oleic acid is most readily esterified with cholesterol during its absorption (33, 34). Furthermore, Swell and Treadwell (34) also reported that the cholesterol ester which accumulated to the greatest extent, irrespective of the fatty acid fed, was cholesterol oleate. Thus, the endogenous cholesterol esters may be rich in oleic acid.

The results obtained indicate that dietary pectin influenced the synthesis of cholesterol esters in the intestinal lumen, thereby providing a possible mechanism by which pectin can influence cholesterol metabolism.

The effectiveness of pectin in lowering cholesterol in tissues can be influenced by many factors: (1) The type of pectin - that with methoxy groups and with high viscosity (35, 36) was reported to be more effective. The results from a comparative study of several types of pectin by Mokady (36) are shown in Table IV. Neither a high degree of methylation nor high-viscosity alone was very effective; (2) the level of dietary fat - with a high-fat diet (2% corn oil plus 18% beef tallow), pectin has no effect on serum cholesterol level (37). Tsai et al. also reported that with

Table IV*
Degree of Methylation and Relative Viscosity of Test Substances and Comparison of Blood Cholesterol with Excretion of Lipids in the Feces

| | Degree of methylation | Relative viscosity | Total blood cholesterol | Fecal lipids[1] | Total fecal sterols |
|---|---|---|---|---|---|
| | (%) | (%) | (mg %) | (g/rat) | (mg/rat) |
| Control | | | 87 ± 5.2[2] | 0.272 | 30.4 |
| Pectin 1 | 7.8 | 2.9 | 79 ± 5.2 | 1.245 | 84.6 |
| Pectin 2 | 8.2 | 6.0 | 66 ± 2.8[3] | 2.000 | 113.3 |
| Pectin 3 | 4.1 | 23.5 | 75 ± 3.0 | 1.060 | 67.6 |
| Pectin S | 7.7 | 2.4 | 72 ± 2.8 | 1.503 | 82.0 |
| Pectin MR | 7.8 | 2.6 | 72 ± 2.8 | 1.300 | 89.0 |
| Algin | --- | 10.2 | 77 ± 3.2 | 1.367 | 38.0 |

[1] Pooled mean value of 8 animals for the entire period of 12 days.

[2] Mean for 8 animals ± SE.

[3] $P < 0.05$.

* After Mokady (36).

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as much as 15% corn oil, pectin showed no apparent cholesterol lowering properties (38); (3) type of dietary fat - corn oil versus lard in a cholesterol-pectin diet. Pectin eliminated the elevation of liver cholesterol in the rats fed ad libitum for 4 weeks by decreasing cholesterol absorption with corn oil and by decreasing absorption and increasing disappearance with lard (39) (Table V). In this experiment, all diets for the last 24 hours of feeding contained the same specific activity of cholesterol-4-$^{14}$C. The total food intake was carefully measured. Then rats were continuously fed their respective diet without $^{14}$C-cholesterol for another 24-hour or 48-hour period, designated as 48-hour and 72-hour feeding periods, respectively. The radioactive cholesterol level (% of intake) in the liver was markedly lower in the pectin-fed than in the cellulose-fed rats, regardless of the type of fat. However, pectin accelerated the disappearance of $^{14}$C-cholesterol during the 48-hour to 72-hour period only in the lard-containing diet (39). When the rats fed the control diet were shifted to a

Table V
The Effect of Pectin and Fat on Serum and Liver Cholesterol Levels and the Recovery of Radioactive Cholesterol (% Intake) in the Liver of Rats 48-Hour and 72-Hour After Feeding Cholesterol-4-$^{14}$C Diet for 24 Hours

| | Corn oil | | Lard | |
|---|---|---|---|---|
| | Cellulose | Pectin | Cellulose | Pectin |
| Serum cholesterol mg/dl (10)[1] | 134 ± 3[b] | 115 ± 5[a] | 160 ± 13[c] | 147 ± 16[c] |
| Liver cholesterol mg/100 g body weight (10) | 65 ± 7[c] | 34 ± 4[b] | 70 ± 5[c] | 21 ± 3[a] |
| Liver Radioactivity % intake | | | | |
| 48-hour (5) | 16.7 ± 1.6[a] | 8.3 ± 1.7[b] | 15.3 ± 0.9[a] | 6.4 ± 1.2[b] |
| 72-hour (5) | 14.5 ± 1.2[a] | 6.3 ± 1.2[b] | 16.0 ± 0.6[a] | 4.6 ± 1.2[b] |

[1] Numbers in the parentheses represent number of animals used.

Means having different superscript letters are significantly different ($P < 0.05$).

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cholesterol-free diet for 2 or 4 days, pectin lowered liver cholesterol and total lipids in the lard diet but not in the corn oil diet (Table VI). Fecal steroid excretion on day 4 of feeding agreed with the degree of reduction of liver cholesterol (Table VII). There were significantly more neutral, free, and esterified cholesterol and bile acids in the feces of pectin-fed rats than in the feces of cellulose-fed rats.

Pectin or other mucilagenous fibers could play an important role in carbohydrate metabolism. The addition of gel-forming fiber in the test meal or in the standard glucose tolerance test solution flattens blood glucose and insulin responses in man (40-42) as well as in rats (28). It was suggested that these actions of fiber are due to its reduction of the gastric emptying rate and decrease in the intestinal absorption rate of the carbohydrates by formation of an additional diffusion barrier at the intestinal surface through the ability of the fibers to produce viscous solutions. Recently in our laboratory (Li and Chang, unpublished observation), we have done preliminary work on the effect of pectin or guar gum on sucrose absorption and observed that there was more fructose, glucose, and sucrose remaining in the lower intestinal content of rats 4 hours after a 2-hour meal feeding with a sucrose diet (61.2% w/w) containing either 5% pectin or 5% guar as compared with 5% cellulose (Table VIII). It seems that these fibers reduced the absorption rate of these simple sugars, especially fructose. At present, we are repeating the experiment and appear to be obtaining confirmatory results.

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Table VI

Effect of Pectin and Type of Fat on the Removal of Accumulated Liver Lipids in Rats

| Type of dietary fat | Liver lipids mg/100 g body wt. | Initial[1] | 2-day of feeding | | 4-day of feeding | |
|---|---|---|---|---|---|---|
| | | | Cellulose | Pectin | Cellulose | Pectin |
| Corn oil | Total lipid | 594 ± 39 (10)[2] | 599 ± 66 | 557 ± 41 | 366 ± 63 | 485 ± 25 |
| | Triglyceride | 73 ± 4 (5) | 63 ± 13 | 71 ± 2 | 30 ± 4* | 55 ± 13 |
| | Total cholesterol | 65 ± 7 (10) | 67 ± 7 | 46 ± 9 | 38 ± 7* | 34 ± 7* |
| Lard | Total lipid | 483 ± 25 (10) | 460 ± 27 | 384 ± 28* | 517 ± 61 | 271 ± 36* |
| | Triglyceride | 79 ± 4 (5) | 63 ± 7 | 56 ± 9 | 82 ± 20 | 46 ± 12 |
| | Total cholesterol | 70 ± 5 (10) | 76 ± 12 | 28 ± 4* | 75 ± 16 | 29 ± 17* |

[1] All rats had been fed cholesterol-cellulose diet either with corn oil or lard for 4 weeks.

[2] Number in parentheses represents number of animals.

* Statistical difference from the initial value is significant at 5% level.

Table VII
Fecal Steroid Excretion of Rats at 4th Day Refeeding Period on Cholesterol-Free Diet

| | Corn oil | | Lard | |
|---|---|---|---|---|
| | Cellulose | Pectin | Cellulose | Pectin |
| Neutral sterol (mg/day) | | | | |
| Total | $34.7 \pm 1.6^{b}$ [1] | $44.9 \pm 4.6^{b}$ | $19.6 \pm 1.4^{a}$ | $34.2 \pm 4.3^{b}$ |
| Esterified | $7.3 \pm 0.4^{b}$ | $18.5 \pm 3.4^{c}$ | $4.6 \pm 0.9^{a}$ | $11.2 \pm 1.7^{b}$ |
| Bile acids (mg/day) | $13.1 \pm 1.2^{a}$ | $31.8 \pm 6.1^{b}$ | $11.3 \pm 1.5^{a}$ | $23.4 \pm 4.6^{b}$ |

[1] Mean $\pm$ SE of 5 rats.

Table VIII
Intestinal Contents of Carbohydrate of Rats Fed a Diet Containing 61.3% Sucrose 4 Hours After 2-Hour Meal Feeding (Data are from a Pooled Sample of Three Rats from the Same Diet Group)

| Carbohydrate* | Intestinal Section** | Diet Cellulose | Diet Pectin | Diet Guar Gum |
|---|---|---|---|---|
| Fructose | U | 1.77 | 1.64 | 1.80 |
| | L | 0.13 | 1.29 | 1.70 |
| Glucose | U | 1.28 | 1.42 | 1.14 |
| | L | 0.05 | 0.38 | 0.31 |
| Sucrose | U | 4.56 | 6.06 | 5.36 |
| | L | 0.02 | 0.99 | 0.71 |

* % = percent of total dry matter

** U = upper half of the intestine; L = lower half of the intestine.

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# 12

# Chitin and Chitosan: Influence on Element Absorption in Rats

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Chitin and chitosan are reported to bind essential elements in vitro. To evaluate these unconventional sources of dietary fiber in vivo, the apparent absorption of six (6) elements (i.e., P, Ca, Mg, Fe, Zn and Cu) from diets containing chitin and chitosan were measured by balance trials in growing rats. At the 5% level, and at three particle sizes, neither chitin nor chitosan affected animal growth or food consumption over a 3 week period compared to a 5% cellulose control diet. Apparent absorption of the six elements were positive in all animals. Increasing both chitin and chitosan to 10% and 20% levels in the diet depressed Fe absorption except in animals consuming 10% chitosan. There was no difference in growth or food consumption between animals groups receiving higher fiber levels. Animals were in positive balance for P, Ca, Mg, Zn and Cu. Greater morphological changes were observed histologically in the small intestine and cecum of animals consuming chitosan and chitin compared to animals consuming cellulose. Increased numbers of neutrophils were seen in the lamina propria of the small intestine and cecum. More crypt epithelial cells of the small intestine were undergoing mitosis. The inability of rats to utilize dietary Fe and lose body Fe is believed to be due to the inflammatory state of the small intestine, caused by chitosan, and to a lesser degree chitin. Reasons for mortality in rats consuming high levels of chitosan, >10%, are also discussed.

Dietary fiber (DF) has taken on a larger meaning than its original definition of plant cell wall material resistant to the digestive process (1) in animals or man.

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Many non-metabolizable materials not obtained from plant sources that are consumed in trace and possibly bulk amounts both intentionally and unintentionally are now recognized. Examples include mucopolysaccharides from animal tissue (2) microbial cell walls (3) exoskeletons of arthopods (4) and synthetic bulking agents (5). Consideration must also be given to intestinal tract residues derived from the processing of foods which Maillard reaction products (6) are an excellent example.

Early in the last decade, two, now almost classic publications, (1, 7) caused a reinvestigation of thought as to the importance of fiber in man's diet. This was not a new revelation (8). What was new, was the recognition of the multiplicity of health-related disorders attributed to the lack of DF in the diet (9). Increased DF consumption is advocated (10), but there may be adverse consequences with excessive intake. The biochemical and/or physiological action of DF in the gut have not been clearly delineated.

One of the benefits attributed to DF has been its ability to sequester bile acids preventing their reabsorption (11). The objective of decreasing plasma cholesterol by altering bile acid homeostasis is strongly championed. However, DF has been implicated in the impairment of essential element absorption (12). A possible nutritional or health status trade-off exists for the host consuming DF.

Two unconventional, but potential sources of DF are chitin and chitosan. Chitin, along with cellulose and collagen, are the principal skeletal or support matrices in all living systems. Reviews of the chitin system in animals and plants (13) and the chemistry of chitin (14) are available.

Commercial chitin is mainly derived from waste products of crustacea (i.e. crab and shrimp) processed for human food. Chitin is similar to cellulose, but is a polymer of N-acetyl-D-glycosamine units, linked by $\beta$ $(1 \rightarrow 4)$ glycosidic bonds. It may have potential for use as DF (15), but it does not have wide application and has not been approved for use in foods. At present, ingestion of chitin by man is only incidental.

Treatment of chitin with strong base causes deacylation, resulting in the product chitosan (Figure 1). This compound reportedly has potential for lowering blood cholesterol in rats (4, 16, 17). Chitosan is being compared to and suggested as a substitute for cholestyramine; this latter compound has proved to be a very effective agent in the treatment of patients with elevated serum cholesterol (18). However, the mechanisms of action may be different. The safety of chitosan or chitin has not been thoroughly established. Both chitin and chitosan have been reported to bind essential elements from solution (19) and chitosan is used to remove suspended organic materials in waste treatment (20). These observations again suggest a reciprocal relationship between cholesterol and essential element status may occur with the ingestion of chitosan.

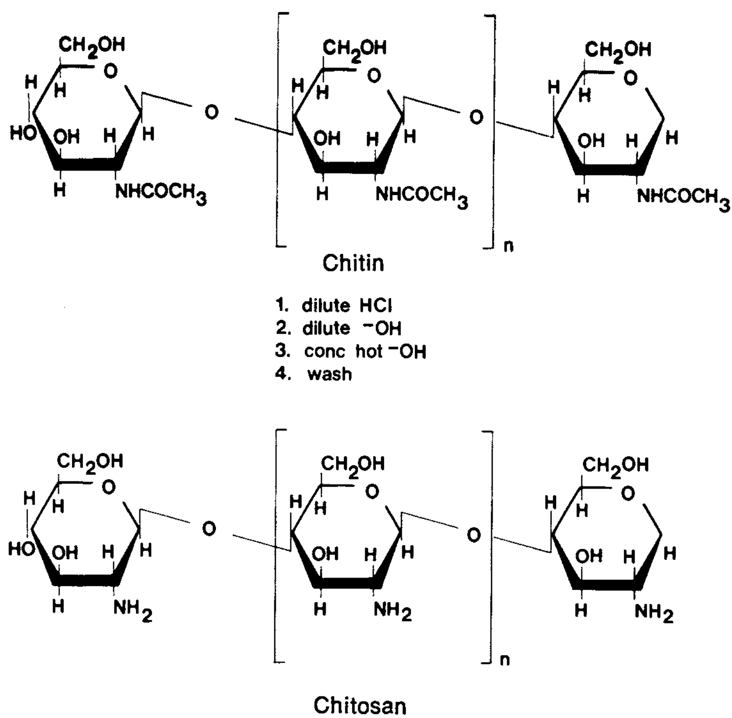


*Figure 1. The chemical structures of chitin and chitosan.*

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The primary objective of this investigation was to examine the effect of chitin and chitosan on essential element absorption in rats. Since previous reports (4, 21) and unpublished observations by one of the authors (D.T.G.) have indicated high levels of chitosan impair growth and cause death in rats, a second objective was established to more thoroughly explain these findings.

## Materials and Methods

Chitin and Chitosan. Chitin and chitosan were received through the courtesy of Kypro Co., Seattle, Washington. After sifting to remove fines and extraneous non-shell flakes, the remaining material was ground in a Thomas Wiley laboratory mill (Model 4, A. H. Thomas Co., Philadelphia, PA) to pass a 2.00 mm screen. Using a Ro Tap Testing Sieve Shaker (Model B, W. S. Tyler, Inc., Mentor, OH), three fractions of each material were collected: <1.00-0.50 mm; <0.50-0.25 mm and <0.25 mm.

Diets. Semi-purified diets were prepared as recommended by the American Institute of Nutrition-AIN (22), and are described in Table 1. Two animal feeding experiments were carried out. In the first experiment (Expt. 1), chitin and chitosan were fed at the 5% level in three particle size ranges. A commercial preparation of the AIN salt mixture was added (U.S. Biochem. Corp., Cleveland, OH) to diets fed in Expt. 1. In the second experiment (Expt. 2), chitin and chitosan of one particle size range 0.50-0.25 mm, were fed at levels of 10 and 20%. The salt mixture used in Expt. 2 was formulated to meet AIN specifications (22). Substitution of increasing amounts of DF in Expt. 2 was made at the expense of dietary carbohydrate. Hydrolyzed cellulose (Celufil; U.S. Biochem. Corp., Cleveland, OH) was incorporated into control diets at levels of 5% (Expt. 1) and 10 and 20% (Expt. 2).

Animals and balance protocal. Male weanling rats, 21 days old, of the Sprague Dawley strain were used in both experiments, 5 animals per diet group. Animals were individually housed in suspended stainless steel cages with wire bottoms in a room maintained at 23$^{o}$C with a 12 hr light-dark cycle. Glass distilled water and food were provided *ad libitum* except for food during pre- and post-balance periods. Ten hr before each period, food was removed. Prior to receiving food again, each animal was administered 0.2 ml 0.03% brilliant blue dye in distilled water by gavage. Food consumption was allowed for 60 hr and then food was removed for another 10 hr. Dye was again administered and the procedure repeated twice more in Expt. 2. Only one balance trial was conducted in Expt. 1. Apparent element absorption was calculated from the equation:

Table 1. Composition of Diets.

| Nutrient | Experiment 1 | Experiment 2 | |
|---|---|---|---|
| | % | | |
| Casein, vitamin free | 20.0 | 20.0 | 20.0 |
| Corn oil (0.01% BHT & 0.01% BHA) | 5.0 | 5.0 | 5.0 |
| Dietary fiber | 5.0 | 10.0 | 20.0 |
| chitin | [1] | [1] | [1] |
| chitosan | ——[1] | ——[1] | ——[1] |
| cellulose | ——[2] | ——[1] | ——[2] |
| | —— | —— | —— |
| Vitamin premix - AIN-76 | 1.0[3] | 1.0 | 1.0 |
| Mineral premix | 3.5 | 3.5 | 3.5 |
| Choline bitartrate | 0.2 | 0.2 | 0.2 |
| DL-methionine | 0.3 | 0.3 | 0.3 |
| Dextrose-monohydrate | 65.0 | 60.0 | 55.0 |

[1] See Material and Methods for particle sizes.

[2] See Material and Methods for type and source of cellulose.

[3] U.S. Biochemical Corp., Cleveland, OH.

Apparent element absorption: mg, μg/60 hr = diet element concentration mg, μg/g X g diet consumed/60 hr − fecal element concentration mg, μg/60 hr

Phosphorus, Ca and Mg are reported in mg/60 hr; Fe, Zn and Cu are reported in μg/60 hr. Food efficiency is the ratio of food intake (g) divided by animal weight gain (g).

Analytical. Chitin and chitosan samples, diets, fecal samples and animal carcasses were wet digested in $HNO_3$-$HClO_4$ prior to element analysis. Determination of Ca, Mg, Fe, Zn and Cu were by atomic absorption spectrophotometry (23). Phosphorus was measured by the method of Fiske-Subbarow (24). Fecal matter was freeze-dried prior to digestion and analyzed in duplicate. Animal carcasses were heated to 115°C (15 PSI) for 2 hr in sealed Mason jars containing one weight volume of of glass distilled water. After homogenization, the total contents were made up to an appropriate volume from which 25 ml aliquots were withdrawn for digestion and element analysis in duplicate. Hemoglobin was determined spectrophotometrically (25) as cyanmethemoglobin.

Statistics. Data for Expt. 1 were analyzed by one-way analysis of variance (26). Differences among means were checked by least significant difference. In Expt. 2 the repeated measurement design (i.e. three balance periods) was subjected to a split-plot analysis of variance as outlined by Gill and Hafs (27). Differences among pooled means were verified by least significant difference.

Histopathology. Three animals from each group receiving 10 and 20% chitin, chitosan or cellulose were histologically examined upon termination of Expt. 2. Rats were on test diets 23 days. Animals were assigned a code number prior to necropsy. Immediately after euthanization with $CO_2$, the abdominal cavity was incised and the intestinal tract examined *in situ* along with the other organs. The entire gastrointestinal tract was removed, perfused with 10% formalin phosphate (0.1M-pH 7.0) buffer for immediate preservation and then placed in buffered formalin solution for complete fixation. Samples of liver, kidney, lung and salivary gland were also preserved in the formalin solution. After 24 hr of fixation, the gut and other tissues were embedded in paraffin and sectioned parallel to the long axis of the gut in 5 μ slices. The tissues were routinely stained with hematoxylin and eosin and were permanently mounted.

Sections of the gut examined histopathologically included: stomach, duodenum, jejunum, ileum, cecum and colon. A scoring system was used to quantify the changes observed in these organs. The two criteria were: (1) inflammatory cell infiltrate--the number of neutrophils in the lamina propria of the intestine and (2) mitotic activity--the number of mitotic figures in crypt cells (i.e. number of crypt cells in metaphase).

Each mounted tissue was examined at ten random locations

with a light microscope at 400 power magnification (x400). Each location was referred to as a high-powered field (HPF). Numbers of mitotic figures are reported as the mean value observed in 10 HPF of each tissue from 3 animals per group. Quantification of neutrophils were enumerated using the same counting protocol. Histological lesions observed in the intestines were recorded.

Microbiology. Culture specimens of cecal contents for microbiological examination of the gut flora were aseptically obtained prior to fixing the entire intestinal tract. Cecal flora was inoculated onto MacConkeys (BBC, Cockeysville, MD) and brilliant green agars (Difco Laboratories, Detroit, MI) and suspended in gram negative broth (Difco). After 24 hr incubation at 37$^{o}$C, colony morphology, carbohydrate utilization, catalase and oxidase production, gram stain reaction and cellular morphology were evaluated (28). Specific organisms tested for included: Actinobacillus sp, Bardetella bronchiseptica, Citrobacter freundii, Escherichia coli, Enterobacter sp, Klebsiella pneumoniae, Protus sp, Pseudomonas aerugenosa and Salmonella sp.

## Results

Experiment 1. At the 5% level of DF consumption, among seven groups of animals only one group showed a lower weight gain (Table 2). Pooled weight gains for the 3 groups of animals (15 rats) consuming one of three particle sizes of chitosan (103.8±15.6 g) were lower, but not significantly, compared to the same number of animals ingesting chitin (113.3±11.8 g); the 5 animals fed cellulose had intermediate weight gains (107.9±7.2 g). It has been reported that finely ground chitosan restrained growth when fed at the 2% level (4).

There were significant differences in apparent element absorption values among animal groups consuming chitin and chitosan in three ranges of particle size (Table 3). A trend toward lower absorption would appear to result with decreasing particle size. Significantly lower apparent absorption of P ($P<0.01$), Ca ($P<0.01$), Fe ($P<0.05$) and Cu ($P<0.05$) were found in animals fed chitosan compared to animals consuming chitin. Only Ca apparent absorption was significantly lower ($P<0.01$) in animals fed chitosan versus cellulose controls. Although differences occurred in apparent element absorption among groups of animals, all animals were in positive balance for all six elements with two exceptions. Two groups of animals consuming chitosan were in negative apparent Cu balance.

The carcasses of the three groups of animals consuming chitin or chitosan of equivalent particle sizes (0.50-0.25 mm) or cellulose were analyzed for element concentration (Table 4). Significantly lower ($P<0.05$) carcass levels of Ca, Fe, Zn and Cu were observed in animals fed chitosan as compared to those fed chitin or cellulose. However, this chitosan diet group

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Table 2. Weight gain and efficiency of food utilization among animals fed chitin, chitosan or cellulose in two experiments[1].

| | | | Experiment 1[2] | | | |
|---|---|---|---|---|---|---|
| Dietary fiber | Particle size (range) | Level | No. of animal | Initial weight | Weight gain | Food efficiency |
| | mm | % | | g | g | g |
| Chitin | 1.00 - 0.50 | 5 | 5 | 58.4±2.0 | 108±3.0[a] | 2.65±0.11[a] |
| | 0.50 - 0.25 | 5 | 5 | 58.6±1.9 | 111.2±7.7[a] | 2.37±0.01[b] |
| | <0.25 | 5 | 5 | 59.3±1.7 | 120.8±2.7[a] | 2.47±0.09[a] |
| Chitosan | 1.00 - 0.50 | 5 | 5 | 58.5±1.9 | 110.3±4.4[a] | 2.57±0.06[a] |
| | 0.50 - 0.25 | 5 | 5 | 59.2±1.6 | 90.2±6.2[b] | 2.70±0.14[c] |
| | <0.25 | 5 | 5 | 59.5±1.8 | 110.7±6.7[a] | 2.54±0.05[a] |
| Cellulose | | 5 | 5 | 60.2±2.0 | 107.9±7.2[a] | 2.50±0.97[a] |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | Experiment 2[3] | | | |
| Chitin | 0.50 - 0.25 | 10 | 5 | 58.6±1.3 | 122.8±7.0 | 2.54±0.11 |
| | | 20 | 5 | 59.2±1.3 | 117.0±7.0 | 2.57±0.11 |
| Chitosan | 0.50 - 0.25 | 10 | 4 | 59.1±1.5 | 113.4±7.9 | 2.54±0.13 |
| | | 20 | 3 | 59.9±1.5 | 102.4±9.0 | 2.75±0.14 |
| Cellulose | | 10 | 4 | 58.8±1.3 | 123.4±7.0 | 2.45±0.11 |
| | | 20 | 5 | 58.2±1.5 | 117.3±7.8 | 2.69±0.13 |

[1]Mean ± S.D.; values in same column in Expt. 1 not sharing a common superscript are significantly different (P 0.05).

[2]Growth on diets for 22 days (total animal age 43 days).

[3]Growth on diets for 23 days (total animal age 44 days).

Table 3. Apparent element absorption among animal groups consuming chitin, chitosan or cellulose at the 5% level.

| | Particle size (range) | Number of animals | P |
|---|---|---|---|
| | mm | | |
| Chitin | 1.00 - 0.50 | 5 | $190.9 \pm 7.0^{a,b}$ |
| | 0.50 - 0.25 | 5 | $193.5 \pm 11.4^{a}$ |
| | <0.25 | 5 | $195.1 \pm 6.5^{a}$ |
| Chitosan | 1.00 - 0.50 | 5 | $166.0 \pm 9.0^{b,c,d}$ |
| | 0.50 - 0.25 | 5 | $143.5 \pm 10.0^{d,e}$ |
| | <0.25 | 5 | $161.2 \pm 6.0^{c,d}$ |
| Cellulose | | 5 | $174.4 \pm 8.8^{a,b,c}$ |

[1]Mean ± S.D.; values in same column not sharing a common superscript are significantly different ($P<0.05$).

[2]Days 19-21.5 (60 hr) during 22 day growth period.

[3]Values in parenthesis are negative.

| Ca | Mg | Fe | Zn | Cu |
|---|---|---|---|---|
| mg/60 hr[1,2] | | ug/60 hr[1,2,3] | | |
| 110.8±10.3[a,b] | 13.8±1.2 | 1,310±225[a] | 536±121 | 83.0±34.9[a] |
| 120.0±9.4[a] | 13.9±1.1 | 1,305±299[a] | 459±61 | 13.8±7.7[b,c] |
| 88.6±10.3[b,c] | 10.7±1.6 | 812±297[a,b] | 454±56 | 26.1±15.7[b,c] |
| 101.0±12.4[a,b] | 14.4±1.4 | 788±215[a,b] | 312±30 | (9.2)±11.2[c] |
| 41.7±9.0[d] | 9.9±2.6 | 337±41[b] | 357±62 | (2.2)±10.0[b,c] |
| 61.1±9.7 | 12.5±1.7 | 715±200[a,b] | 447±26 | 41.0±10.6[a,b] |
| 112.8±7.6[a,b] | 14.4±1.3 | 956±84[a] | 498±59 | 44.8±8.8[a,b] |

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Table 4. Whole body element concentration per g animal weight of rats maintained on diets containing chitin, chitosan or cellulose at the 5% level for 22 days (animal age 43 days).

| Dietary fiber | Animal weight g | Number of animals | P | Ca |
|---|---|---|---|---|
| | | | mg/g[2] | |
| Chitin[1] | 178.3±19.1 | 5 | 5.19±0.40 | 8.42±0.49[a] |
| Chitosan[1] | 160.8±15.1 | 5 | 4.94±0.26 | 7.01±0.19[b] |
| Cellulose | 175.7±20.3 | 5 | 5.42±0.58 | 9.15±0.77[a] |

[1]Particle size range: 0.50 - 0.25 mm (see Table 2).

[2]Mean ± S.D., values in same column not sharing a common superscript are significantly different ($P<0.05$).

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| Mg | Fe | Zn | Cu |
|---|---|---|---|
| | ug/g[2] | | |
| 0.41±0.05 | 57.5±9.2[a] | 26.3±1.5[a] | 5.4±1.9[a] |
| 0.34±0.05 | 41.9±4.0[b] | 24.5±1.1[b] | 3.1±0.2[b] |
| 0.41±0.06 | 44.4±3.6[b] | 25.6±3.4[c] | 3.8±0.4[b] |

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also exhibited significantly lower weight gains (Table 3) which would account for the lower element values derived from carcass analysis.

Experiment 2. There were no significant differences in animal weight gains or efficiency of food utilization among animal groups fed chitin, chitosan or cellulose at levels of 10% and 20% (Table 2). In each DF diet group, weight gains were lower in animals at the 20% DF level than at the 10% DF level, but not significantly.

Apparent element absorption values for three, 60 hr balance periods for P, Ca, Mg, Fe, Zn and Cu in the six diets are illustrated in Figures 2-7, respectively. Among animal groups fed the different DF diets at two levels, the amount of each element absorbed increased from balance period 1 to balance period 3 with a few exceptions. Animals consuming 20% chitin in their diets had declining apparent absorption values for Mg (Figure 4) and Cu (Figure 7) over 23 days. Animals fed 10 and 20% chitin and 20% chitosan were in constant negative Fe balance; the magnitude of which increased in each successive balance period (Figure 5). Apparent Cu absorption in animals consuming 20% chitosan was very high and especially during the 3rd balance period. This high apparent absorption can be explained in part by the high level of Cu in this diet, 11 μg/g, versus a mean of 7.4 μg/g in the 5 other diets.

Using data illustrated in Figures 2-7, the apparent element absorption levels in each period for each animal group were pooled and are shown in Table 5.

A second criteria resulting from histopathological examination of tissues altered by DF was the number of mitotic figures in crypt epithelial cells which may indicate cell turnover from functional or morphological cell loss. Animals on chitosan diets had the largest number of cells in metaphase transition. An example of increased number of mitotic figures found in crypts of the duodenum section of the small intestine of animals on a 20% chitosan diet is shown in Figure 8B. An increased nuclear division of crypt epithelial cells can only suggest early loss of cells with production and movement of less mature epithelial cells along the villus. Villi of the duodenum from rats consuming chitosan had the appearance under a HPF of being blunt or flattened compared to villi from animals ingesting cellulose (Figure 8A).

By combining results of inflammatory response (Table 6) and the number of mitotic figures (Table 7) found in intestinal tracts, a more realistic comparison of histopathological observation and possible physiological action caused by DF is best quantified. Using both indices, total abnormalities in animal intestines caused by DF can be arranged in the decreasing order of insult: 20% chitosan > 10% chitosan > 20% cellulose > 10% chitin > 10% cellulose > 10% chitin. Another morphological change observed in animals consuming 20% chitosan included

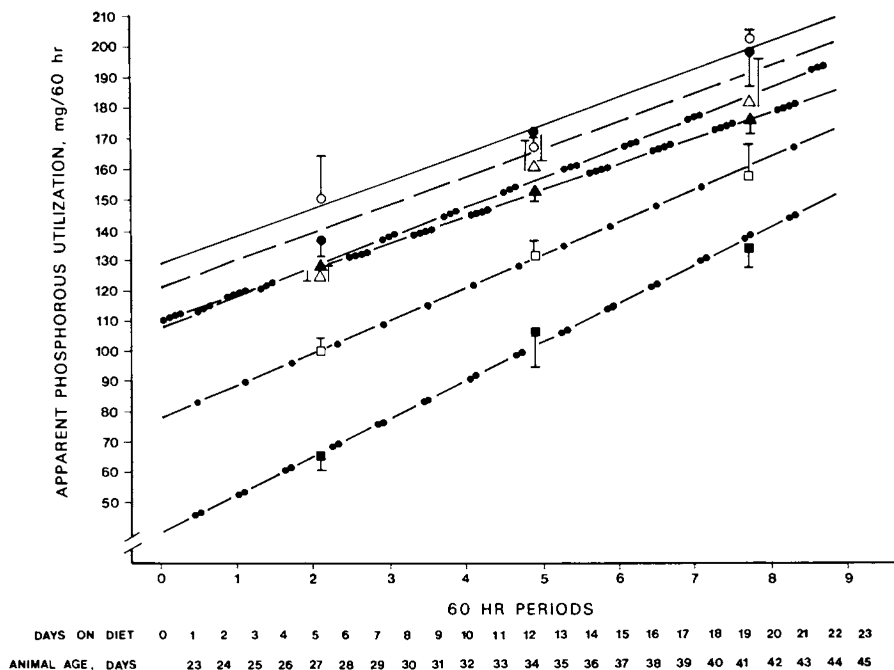


*Figure 2. Apparent utilization (i.e., absorption) of P by rats consuming three dietary fibers at two levels. Key ○–○, 10% chitin; ●–●, 20% chitin; □–•–□, 10% chitosan; ■–• • –■, 20% chitosan; △– • • • –△, 10% cellulose; and ▲– • • • –▲, 20% cellulose. Each point represents the mean absorption ± SEM for a 60 h period. Days on the diet and animal age (in days) are also indicated on the x-axis.*

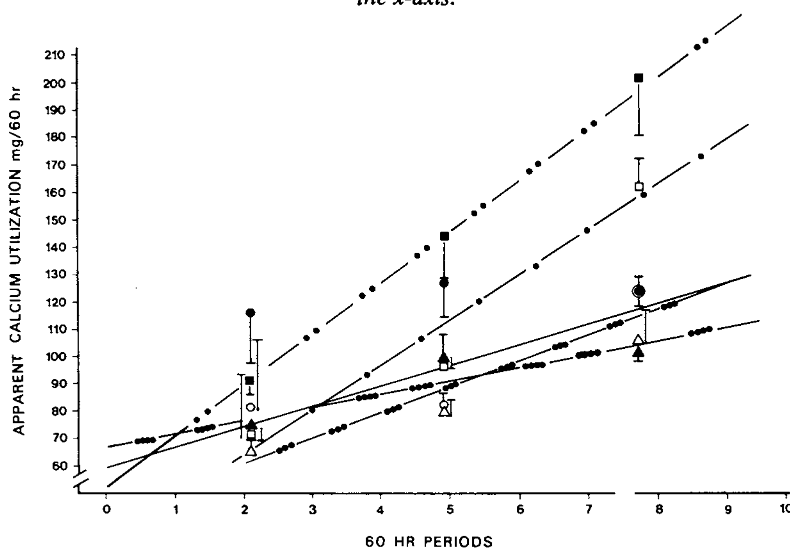


*Figure 3. Apparent utilization of Ca by rats consuming three dietary fibers at two levels. Key is the same as in Figure 2.*

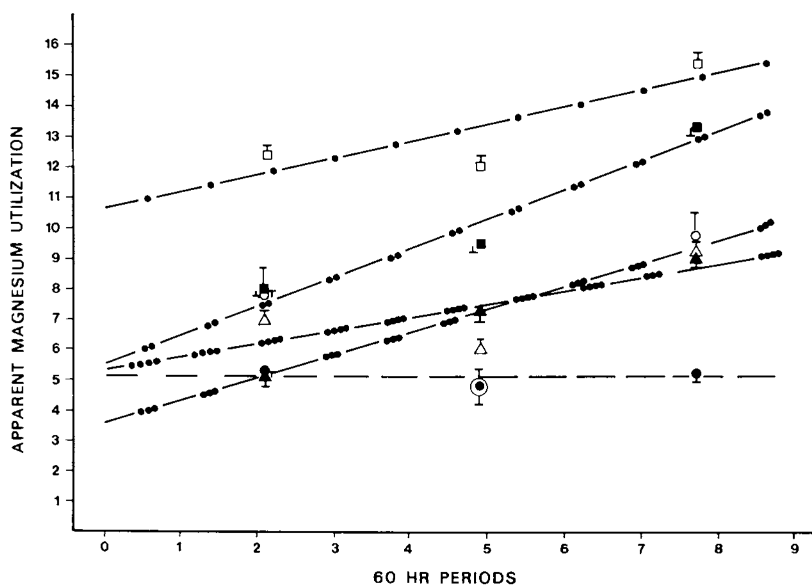


*Figure 4. Apparent utilization of Mg by rats consuming three dietary fibers at two levels. Key is the same as in Figure 2.*

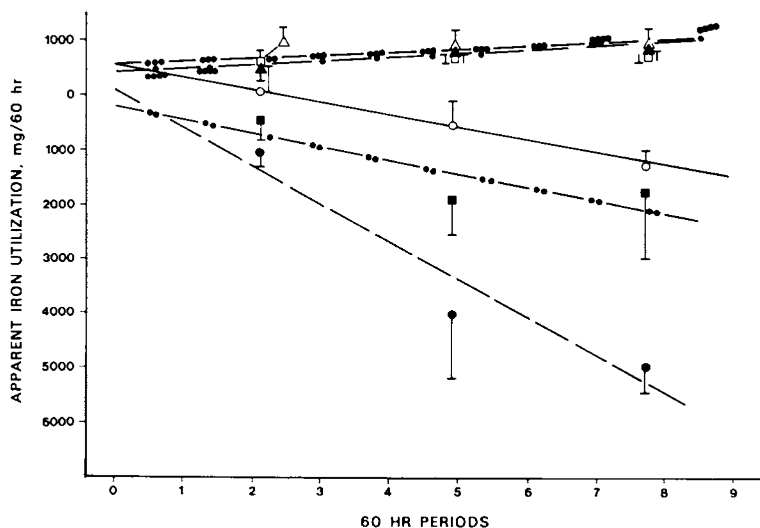


*Figure 5. Apparent utilization of Fe by rats consuming three dietary fibers at two levels. Key is the same as in Figure 2.*

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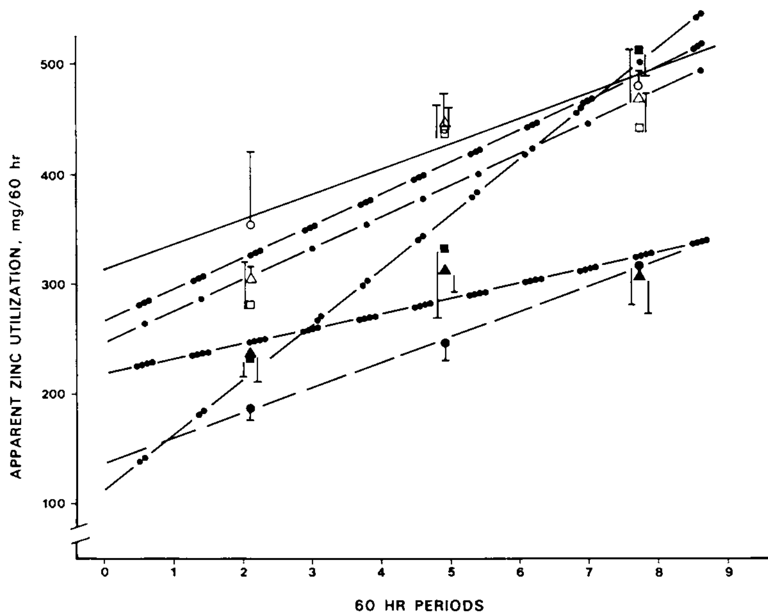


*Figure 6. Apparent utilization of Zn by rats consuming three dietary fibers at two levels. Key is the same as in Figure 2.*

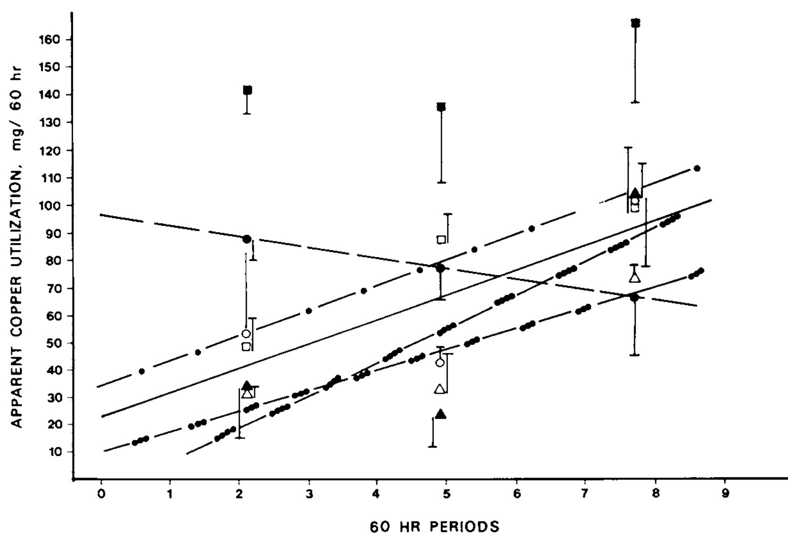


*Figure 7. Apparent utilization of Cu by rats consuming three dietary fibers at two levels. Key is the same as in Figure 2.*

Table 5. Mean apparent element absorption among animal groups consuming chitin, chitosan or cellulose at levels of 10 and 20%.[1]

| Dietary fiber | Level | Number of animals | P | Ca |
|---|---|---|---|---|
| | % | | ---------------- mg/60 hr[2] | --- |
| Chitin | 10 | 5 | $174.1 \pm 3.6^{a,b}$ | $96.0 \pm 4.8^{a,e,f}$ |
| | 20 | 5 | $166.3 \pm 3.4^{a,b}$ | $122.2 \pm 4.5^{b,c}$ |
| Chitosan | 10 | 4 | $130.0 \pm 3.8^{b}$ | $110.7 \pm 5.1^{b,c}$ |
| | 20 | 3 | $101.8 \pm 5.0^{c}$ | $141.3 \pm 6.5^{c,d}$ |
| Cellulose | 10 | 4 | $156.0 \pm 3.4^{d}$ | $87.2 \pm 4.5^{a,e,f}$ |
| | 20 | 5 | $152.0 \pm 3.8^{d}$ | $90.5 \pm 5.1^{a,e,f}$ |
| Chitin vs chitosan[4] | | | ** | * |
| Chitin vs cellulose[4] | | | ** | * |
| Chitosan vs cellulose[4] | | | ** | ** |

[1]Mean of three balance periods.

[2]Mean ± S.D.; values in same column not sharing a common superscript are significantly different (P 0.05).

[3]Values in parenthesis are negative.

[4]Pooled diet comparisons; *(P<0.05); **(P<0.01).

| Mg | Fe | Zn | Cu |
|---|---|---|---|
| | ug/60 hr[2,3] | | |
| 7.5±0.5[a,e,f] | (556)±285[a,d] | 424±14[a,c,e] | 66±9[a,b,c,e,f] |
| 5.1±0.5[b] | (3,341)±2,063[b] | 249±13[b,e,f] | 77±9[a,b,c,f] |
| 13.3±0.5[c] | 771±300[c] | 385±15[a,c,d,e] | 79±10[a,b,c,f] |
| 10.2±0.7[d] | (1,374)±388[a,d,e,f] | 358±19[c,d] | 196±13[d] |
| 7.5±0.5[a,e,f] | 843±269[c,e,f] | 407±13[a,c,e] | 46±9[a,e,f] |
| 7.2±0.5[a,e,f] | 791±301[c,e,f] | 284±15[b,f] | 54±9[a,b,c,e,f] |
| ** | ** | * | ** |
| N.S. | ** | N.S. | ** |
| ** | ** | N.S. | ** |

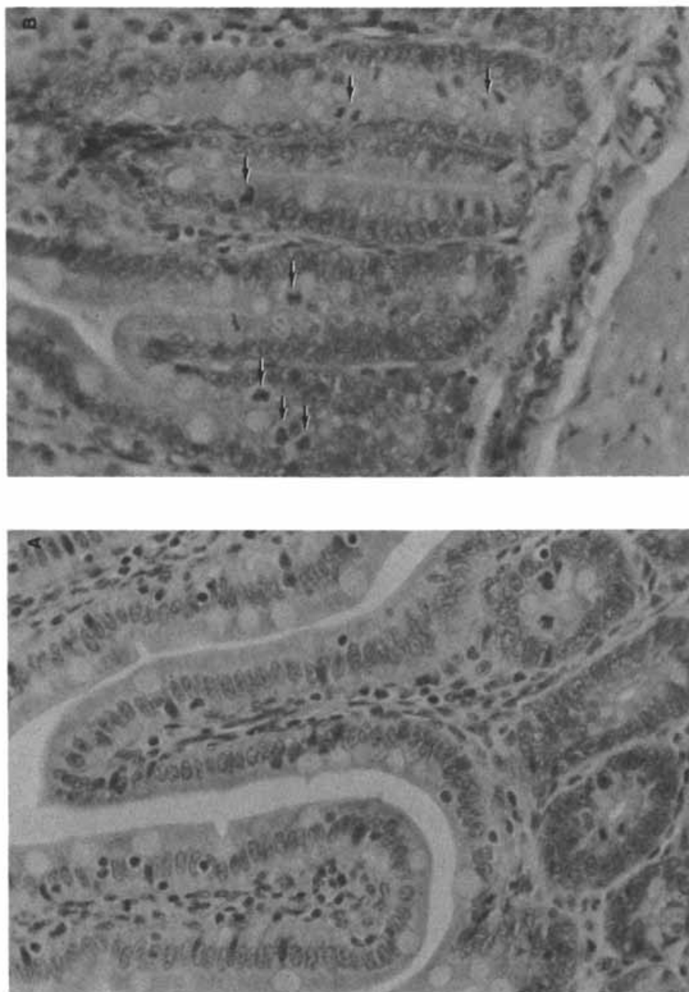


*Figure 8. Photomicroscopy of duodenum sections from two rats, one consuming 20% cellulose (A) and the second, 20% chitosan (B) 220×. Cross-section of crypt and longitudinal section of villus illustrated in A, and longitudinal section of crypt area only illustrated in B. Arrows indicate mitotic figures.*

Table 6. Inflammatory cell infiltrate of intestinal tracts from rats consuming chitin, chitosan or cellulose at levels of 10 and 20%.

| Dietary fiber | Level % | Duodenum | Jejunum | Ileum | Cecum |
|---|---|---|---|---|---|
| | | Neutrophils/HPF[1,2,3] | | | |
| Chitin | 10 | 3.7±1.1 | 3.6±0.8 | 5.4±1.4 | 4.9±2.1 |
| | 20 | 2.7±1.3 | 3.3±1.4 | 3.5±1.4 | 2.5±1.0 |
| Chitosan | 10 | 6.3±2.3 | 5.8±1.1 | 4.9±1.1 | 4.4±1.4E |
| | 20 | 3.3±1.1 | 4.6±1.6 | 6.1±1.6 | 8.0±2.6E |
| Cellulose | 10 | 3.7±1.3 | 3.7±1.1 | 3.3±0.8 | 4.1±1.2 |
| | 20 | 7.1±1.4 | 3.6±1.3 | 2.8±1.0 | 3.7±1.4 |

[1]See Material and Methods for explanation of determining number of neutrophiles per high powered field (HPF).

[2]Mean ± S.D.; differences between means were not statistically tested.

[3]E = edema.

Table 7. Mitotic activity in crypts of intestinal tracts from rats consuming chitin, chitosan or cellulose at levels of 10 and 20%.

| Dietary fiber | Level % | Duodenum | Jejunum | Ileum | Cecum |
|---|---|---|---|---|---|
| | | ------- Mitotic figures/HPF[1,2,3] | | | --------- |
| Chitin | 10 | 3.0±1.0 | 3.2±1.0 | 3.4±1.0 | 1.1±0.6 |
| | 20 | 4.1±1.4 | 3.7±1.3 | 3.4±1.3 | 0.7±0.6 |
| Chitosan | 10 | 6.5±1.2 | 6.7±1.1 | 3.9±0.9 | 1.3±0.9E |
| | 20 | 6.1±1.5 | 5.0±1.5 | 6.9±1.9 | 1.0±0.7E |
| Cellulose | 10 | 2.8±1.0 | 3.5±0.9 | 3.2±1.2 | 0.7±0.7 |
| | 20 | 4.7±1.4 | 4.5±1.3 | 3.3±0.9 | 1.0±0.7 |

[1]See Material and Methods for explanation of determining number of mitotic figures per high powered field (HPF).

[2]Mean ± S.D.; differences between means were not statistically tested.

[3]E = edema.

lacteal dilation or edema of the lamina propria of the cecum (Figure 9B). This lesion was not observed in ceca of the animals in the 20% chitin or cellulose groups (Figure 9A). For all animals, the entire small intestine showed the greatest changes with respect to dietary fiber.

Two bacteria isolated from the ceca of all test animals were *Escherichia coli* and *Enterobacter* sp. No pathogenic bacteria (e.g. *Salmonella typhimurium*) were identified. The absence of this latter organism helped rule out enterotoxemia as a cause of death in one animal and impaired growth in a second animal consuming 20% chitosan in their diets. Chitosan and chitin did not appear to change the intestinal flora population either quantitatively or qualitatively. Screening of anaerobic bacteria was not performed.

Gross and histopathological examination of lungs, kidneys, livers and salivary glands from all animal groups revealed no evidence of naturally occurring infectious diseases.

## Discussion

Chitosan is being considered as a possible blood cholesterol lowering agent. Only at levels above 5% in the diet of rats does chitosan and its parent compound chitin appear to cause harmful effects. As commercially available, neither compound can be considered homogenous. Extensive cleaning and standardization of both materials, and specifically chitosan, may be necessary for future evaluation. Results observed in this study can possibly be attributed to some unknown constituent(s) in chitin or chitosan, as may be the case when using natural products.

Animals ingesting chitin and chitosan at levels of 5, 10 and 20% did not show any significant decrease in growth after 23 days compared to animals fed cellulose at equivalent levels. Earlier reports have indicated impaired growth in rats when levels of chitosan in the diet exceeded 10% (4, 21).

At levels of 5%, different particle sizes of chitin or chitosan did not alter element absorption to any significant degree. Animals were in positive balance for each of the six elements examined. Body element composition varied, but, again, differences were small. At the 5% level in the diet and below, further long term studies are needed to establish if chitin or chitosan have any adverse effects on animal health.

Originally this experiment was designed to determine the degree of positive or negative absorption of elements as influenced by chitin and chitosan. Dietary element levels could not be standardized among diets because of the variation in amounts provided by the test DF sources. Although diets were adequate in all elements, differences observed in positive balance values may only reflect differences in amounts consumed. An example of the difference between dietary levels and

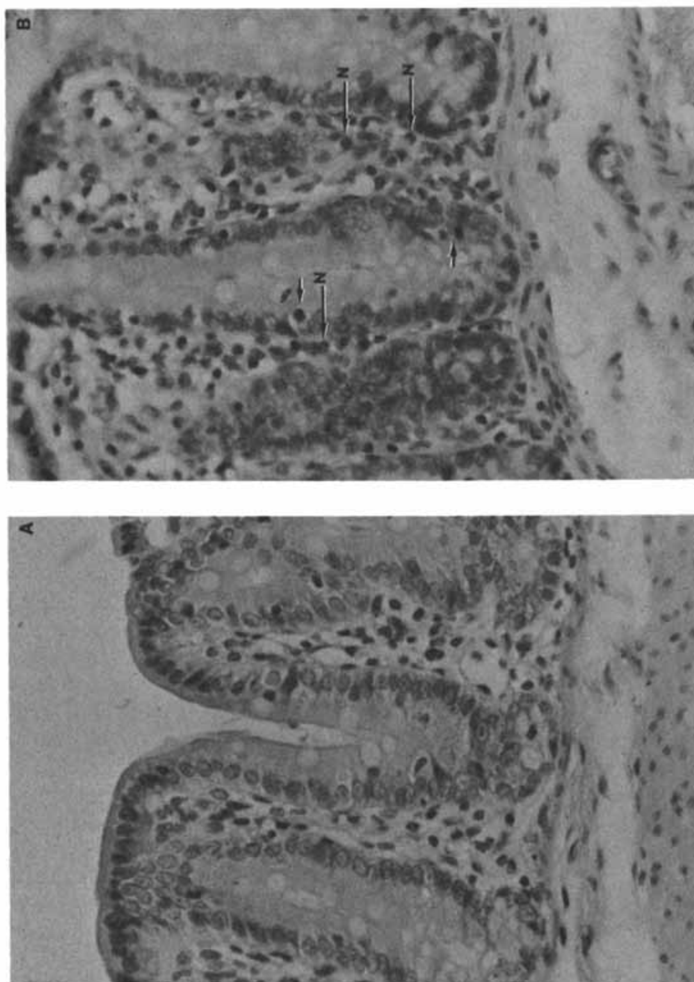


*Figure 9. Photomicroscopy of longitudinal sections of cecum from two rats, one consuming 20% cellulose (A) and the second, 20% chitosan (B) 220×. Arrows with N indicate neutrophils, short arrows indicate mitotic figures.*

absorption is discussed for Ca. Animals consuming 20% chitosan had the lowest mean absorption of Ca (102 mg/60 hr) while dietary levels were the highest (11.24 mg/g). Dietary Ca levels in the 20% cellulose control diets were lowest (4.95 mg/g), but absorption was significantly higher (152 mg/60 hr) than in the 20% chitosan fed animals. Since both diets contained the same amount of AIN salt mixture, it appears that chitosan may impair Ca absorption. However, caution must be exercised in making this assumption. Growth was lower in animals fed chitosan (20%) and this may account for the lower needs as measured by balance in this study. Further work is needed to evaluate the accuracy of balance techniques in measuring element absorption at low, adequate and excessive element intakes as employed in this study.

For the elements P, Ca, Mg, Zn and Cu, variation in apparent absorption levels existed among animal groups ingesting chitin, chitosan or cellulose at various levels. A clear trend of one DF source exhibiting an inhibitory or enhancing effect on the absorption of any one of these 5 elements was not clearly delineated. Differences existed in absorption of these 5 elements, but none of these changes resulted in animals being in marginal balance or even near negative balance.

The most striking result of this study was the consistently negative Fe balance over time observed in three of the four animal groups fed chitin or chitosan. The positive Fe balance in which animals consuming 10% chitosan maintained themselves contradicts the observations found with the other three diets. This cannot be explained considering other information obtained from the same group. Since all test animal groups had lower hemoglobin levels than cellulose controls (Table 8), the 10% chitosan fed animals would also have been expected to be in negative Fe balance.

Conflicting results were also observed in animals consuming 20% chitin. Their hemoglobin value (13.7±1.1 g Hb/dl) would have been expected to be lower considering the manner in which animals voided Fe via fecal excretion over time (Figure 5). At the rate of Fe loss observed, total depletion of Fe was probable after 23 days on the diet or shortly thereafter.

During the course of Expt. 2, one animal on a chitosan diet at the 20% level died on day 6. Histological observations of the intestinal tract were inconclusive because of the time delay between death and examination (ca. 12 hr). Microbiological survey of internal organs indicated the presence of Proteus sp in the lung and extensive septicemia. This latter condition was diagnosed as the cause of death. A second animal in this group began to show signs of decreased food intake and growth. The animal was maintained on the diet until euthanized and immediately necropsied on day 9. Gross pathology indicated the intestine to be greatly distended with very firm luminal contents appearing to produce constipation. Mild enteritis and

Table 8. Final hemoglobin level in animals consuming chitin, chitosan or cellulose at levels of 10 and 20% for 23 days.

| Dietary fiber | Level % | No. of animals | Hemoglobin g/dl[1] |
|---|---|---|---|
| Chitin | 10 | 5 | 12.7±0.6[a] |
| | 20 | 5 | 13.7±1.1[a] |
| Chitosan | 10 | 4 | 13.5±0.9[a] |
| | 20 | 3 | 13.5±0.9[a] |
| Cellulose | 10 | 4 | 14.5±0.7[b] |
| | 20 | 5 | 14.4±1.1[b] |

[1]Mean ± S.D.; values not sharing a common superscript are significantly different ($P<0.05$).

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cecitis were observed, apparently caused by the physical nature of the diet. The swelling and viscous properties of chitosan in acid have been documented (29) and are considered to be some of the major factors contributing to the morbidity and mortality in animals eating this compound. Gross and histological changes observed in the animal examined on day 9 were similar to those seen in animals sacrificed on day 23.

It is not known when, or at what rate, morphological changes take place in the intestine upon ingestion of any DF. However, adaption of the gut to the type and level of DF may account for some of the conflicting results in Fe balance and hemoglobin levels previously mentioned. Early insult to the intestinal wall could lead to immediate interference with Fe absorption and negative balance as was observed with three test diets but not the fourth (i.e. 10% chitosan). Longer feeding trials with balance measurements may be necessary to ascertain if Fe status will be compromised in animals consuming cellulose at high levels. It would appear advantageous to examine the rate of intestinal cellular change in greater detail as well as the ability of this animal model to adapt to type and level of DF.

Histopathologic examination of the intestinal tract of all animals, but especially those consuming chitosan, indicates a possible mechanism to explain the results observed with Fe absorption. The physical nature of chitosan, and to a lesser degree, chitin, when ingested at levels above 5%, seemed to produce a mechanical abrassive effect on the intestinal mucosa. In addition to the increased number of neutrophils in the intestinal lamina propria, the active mitoses of the crypt epithelial cells suggested premature loss of cells from the villi. If the "intestinal mucosal block" (30, 31) mechanism was impaired, this could explain both the animals inability to absorb dietary Fe plus the negative Fe balance observed. Immature mucosal cells do not have sufficient Fe levels to regulate Fe intake from the gut lumen and their early sloughing leads to continued loss of Fe supplied from body stores. In support of this theory, the results of a scanning electron microscopy study which evaluated the topographical changes of the jejunum and colon of rats consuming various types of fiber suggested that alterations in the gut ultrastructure caused by DF may be associated with altered bile acid reabsorption (32).

The chemical properties of chitosan and chitin may provide another mechanism for decreased element absorption and specifically Fe. Chitosan differs from chitin in having free amino groups causing partial solubilization in stomach acid and resulting in viscous intestinal lumen contents. This chemical change does not result with chitin ingestion to any significant degree. However, since chitin may contain some free amino groups, the action of this functional group on the gut needs to be examined further. The free amino groups of chitosan are

believed to bind bile acid and/or alter micelle formation (4). Through one or both of these mechanisms, consumption of chitosan by rats lowers their blood cholesterol (4, 16, 17). Because of the viscous nature of the intestinal contents when chitosan is partially dissolved with stomach acid, some type of element "trapping" could be envisioned in the intestine. In this respect, Fe may be prevented from reaching absorptive sites. However, Fe would have to be selectively "trapped" or chelated since the utilization of other elements were not impaired to the same degree as observed with Fe. An interpretive finding of this study may be supportive evidence of how certain fibers, at high levels of consumption, are responsible for impaired Fe uptake observed in dogs (33) and human experiments (34, 35, 36).

## Conclusion

Chitin and chitosan at very high levels of intake may cause physical insult to the intestinal tract of rats. Also, apparent absorption of Fe is impaired when chitin and chitosan are fed at dietary levels of 10% and 20%. However, such high levels of intake are atypical. At dietary levels below 5%, neither compound appears to compromise element absorption.

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# Citrus Pectic Polysaccharides—Their in Vitro Interaction with Low Density Serum Lipoproteins

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Dietary fiber," the skeletal remains of plant cells that are resistant to digestion by enzymes of man" (1) include at least five different known components: lignin, cellulose, hemicellulose, gums and pectins. Lignin is a non-carbohydrate component and appears to have no known role in human nutrition. Cellulose and hemicellulose affect large bowel function and cause an increase in stool weight and decrease in transit time. Gums and pectins can also increase intestinal transit time, adsorb fecal water, influence glucose and lipid metabolism, mineral absorption and, in addition, may possibly play a role in colon cancer.

With the exception of cellulose, a polymer of glucose, the chemistry of hemicelluloses, gums and pectins is extremely complex (2). Pectin, a heterogeneous mixture of a number of complex pectic polysaccharides, appears early during plant cell wall biogenesis. A series of complex biochemical steps result in the formation of cell plate followed first by its growth in area (primary cell wall) then in thickness (secondary cell wall). Exclusive of randomly oriented cellulose fibrils, primary cell wall is composed mainly of pectic polysaccharides (3). These pectic polysaccharides are rich in D-galacturonic acid, D-galactose and L-arabinose residues. With growth in thickness, there occurs a replacement of pectic polysaccharide deposition with polysaccharides rich in D-glucuronic acid, D-xylose and D-glucose residues. These polysaccharides which add to the growth in thickness of the plant cell wall are classified as alkali-soluble hemicelluloses. Previous studies (4) the chemistry of pectin suggested the presence of three major polysaccharides in pectin, namely 1) a glacturonan polymer containing 1,4 linked α-D-galacturonic acid residues, 2) a galactan polymer containing chains of 1,4 linked α-D-galactopyranose residues and 3) and arabinan a branched polymer containing 1,5 and 1,3 linked L-arabinofuranose residues. Comparatively recent studies (2-3,5-6), however, suggest that the chemistry of pectin is much more complex than previously

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believed. Presence of covalantly-linked neutral sugars especially galactose, rhamnose and arabinose to polymers of galacturonic acid residues was demonstrated. Also, demonstrated was the presence of L-fucose, D-apiose and D-xylose covalently linked to three major polysaccharides, i.e., galacturonans, arabinans and galactans as mentioned earlier. The demonstrated covalently-linked amino acids such as hydroxy proline within the structural framework of various pectic polysaccharides added further complexity to the chemistry of pectin.

Cholesterol Lowering Influence of Pectin: Several investigators (7-9) have clearly suggested that pectin when supplemented in the diet of laboratory animals and/or human volunteers causes significant reduction in serum cholesterol levels. This, indeed, is an important observation, as it has been shown that the etiology of atherosclerosis and coronary artery disease is associated with elevated serum cholesterol levels, and the observed reduction of serum cholesterol level by pectin holds promise for the treatment and/or prevention of these diseases. It is relevant here to point out that dietary pectin has shown to retard induced avian atherosclerosis (10).

Pectin Bile Salt Interaction. The biochemical basis by which pectin causes lowering of serum cholesterol level remains elusive. It has been suggested that dietary fibers in general cause lowering of cholesterol levels by binding with bile salts and consequently causing a break in the enterohepatic circulation of bile acids and increasing cholesterol turnover (11-13). This explanation is similar to one where cholestyramine, an ion exchange resin (Dowex 1x2 $Cl^-$), used as a drug (Cuemid, Questran, Quantalan) to affect the lowering of serum cholesterol levels when given to patients with hypercholesterolemia. Critical evaluation of data suggesting the binding of bile salts to dietary fiber reveals that the observed *in vitro* affinity of various dietary fibers to bile acids is much lower when compared with binding of bile acids with cholestyramine. The *in vitro* affinity of cholestyramine for acids is 1.0 mg bile acid/mg of resin estimated at pH 6.4 in 0.9% saline (14). On the other hand, the *in vitro* binding of bile acids to fibers such as wheat straw, sugar cane pulp, bran, oat, etc., ranges from 0.007 mg to 0.024 mg of bile acid per mg of fiber in normal saline. These values amount to 41-142 times less than the affinity of bile acids to cholestyramine. Although alfalfa, guar gum and pectin, unlike the various fibers mentioned above, showed better affinity for bile acids, this affinity is about 5 times less than the affinity of bile acids to cholestyramine. For a physicochemical phenomenon to be of biological significance, the reactants mush exhibit a stoichiometric relationship. In the studies mentioned above (12,13), although increasing the amount of various fibers in the incubation

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mixture did cause an increase in the amount of bile acid bound to the various fibers studied, this observed increase was not stoichiometric. Furthermore, no binding of bile acids to purified dietary fibers was observed by these investigators. It is of interest to mention the results of a recent study (15) which failed to demonstrate the *in vitro* binding and/or interactin of pectin with bile acids when examined by utilizing NMR spectroscopy. The observations made by these investigators are similar to those where interaction of bile acids and dietary fibers from a variety of sources was studied *in vitro* by utilizing labelled bile acids, equilibrium dialysis and/or centrifugation techniques (16). Increased excretion of bile salts has also been presented as an evidence for the binding of bile acid to dietary fibers consequently resulting in hypocholesterolemia (17,18). However, in a recent study (19) where fecal bile acid excretion was studied in response to intake of a variety of fibers, no significant alterations in the fecal bile acid content was observed. It is evident from the above that our current understanding of the biochemical basis by which dietary fiber causes lowering of cholesteol levels is far from complete, and therefore it needs to be researched and re-evaluated.

*Pectin Lipoprotein Interaction.* Cholesterol in serum is not found free but is carried by a number of lipoproteins found in serum. Low density lipoprotein (LDL) is the major carrier of circulating cholesterol. Elevated levels of LDL in the serum contribute significantly to the risk of coronary heart disease, and development of atherosclerosis. Atherosclerotic lesions are characterized by intimal proliferation of smooth muscle cells accompanied by accumulation of large amounts of connective tissue components such as collagen, elastin and glycosaminoglycans. In atherosclerosis, presence of large amounts of LDL and glycosaminoglycan complexes in plaques have been demonstrated (20,21). Glycosaminoglycans, like pectin, are polyanionic complex carbohydrates found in the connective tissue of animals.

In view of the fact that pectin lowers serum cholesterol levels and cholesterol is carried by lipoproteins in the serum, we investigated the interaction of pectin obtained from grapefruit albedo with various human serum lipoprotein fractions *in vitro* (22). It was observed that pectin interacted specifically with LDL, while no interaction was observed between pectin and high density or very low density lipoproteins. The biological significance of this observed *in vitro* interaction of pectin with LDL remain unclear, primarily because of lack of evidence suggesting entry of pectin or some component of it into the blood streatm. Therefore, unless such entry and/or absorption of pectin or one of its components is demonstrated, the biological implication of this observed interaction

resulting in the formation of pectin-LDL complexes and the metabolic fate of such complexes if found remains hypothetical in nature.

Our ongoing studies aimed to gain an insight into the biochemical basis of this observed interaction between LDL and pectin have revealed that selective modification of basic amino acid residues such as histidine, arginine and lysine, found in LDL caused a significant loss of interaction between pectin and LDL. These results suggest that various basic amino acids are involved in the observed interaction between pectin and LDL (23).

Results from our recent experiments designed to evaluate the comparative effectiveness of cholystyramine and pectin in lowering serum cholesterol levels in rats (24), clearly suggest that pectin is comparatively as effective as cholestyramine in lowering serum cholesterol levels and this observed lowering of serum cholesterol by pectin is mediated through an exclusive lowering of cholesterol associated with LDL. Selective lowering of LDL by pectin (25) and by other dietary fibers such as oat bran (26) has also been reported by other investigators. Based on these observations, it could be concluded that the mediation of serum cholesterol lowering effect of pectin is accomplished by lowering of cholesterol associated with serum low density lipoproteins by mechanisms which remain elusive. Furthermore, experimental attempts to elucidate the possible mechanism by which pectin causes lowering of cholesterol level must take into account the heterogeneous nature of pectin, for a unique pectic polysaccharide found in pectin may be solely responsible for the observed hypocholesterolemic activity of pectin.

Selective Interaction of various Pectic Polysaccharides with Low Density Lipoproteins. As mentioned earlier, *in vitro* interaction between unfractionated pectin and LDL results in the formation of insoluble complexes (22). Further insight into this observed interaction was gained by examining this interaction between isolated pectic polysaccharides found in pectin and human LDL. Various pectin polysaccharides found in citrus pectic were resolved by employing DE-52 cellulose ion exchange column chromatography techniques (27). Four chemically distinct pectic polysaccharides were resolved following sequential elution of column bound pectin with 0.025, 0.1, 0.25 and 0.5 M sodium phosphate buffer, pH 6.0, and composed of 13% (Fraction I), 5% (Fraction II), 62% (Fraction III), and 20% (Fraction IV), respectively, of the total pectin subjected to ion exchange chromatography. The galacturonic acid contents of these individual pectic polysaccharide fractions were not strikingly different and ranged between 70-80% of the total carbohydrate contents. However, the neutral sugar composition of these pectic polysaccharides was found to be different. A progressive decrease in the degree of methylation of these pectic

polysaccharides was also observed in that pectic polysaccharide eluted with 0.1 M salt (Fraction II) was highest in its methoxy content (14.85%), whereas pectic polysaccharide eluted with 0.5 M salt (Fraction IV) was lowest in its methoxy content (4.42%). The methoxy content of Fraction I, the fraction eluted with 0.025 M salt, and not retained by ion exchange resin was found to be similar to unfractionated pectin (8.9%). Examination of neutral sugar composition of various pectic polysaccharides revealed that Fraction I and Fraction IV were rich in rhamnose content, when compared with other pectic polysaccharides (28).

Examination of interaction between these pectic polysaccharide fractions and LDL *in vitro* by employing experimental techniques described earlier (22), revealed the selective nature of this interaction. Pectic polysaccharides I and IV, i.e., polysaccharides rich in rhamnose content were the only polysaccharides which interacted specifically with human LDL, causing their precipitation *in vitro*. No or little interaction between LDL and pectic polysaccharide Fractions II and III was observed. These observations suggest that pectic polysaccharides rich in rhamnose sugar residues, specifically interact exclusively with human LDL, and this interaction appears to be independent of the methoxy content of the polysaccharides.

In summary, studies demonstrating *in vitro* interaction between a variety of dietary fiber and bile acids (12,13) may be of importance in part in explaining the biochemical basis by which ingestin of dietary fiber causes lowering of serum cholesterol levels. However, in view of results obtained from comparatively recent *in vitro* (15,16) and/or *in vitro* (19) studies indicating lack of interaction between dietary fiber and bile salt, the existance of a much more complex mechanism by which dietary fiber causes lowering of serum cholesterol level could not be overruled. The finding of specific *in vitro* electrostatic interaction between pectin and human low density lipoprotein (22) adds further complexity to the fiber and serum cholesterol connection. Further complexity is added to this connection by observations suggesting heterogeneous nature of dietary pectin, and selective interaction of rhamnose rich pectic polysaccharides with human low density lipoprotein. It is, therefore, safe to conclude that, only through studies aimed towards defining the psysicochemical nature, metabolism and cholesterol-lowering capability of well defined individual polysaccharides found in dietary fiber, can an insight into the elusive biochemical basis by which dietary fiber causes lowering of serum cholesterol be gained.

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# 14

# Washed Orange Pulp: Characterization and Properties

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Hamlin and Valencia washed orange pulp from several crop years were dried and analyzed. The range in values of total pectin, neutral detergent fiber and crude fiber were 26.0-45.6%, 15.8-31.0% and 9.9-20.6% respectively. In addition, detailed nutrient and dietary fiber analyses were obtained for selected samples. Functional properties such as flavor muting, chromatophore formation and oil holding properties were noted. Food processing modification yields a novel form of the hydrated pulp with extended functional properties.

Interest in dietary fiber has refocused attention on a known but underutilized fiber source: washed orange pulp. Its precursor, orange pulp, is that tissue produced upon expression of orange juice from the juice vesicles. With further extraction(s) of residual soluble sugars from the pulp, an osmotic re-equilibrium occurs to yield the washed orange pulp. This washed orange pulp is obtained as a hydrated complex of vesicular carbohydrates at 4-10% solids. As a byproduct of the juice concentrate industry, an estimated 500,000 to 1,000,000 tons of wet pulp are produced each crop year. Unlike suggested more exotic sources of dietary fiber, orange pulp is already an established, commonly consumed food component in the American diet. The functional and nutrient properties of washed orange pulp are intimately related to a series of factors. The foremost of these is the orange species but also significant are climatic and crop year variants, harvest maturity, expression and extraction processing variables, uncontrolled enzymatic and microbial activity as well as method of drying. Because of variations in the material, application and development as a fiber has been limited.

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## Methods and Materials

Valencia and Hamlin washed orange pulp were obtained from central Florida cooperatives in the 1978, 1979 and 1980 crop years. The pulp was steam injected to give a minimum temperature of 95°C, then packed and frozen. Samples were thawed immediately before experimental use. Samples of drum dried, freeze dried and spray dried washed orange pulp were prepared in the pilot plant facilities of the General Mills James Ford Bell Technical Center as needed. Compositional and nutrient analyses were performed by Medallion Laboratories according to standard AOAC and AACC procedures. Sugar analyses were determined by HPLC according to DeVries *et al.* (1). Water holding (and oil holding) capacities were determined according to Porzio and Baumann (2). In this procedure, a weighed sample, approximately 1 gram and of uniform particle size, was suspended in an excess of liquid, stirred at ambient temperature for 30 minutes and transferred to a vacuum filtration apparatus. Excess liquid was removed by filtration at 60-100 mm Hg pressure for 3 minutes and the weight gain of liquid reported as water (or oil) holding capacity in grams per gram of sample.

Total pectin is the sum of soluble pectin and protopectin. Soluble pectin was determined by the following procedure: macerated tissue was extracted at 80-90°C in distilled water for 1 hour, the supernatant separated by centrifugation and an additional extraction-centrifugation step repeated. The combined supernatants were brought to 0.5 *N* NaOH, saponified for 4 hours at 26°C, neutralized with acetic acid to pH 5 and precipitated as the calcium pectate at a final calcium concentration of 0.3% (w/v) by addition of $CaCl_2$. The heat flocculated precipitate was filtered on a glass wool support in a 50 ml fritted crucible, rinsed free of calcium salts with distilled water, then washed with acetone to remove remaining lipids and moisture. The precipitate was dried at 70°C in a vacuum oven overnight and reported as calcium pectate. Protopectin was determined on recovered fiber solids from the soluble pectin determination. Fiber solids were hydrolyzed in 0.15 *N* HCl for 1 hour at 80°C. The sample was centrifuged and supernatant retained, tissue rewashed with distilled water and centrifuged. The combined supernatants were neutralized to pH 7, saponified as described above and calcium pectate determined. The acid extracted solids fraction was adjusted to 0.05 *N* NaOH and saponified for 4 hours at 26°C. After the pH was brought to 5 with acetic acid, the sample was centrifuged to yield neutralized supernatant which was then precipitated at 0.3% calcium chloride. The two precipitates from the acid and base extractions were combined, rinsed with distilled water, dried, and reported as protopectin.

Solvent extractions were performed by treatment of 100-150 grams of 93% moisture washed orange pulp in a Soxhlet apparatus charged with 600 ml of acetone or isopropanol. The extraction was continued 3-6 hours by which time no additional yellow pigment could be observed in the extracting solvent. The tissue mass was dried at ambient temperature until no detectable residual solvent was evident. Aqueous extractions of the 93% moisture washed orange pulp were performed with 0.4 M sodium citrate, 0.2 M EDTA, 0.1% $H_3PO_4$, 0.5% $Na_2CO_3$ and 5% NaOH solutions in respective experiments. In each case 200 ml of extracting solution was added to 100 grams of pulp and stirred for three hours of 25°C. After filtration the extracted tissue was washed three times with 200-250 ml of 50°C distilled water and filtered. Those samples for OHC or WHC were then freeze dried. Samples for microscopic examination were kept in the hydrated state.

Soluble and insoluble dietary fiber analyses were performed according to Furda (3).

Phenolic(s) were analyzed colorimetrically utilizing reagent 2,6-dibromo-N-chlorobenzo-quinoneimine. Catechin was the reference standard and results are reported as mg "catechin equivalent" per gram sample.

## Results and Discussion

Washed orange pulp, obtained from FMC juice finishers is a relatively homogeneous fraction of the juice vesicle (4). This material also contains chromoplast lipids and cytoplasmic materials such as proteins, bioflavanoids, soluble carbohydrates, limonoids and hydrolytic enzymes (5). The processor can control the uniformity of the material but care is necessary to prevent indigenous pectinesterases from rapidly altering the character of the tissue. Microbial activity can also lead to undesirable changes. Therefore steam injection to pasteurize and stabilize the material is extremely desirable. Washed orange pulp has a membranous structure which is shown in Figure 1A. A view of the surface is presented in Figure 1B where the fibrillar organization of the cell wall pectins is evident at the higher magnification. Figure 1C shows an SEM photomicrograph of an identical sample of washed orange pulp.

Hamlin and Valencia washed orange pulps were obtained and analyzed and the results are presented in Table I. Because of low solids content of wet pulp, analyses were performed on the drum dried product and the reported moisture level reflects the processing conditions of drying. The change in tissue morphology upon drum drying is shown in SEM photograph Figure 1D where the surface results from contact of the pulp and its soluble solids with the hot drier surface.

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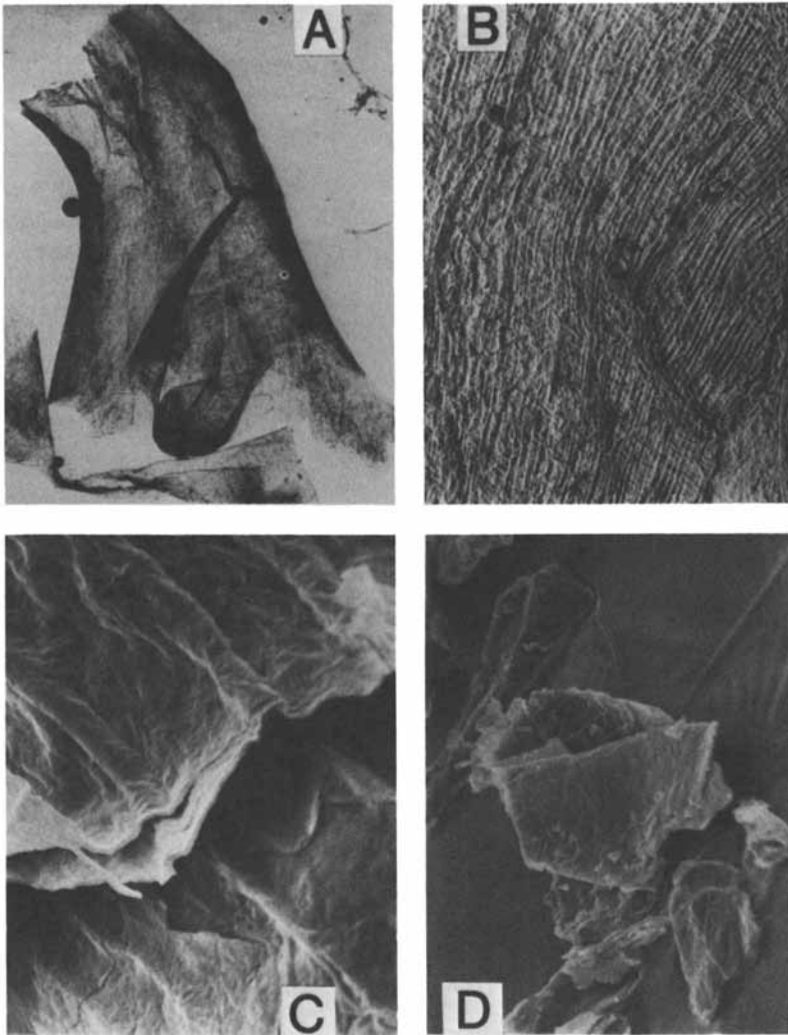


*Figure 1. Washed orange pulp (juice vesicles). Key: a, whole juice vesicle, 13×; b, enlarged view of vesicle surface showing pectin ridges, 140×; c, scanning electron microscopy (SEM) of untreated juice vesicle, 200×; and d, scanning electron microscopy (SEM) of drum-dried washed orange pulp, 220×.*

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TABLE I

COMPONENT COMPOSITION OF WASHED ORANGE PULP (a,b)

| | ORANGE VARIETY (YEAR) | | | |
|---|---|---|---|---|
| | HAMLIN (1978-79) | HAMLIN (1977-78) | VALENCIA (1978-79) | VALENCIA (1979-80) |
| MOISTURE | 2.89 | 3.00 | 9.67 | 7.44 |
| PROTEIN (N*6.25) | 8.89 | 9.63 | 7.82 | 7.47 |
| SUGARS, TOTAL | 35.51 | 11.11 | 18.91 | 8.59 |
| SUGARS, REDUCING | 18.10 | 7.92 | 9.83 | 5.30 |
| FAT | 0.91 | 0.47 | 0.84 | 0.73 |
| ASH | 3.27 | 1.85 | 2.08 | 3.11 |
| NDF | 15.80 | 26.08 | 22.94 | 31.02 |
| ADF | 13.76 | - | - | - |
| CRUDE FIBER | 9.92 | 16.88 | 15.40 | 20.55 |
| PECTIN, TOTAL | 26.00 | 38.84 | 34.39 | 45.63 |
| PECTIN, PROTO- | 17.89 | 26.31 | 28.29 | 36.46 |
| PECTIN, SOLUBLE | 8.11 | 12.53 | 6.10 | 9.17 |

(a) Grams/100 grams.
(b) Samples were drum dried and milled before analysis; NDF, neutral detergent fiber; ADF, acid detergent fiber

Analyzed sugar values mainly reflect the efficency of an individual processor in removing the soluble juice sugars. The 8.6% sugar value probably represents the lowest practical limits of commercial processing efficency and economics. In Table II the component compositions of Table I have been recalculated to a moisture and sugar free basis. Agreement of pectin, protein and neutral detergent fiber values now reflect inherent insoluble cell component composition. A more accepted method in the analysis of fiber components for the sugar rich washed orange pulp utilizes preparation of alcohol insoluble solids as reported by Ting (6). In our case the drum dried pulp is more representative of a food fiber ingredient and so analyzed. Dietary fiber composition of washed orange pulp by the method of Furda (3) yields both soluble and insoluble dietary component. These values are found in Table III along with selected reference fiber materials. A more complete nutrient analysis of drum dried washed orange pulp is presented in Table IV along with a reference fiber-wheat bran. The significant difference between these two fibers is noted in the large component of starch and low pectin in bran as compared to a much greater equivalent of pectin of the pulp. Two heat sensitive vitamins, A & C, were determined additionally on a freeze dried washed orange pulp sample. The expected larger values reflect original levels in the tissue before drum drying.

Pectin has become a subject of increasing interest with elucidation of the role of dietary fiber in cholesterol reduction (7). As noted, washed orange pulp is a rich source of pectin(s). We have reported pectin as soluble pectin as well as protopectin, i.e. large molecular weight, methoxylated insoluble pectin. In our procedure protopectin values may include some hemicelluose material as a function of the analysis. Distinction between the soluble and protopectin can be seen with microscopic examination of extracted tissue. Figure 2A shows a washed orange pulp juice vesicle after mild extraction in dilute sodium carbonate solution at ambient temperature. Degredation of the pectinaceous fibril surface is evident. There is retention of the inherent tissue organization even while segments of soluble pectin are removed. Upon more rigorous extraction with 5% NaOH, complete loss of pectinaceous material results leaving the inner lamella composed of relatively pure cellulose (Figure 2B).

The property of dried citrus pulp to be "wet" by oils has been described by Kesterson and Braddock (8). Washed orange pulp solids normally contain 0.5-1.5% ether extractible lipids. Also present is cuticular wax which gives lipophilic character to the pulp solids (9). Both the water holding capacity (WHC) which encompasses sorption and hydration phenomena, and oil holding capacity (OHC) which can be described in terms of adsorption and capillary entrapment of

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TABLE II

COMPONENT COMPOSITION OF WASHED ORANGE PULP

ADJUSTED TO MOISTURE AND SUGAR-FREE BASIS (a)

| | ORANGE VARIETY (YEAR) | | | |
|---|---|---|---|---|
| | HAMLIN (1978-79) | HAMLIN (1977-78) | VALENCIA (1978-79) | VALENCIA (1979-80) |
| PROTEIN | 14.43 | 11.21 | 10.95 | 8.90 |
| FAT | 1.48 | 0.55 | 1.16 | 0.86 |
| ASH | 5.33 | 2.15 | 2.91 | 3.70 |
| NDF | 25.77 | 30.38 | 32.08 | 36.92 |
| CRUDE FIBER | 16.15 | 19.65 | 21.56 | 24.41 |
| PECTIN, TOTAL | 42.41 | 45.22 | 48.15 | 54.34 |
| PECTIN, PROTO- | 29.18 | 30.63 | 39.61 | 43.42 |
| PECTIN, SOLUBLE | 13.23 | 14.59 | 8.54 | 10.92 |

(a) Calculated from data in Table I; reported as grams/100 grams

TABLE III

COMPOSITIONAL AND NUTRIENT ANALYSIS OF DRUM DRIED VALENCIA WASHED ORANGE PULP AND AACC REFERENCE WHEAT BRAN (a,b)

| COMPONENT | WASHED ORANGE PULP | WHEAT BRAN | UNITS |
| --- | --- | --- | --- |
| PROTEIN (N* 6.25) | 8.61 | 15.96 | G/100 G |
| FAT | 1.12 (c) | 5.83 (d) | " |
| ASH | 3.70 | 5.71 | " |
| SUGARS, TOTAL | 16.81 | 7.86 (e) | " |
| SUGAR, FRUCTOSE | 4.15 | - | " |
| SUGAR, GLUCOSE | 3.59 | - | " |
| CRUDE FIBER | 18.97 | 9.94 | " |
| NEUTRAL DETERGENT FIBER | 32.89 | 44.87 | " |
| PECTIN, TOTAL | 48.47 | 3.30 | " |
| PECTIN, PROTO- | 36.34 | - | " |
| PECTIN, SOLUBLE | 12.13 | - | " |
| STARCH | (f) | 19.4 | " |
| VITAMIN A | 285. [796,g] | - | I.U./100 G |
| VITAMIN B1 | 0.45 | 0.87 | Mg /100 G |
| VITAMIN B2 | 0.16 | 0.44 | " |
| VITAMIN B6 | 0.23 | 0.65 | " |
| VITAMIN C | 22.30 [88.8,g] | - | " |
| NIACIN | 1.71 | 23.3 | " |
| CALCIUM | 528.5 | 1300. | " |
| MAGNESIUM | 123.6 | 480. | " |
| SODIUM | 65.8 | 112. | " |
| POTASSIUM | 466.2 | 1540. | " |
| IRON | 0.3 | 0.14 | " |
| ALUMINUM | 0.3 | < 0.01 | " |

(a) Values reported on a moisture free basis; (b) AACC wheat bran standard, R07-3691; (c) ether extractable; (d) by acid hydrolysis; (e) as invert sugars; (f) not normally analyzed; (g) freeze dried sample.

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TABLE IV

FIBER ANALYSIS OF EEC/IARC REFERENCE SAMPLES AND HAMLIN WASHED ORANGE PULP, % DRY MATTER [a,b]

| MATERIAL | $H_2O$ | FAT | NDF | TDF | IDF | SDF | | | TOTAL PECTINS IN SAMPLE |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | TOTAL | % PECTINS | % N.P.[b] | |
| WHEAT BRAN | 9.0 | 2.8 | 42.1 | 43.0 | 37.0 | 11.0 | 26.0 | 74.0 | 2.9 |
| APPLE PULP | 8.5 | 0.3 | 8.7 | 12.7 | 9.1 | 3.6 | 85.8 | 14.2 | 3.1 |
| POTATO POWDER | 9.2 | - | 2.9 | 10.5 | 6.0 | 4.5 | 57.6 | 42.4 | 2.6 |
| CITRUS PECTIN | 8.7 | 0.1 | 0.3 | 92.5 | 0.3 | 92.5 | 92.1 | 7.9 | 84.9 |
| WASHED ORANGE PULP[c] | 4.5 | 1.2 | 32.9 | 46.5 | 21.9 | 24.6 | 8.1 | 91.9 | 26.6 |

a Reprint from reference 3, pg. 169, by courtesy of Marciel Dekker, Inc.
b N. P. - neutral polysaccharides; IDF - insoluble dietary fiber; SDF - soluble dietary fiber.
c Hamlin

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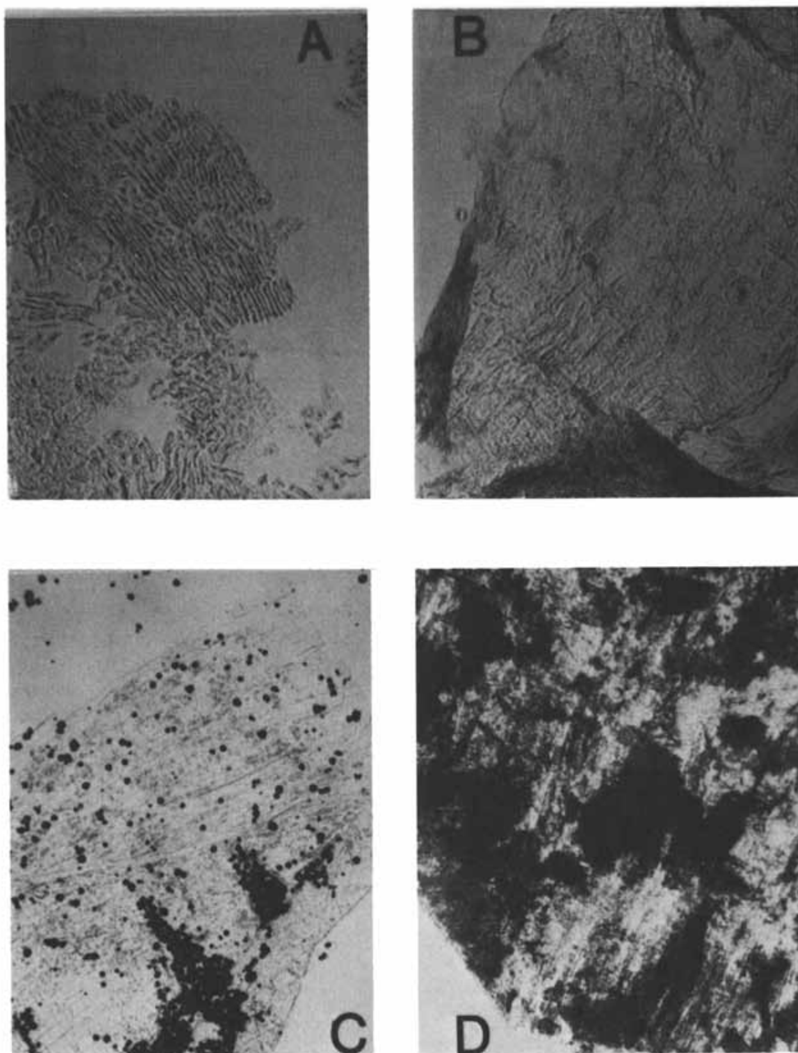


*Figure 2. Structural characterization of juice vesicles. Key: a, extracted juice vesicle showing partially removed pectin, 100×; b, cellulose inner lamella of juice vesicle following 5% NaOH extraction of A, 110×; c, starting material in processed base with iodine stained starch granules, 82×; and d, finished processed base with starch bound to juice vesicle, 82×.*

the oil were determined for different dried and extracted samples (Table V). Density of the fiber particulates was found to have a reciprocal relationship with OHC. Solvent extracted samples contain less than 0.2 % lipids yet exhibited increases in OHC. The extraction is a low temperature drying and yields fiber without major changes in structure. This could explain in part the increase in OHC. However, freeze dried samples of approximate equal density (without extraction) do not show an equivalent oil holding capacity. Tissue bound lipoproteins may remain after extraction to impart surface lipophilicity.

Samples extracted with calcium complexing agents such as EDTA or sodium citrate show extensive loss of soluble pectin. This loss is reflected in the density of the dried fiber solids. In Table V the increase in OHC and WHC of extracted samples are evident as well as changes in density. The loss of pectin gives a porous matrix upon drying so the method used for OHC reflects an increase of entrained oil.

Citrus pulp, like other plant tissue, are rich in bioflavanoids and phenolics. These compounds are found as the substituted flavanones, 3-hydroxyflavanones, flavones, 3-hydroxyflavones, and their glycosidic derivatives as well as the simpler phenolics (10). Colorimetric analysis of washed orange pulp yields 19.9 ± 0.3 mg 'catechin equivalents'/ gram of pulp. This level is sufficient for two specific reactions to occur: formation of green chromatophores in the presence of Fe(+3) at neutral pH and the production of a 'flavor muting' complex. This latter property is a manifestation of a reaction noted in a patented process for production of a "material to remove odor from air" derived from citrus tissue (11). Plant phenolics react with polyols catalyzed by light or radical initiators. This reaction was verified to occur under food processing conditions. A model system reacted fresh washed orange pulp, starch, and sugars in presence of glycerol and flavor concentrates. Samples were prepared and dried, then tested organoleptically. In each case where glycerol and pulp were present together, a significant lowering of flavor intentsity was observed in the model product.

Utilization of washed orange pulp in food processing and product applications was explored. In one specific case, the hydrated pulp was combined with ungelatinized starch, edible acids, nutritive sweeteners and additional water. The mixture was heated and the system brought to 48-53% solids from the 30-40% solids starting level. This process leading to an ingredient is the basis of U.S. patent 4,232,049 (12). The processing of the pulp initiates multiple reactions. They include hydrolysis of sucrose, hydration and gelatinization of starch, conversion of protopectin to the soluble

TABLE V

OIL AND WATER HOLDING CAPACITIES
OF WASHED ORANGE PULP

| SAMPLE TREATMENT (a) | DENSITY (G/CC) | WHC (G/G) | OHC (G/G) |
|---|---|---|---|
| DRUM DRIED | 0.22 | 3.0 | 0.7 |
| FREEZE DRIED | 0.11 | 3.1 | 1.3 |
| SPRAY DRIED | 0.33 | 5.2 | 0.2 |
| ISOPROPANOL EXTRACTED | 0.09 | 4.8 | 2.2 |
| ACETONE EXTRACTED | 0.13 | 5.5 | 1.3 |
| 0.2 M EDTA EXTRACTED | 0.02 | 6.7 | 11.6 |
| 0.4 M CITRATE EXTRACTED | 0.03 | 13.3 | 8.4 |
| 0.1% $H_3PO_4$ EXTRACTED | 0.02 | 9.6 | 4.5 |

(a) solvent extracted samples were air dried overnight; aqueous extracted samples were freeze dried

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pectin, sucrose-pectin gel interactions, volatilization of citrus oils and microbial inactivation. In addition, it was observed that the hydrated, gelatinized starch interacts and is deposited on the vesicle surface of the washed orange pulp. Figure 2C shows the unheated starting mixture where the starch granules are stained with iodide. Figure 2D shows the same sample at the conclusion of the heating process. Starch associates with the vesicle and can be compared with the equivalent process of "sizing" reported in paper manufacturing. The washed orange pulp was then utilized in a series of food products including frostings (12) and a nondairy ice cream analogue (13).

## Conclusion

Washed orange pulp was evaluated as an unconventional source of dietary fiber. The pulp is an excellent source of soluble and insoluble dietary fiber. Dried samples generally contain 25-45% pectins and 9-20% crude fiber. Several properties of the pulp are a function of its cellular components and must be considered in utilization as a food ingredient. Additional functional properties can be obtained by processing the hydrated pulp and have extended the potential use of this byproduct of the juice concentrate industry.

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# Dietary Fiber from Citrus Wastes: Characterization

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Nearly 5 million metric tons of citrus peel and pulp waste materials are produced annually in the manufacturing of concentrated citrus juices in Florida. Although these materials are currently processed into cattle feed, they are rich in polysaccharides and can be considered as a source of dietary fiber. Alcohol-insoluble solids (AIS) of 3 different fractions of the processing residues discharged from commercial juice manufacturing equipment were analyzed for pectin, easily hydrolyzable polysaccharides, and cellulose. Two varieties of orange and one of grapefruit that are the predominant citrus fruit used in juice production were included in this study.

Between 50-60% of the AIS of all samples was removed by a 0.05 M NaOH treatment and about 10-12% by a subsequent acid hydrolysis of the residue. The "cellulose" fraction remaining was nearly completely dissolved in 12 M $H_2SO_4$. Protein and ash comprised about 10% of the AIS and were found mostly in the dilute alkali extract. The neutral sugars were determined by HPLC and GLC of their silylated derivatives of the hydrolysate after removal of acid. Arabinose and galactose were found in the dilute alkali extract. Xylose, arabinose, galactose and glucose were all present in the easily hydrolyzable fraction. Glucose was the main sugar component in the fraction dissolved in the 12 M $H_2SO_4$. Carbazol colorimetric method was used to determine the uronic acids in the fractions.

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Citrus juice processing is one of the more important food industries in the world. Annual production of frozen concentrated orange juice in the United States and Brazil, the two leading citrus concentrate producing countries, utilize nearly 15 million tons of citrus fruit with an enormous quantitity of processing residue. Only about 45-50% of grapefruit and 50-55% of oranges are recovered as juice (1). The residue from the fruit after the juice is removed by mechanical extractors consisted of the peel, the central core and the membrane, and the coarse pulp which is removed by a screen-type finisher. Seeds are included in the residue when seedy varieties of citrus are processed. A distribution of component parts or oranges and grapefruit is shown in Table I.

Table I. Average distribution of component parts of citrus fruits.

| Fruit and variety | Year | Peel (Flavedo & albedo) | Membrane, core & seeds (Rag) | Juice sacs (pulp) | Juice |
|---|---|---|---|---|---|
| 'Pineapple' | 1960-61 | 19.9 | 17.5 | 23.6 | 39.0 |
| orange | 1961-62 | 22.7 | 17.7 | 21.9 | 37.7 |
| 'Valencia' | 1959-60 | 19.2 | 9.8 | 19.9 | 51.1 |
| orange | 1960-61 | 20.8 | 12.8 | 23.7 | 42.7 |
| 'Marsh' | 1961-62 | 27.2 | 13.4 | 21.5 | 37.9 |
| grapefruit | 1962-63 | 29.2 | 14.7 | 19.1 | 37.0 |

Rouse et al. (2, 3, 4).

Most of the citrus residues are now manufactured into animal feed. The production of this product is over a million tons annually (1). Although the processing of these residues to such by-products gives a fair return to the producers, more sophisticated products can be obtained from them which are suitable as human food.

Between 45-75% of the total solids in the citrus peel and membrane has been found to be insoluble in alcohol (Table II), and the main portion of this alcohol-insoluble material is complex carbohydrate (5). The alcohol-insoluble solids (AIS) of the coarse pulp is also largely complex carbohydrate, and it also contains proteins and inorganic salts (6). Of the complex carbohydrates, pectin is by far the most important fraction. Rouse et al. (7) divided the pectic substances into water soluble, oxalate soluble, and sodium hydroxide soluble fractions in their study. Eaks and Sinclair (8) found a difference in the composition of the hemicellulose-cellulose fraction between the mature and immature 'Valencia' orange peel AIS.

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Table II. Proximate composition of citrus waste material.

| Fruit and variety | Component | Water | Total solids | Alcohol insoluble solids (AIS) | Total protein as % AIS | Ash as % AIS |
|---|---|---|---|---|---|---|
| | | | (g/100 g) | | | |
| 'Pineapple' orange | Peel | 77.1 | 22.9 | 13.0 | 5.2 | 2.7 |
| | Rag | 81.2 | 18.8 | 10.2 | 8.1 | 2.0 |
| | Pulp | 89.5 | 10.5 | 3.6 | 11.5 | 1.6 |
| 'Valencia' orange | Peel | 76.6 | 23.4 | 15.2 | 5.6 | 2.9 |
| | Rag | 81.6 | 18.4 | 10.4 | 9.7 | 1.8 |
| | Pulp | 84.2 | 15.8 | 6.4 | 9.6 | 1.5 |
| 'Marsh' grapefruit | Peel | 83.4 | 16.6 | 8.5 | 6.2 | 2.3 |
| | Rag | 82.0 | 18.0 | 9.3 | 6.8 | 1.8 |
| | Pulp | 90.0 | 10.0 | 2.5 | 14.0 | 1.5 |

The water-soluble pectins include the more highly methoxylated pectic acid, while the oxalate-soluble pectic compounds are the calcium pectates of the middle lamellas of the cell wall. Sodium hydroxide easily hydrolyzes both the methoxylated pectates and the calcium pectate. Thus, extraction with dilute alkali of the AIS would include all these pectic fractions.

The hydrophilic nature of these carbohydrate constituents could be an important factor in considering them as a source of dietary fibers. Ferguson and Fox (9) studied the possibilities of manufacturing a high fiber food product from citrus waste. Belshaw (10) suggested the use of a flour manufactured from the peels of oranges and grapefruit in bread to increase the fiber content. Such flour can also incorporate some desirable flavor. Recently, Braddock and Graumlich (11) analyzed the insoluble carbohydrates of different fruit parts of orange and grapefruit and discussed the possible utilization of citrus peel as a source of dietary fiber.

The purpose of this work is to study the composition of the residues from citrus processing and to devise a method for separating and estimating the nature and quantities of various fractions of the complex carbohydrates from such materials.

## Analyzing Citrus Residue

Processing residues. The processing residues used in this study were collected from an FMC Model 091 in-line juice extractor and from an FMC Model 035 juice finisher (FMC Corporation, Citrus Machinery Division, Lakeland, FL). The juice extractor discharges the peel partially shredded, while the central core, section membrane and the entire inner part of the fruit are pressed in the central extraction tube. After the juice is expressed and passed through a prefinisher in the central extraction tube, the residue discharged is collectively called the rag. The prefinished juice then passes through a fine screen (.020 in.) in the finisher which removes the pulp. The pressure applied to the finisher which is adjustable can determine the amount of liquid retained by the pulp. Two cultivars of orange, 'Pineapple' and 'Valencia' and one of grapefruit, 'Marsh' were used in this study as they are the important cultivars in juice manufacturing.

Sample preparation. Approximately 2 kg of each residue component was obtained from each cultivar. The peel and rag were separately passed through a hand-operated grinder. After thorough mixing, duplicate 100 g aliquots of each of these components were weighed out and placed in separate quart Mason jars each containing about 400 ml of 95% ethanol. The pulp

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samples were treated in the same manner but without prior grinding. These alcohol-preserved samples were used in the preparation of AIS. Total solids was determined by drying the samples in a vacuum oven at 75°C for 16 hours followed by 2 hours at 100°C in a regular forced-draft drying oven.

Preparation of alcohol-insoluble solids. The sample preserved in alcohol was placed in a Waring blender and macerated at high speed for 10 minutes and filtered through a Whatman No. 1 filter paper in a Buchner funnel under vacuum from a water aspirator to remove the alcohol solubles which were mostly sugars and organic acids. The residue on the filter was returned to the Waring blender with another 400 ml of 80% ethanol. It was again ground for 10 minutes and filtered. This operation was repeated, and the residue finally extracted with 200 ml of acetone. The white residue was air dried and finally dried in an oven at 75°C overnight. The weight was considered as percent AIS. Each sample was ground in a Wiley mill to pass a 40 mesh screen and stored in tightly stoppered bottles until analyzed.

Total nitrogen of the AIS was determined by a semi-micro Kjeldahl procedure (6) on a 0.50 g sample and the protein content calculated using the factor 6.25. The ash content was determined on a 0.5 g sample of the AIS ignited in a muffle furnace at 550°C for 16 hours.

Extraction of pectic substance fraction. A one-half gram of the AIS was placed in a 100 ml volumetric flask, moistened with about 1 ml of 95% ethanol and made to volume with 0.05 NaOH. The contents were allowed to stand at room temperature for 2 hours with occasional shaking. The mixture was poured onto a filtering cloth (Mira-Cloth, American Can Co.) supported on a Buchner funnel. Fifty ml of the filtrate was transferred into a 100 ml volumetric flask and acidified with 2.5 ml of conc. acetic acid. The content was made to volume and aliquots were used in the analysis of pectin and the accompanied monosaccharides. The residue was washed with a copious amount of water followed by 95% ethanol. It was rinsed from the filter cloth with a stream of acetone into a beaker and the mixture filtered through a tared sintered glass funnel. After air-dried, it was dried in an oven at 75°C and weighed. The difference in weight was considered as that removed by the dilute alkali. A flow diagram describing the fractionation, hydrolysis and subsequent analysis of the fractions are shown in Figure 1.

Uronic acid analysis. Carbazol colorimetric method (7) was used for the determination of total pectins in the dilute alkali extract of the alcohol-insoluble solids. The solution has to be diluted in order for the concentration to fall within the range of determination by this method.

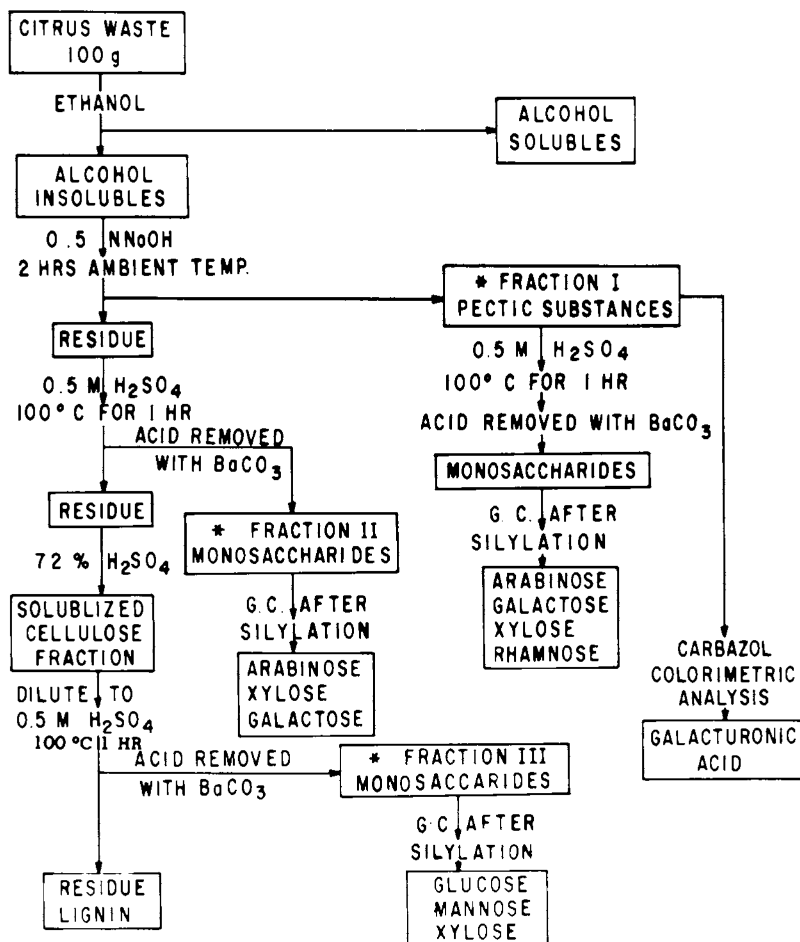


*Figure 1. Preparation, fractionation, hydrolysis, and analysis of citrus processing waste.*

Hydrolysis of the dilute alkali extract. Five ml of the acidified extract was combined with an equal volume of 1 M $H_2SO_4$ in a 15 x 125 mm screw capped culture tube. The mixture was heated for 1 hour in a boiling water bath. After cooling, 0.8 g of $BaCO_3$ was added, and the tube was sonicated for 10 minutes and allowed to stand overnight. After centrifugation, 5 ml of the neutralized supernatant fluid was used for gas chromatographic analysis.

Easily hydrolyzable polysaccharides. The residue after the dilute alkali extract was hydrolyzed with 10 ml of 1 M $H_2SO_4$ in a 15 x 125 mm screw-capped culture tube in a boiling water bath for 1 hour. The hydrolysate was decanted, after centrifuging, into a 125 ml Erlenmeyer flask, and the residue in the tube washed with 5 ml of water. The washing after centrifuging was combined with the hydrolysate. The residue was washed once with ethanol followed by acetone. Each time it was centrifuged and the washings discarded. The residue was dried, weighed and considered as the "cellulose" fraction. The hydrolysate was neutralized with 1.5 g $BaCO_3$, filtered to remove $BaSO_4$ and evaporated to dryness. Twenty-five ml of water was added to dissolve the solids and 1 ml aliquots of the solution used for chromatograpic analysis.

Hydrolysis of the "cellulose" fraction. The residue from the dilute acid extraction was suspended in 1 ml of 72% $H_2SO_4$ and allowed to stand at room temperature for 2 hours. The contents were transferred into a 25 x 400 mm test tube with 50 ml of water. The tubes are placed in a boiling water bath for 1 hour. After cooling the large portion of the clear hydrolysate was transferred over to a 100 ml volumetric flask and the remainder was transferred into a 50 ml centrifuge tube. After centrifuging, the supernatant fluid was combined with the previous portion in the volumetric flask. The residue was washed once in the centrifuge tube and the washings combined with the hydrolysate. The dark precipitate was washed with acetone, air dried and, finally, oven-dried and weighed. The hydrolysate was made to volume and a 1-ml aliquot was used for gas chromatography.

Gas chromatographic and HPLC analysis of monosaccharide. The sugars in each fraction were analyzed as their silylated ether (12) by gas chromatography. The aliquot of the hydrolysate was first evaporated to dryness and treated with 1 ml of Trisil (Pierce Chemical Company, Rockford, IL). A Hewlett Packard Model 5730 gas chromatograph with a 1/4 inch glass column packed with 3% SE 30 on Chromosorb (Supelco Co., Bellefonte, PA) was used. Temperature was programmed from 100

to 220°C at 4° per minute. Both injection and detection temperatures were set at 300°C. Injections were made with the aid of a Hewlett Packard Model 7672A autosampler, and the concentrations of each sugar determined on a Hewlett Packard Model 3385A calculating intergrator using inositol as the internal standard and appropriate standards.

The sugars were also analyzed by HPLC using a Waters HPLC system with a carbohydrate column. The monomers can be separated but the concentrations of the minor constituents in the mixtures were too low for the refractive index (RI) detector.

## Composition of Citrus Processing Residue

The proximate composition of these citrus processing residue components are shown in Table II. The peel contained about 10-15% AIS on fresh weight basis or about 50 to 65% of the dry weight. Grapefruit had lower AIS and total solids than the 2 varieties of oranges. 'Valencia' oranges had higher alcohol-insoluble solids in the pulp than either the 'Pineapple' orange or the grapefruit. The AIS of the rags showed the least differences among the cultivars.

Protein is higher in the pulp and rag than in the peel. The peel had between 5 and 6% protein, whereas the pulp had more than twice that value. The ash content is slightly higher in the peel.

The dilute alkali removed 46 to 53% of the AIS of the peel, 50 to 60% of that of the rag and 62 and 64% of that of the pulp of 'Pineapple' orange and 'Marsh' grapefruit. In the pulp of 'Valencia' oranges, which has much higher AIS content, only 46% was removed (Table III).

The dilute acid hydrolysis of the residue following the first extraction removed about 25% of the AIS of orange peel but only 17% of that of grapefruit peel. The rag and pulp components of the 'Valencia' oranges, which had less amounts removed by the dilute alkali extract, were much higher in this fraction than those of either the 'Pineapple' orange or the 'Marsh' grapefruit. The "cellulose" fraction of the peel was about 28% in all three kinds of fruit but only about 15 to 20% in their pulp components, with the rags occupying the values in between.

The use of a 0.05 M NaOH to extract the AIS of citrus tissues removes all the pectins (7). In all three types of citrus waste components these hydrolysates contained mostly uronic acids, but arabinose, galactose and small amounts of xylose were also present (Table IV). Arabinose is generally 2 to 3 times higher than galactose. In some samples, especially in the peel of 'Pineapple' orange, considerable amounts of rhamnose were found. Only a small amount of rhamnose was found in the 'Valencia' peel sample and none in the grapefruit

Table III. Percent distribution in fractionation of AIS of citrus waste components

| Fruit and variety | Waste component | 0.05N NaOH Extract (pectin containing) | 1 M $H_2SO_4$ Hydrolysate | Cellulosic fraction | Residue after 72% $H_2SO_4$ solubilization | Total |
|---|---|---|---|---|---|---|
| 'Pineapple' orange | peel | 46.0 | 24.0 | 28.8 | 1.0 | 99.8 |
| | rag | 60.0 | 18.0 | 22.6 | 0.7 | 101.3 |
| | pulp | 64.0 | 14.0 | 16.0 | 3.3 | 97.3 |
| 'Valencia' orange | peel | 49.0 | 24.0 | 27.4 | 1.2 | 101.6 |
| | rag | 49.0 | 25.0 | 24.1 | 1.4 | 99.5 |
| | pulp | 46.0 | 27.0 | 20.6 | 3.4 | 97.0 |
| 'Marsh' grapefruit | peel | 53.0 | 17.0 | 28.5 | 1.6 | 100.1 |
| | rag | 55.0 | 17.0 | 26.0 | 1.3 | 99.3 |
| | pulp | 62.0 | 17.0 | 15.1 | 4.3 | 98.4 |

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Table IV. Neutral sugars and uronic acid in the 0.05 N NaOH extract of the AIS of citrus processing waste.

| Fruit and variety | Type of residue | Carbohydrate monomers | | | | | | | % of Extracted material |
|---|---|---|---|---|---|---|---|---|---|
| | | Arab. | Galac. | Rham. | Xyl. (mg/g AIS) | Glu. | Uronic acids | Total | |
| 'Pineapple' orange | peel | 21.3 | 8.2 | 30.0 | 5.6 | -- | 200.0 | 265.1 | 57.6 |
| | rag | 45.6 | 11.5 | -- | 3.4 | 23.0 | 324.4 | 407.9 | 68.0 |
| | pulp | 74.8 | 30.8 | -- | 6.3 | -- | 320.6 | 432.5 | 67.6 |
| 'Valencia' orange | peel | 30.0 | 7.9 | 3.2 | 3.0 | 4.6 | 236.4 | 285.1 | 58.2 |
| | rag | 46.2 | 12.8 | -- | 8.3 | 8.5 | 262.4 | 338.2 | 69.0 |
| | pulp | 59.0 | 20.1 | -- | 5.3 | -- | 192.4 | 276.5 | 60.1 |
| 'Marsh' grapefruit | peel | 17.0 | 6.0 | -- | -- | -- | 280.4 | 303.0 | 57.2 |
| | rag | 20.3 | 11.3 | -- | 1.8 | 1.0 | 356.0 | 390.4 | 71.0 |
| | pulp | 32.4 | 15.2 | -- | 3.4 | -- | 287.6 | 338.6 | 54.6 |

peel sample. Xylose was found in all samples except in that of the grapefruit. Glucose was found in all rag samples, but in that of grapefruit the amount was smaller than those in the corresponding components of the oranges.

The uronic acid content of the total monomers amounted to 70% in the pulp of 'Valencia' orange to 92% in that of grapefruit peel. Grapefruit peel is known to produce better pectin than orange peel (13) and the ratio of its uronic acid to the sugar monomers is much higher. The sum of all monomers of all components only accounted for 55 to 70% of the total amount extracted. These values did not vary much from those reported earlier (5). Some of these differences probably were due to protein and minerals. The incomplete hydrolysis could be another explanation of the low recovery.

Arabinose, galactose, xylose and glucose were also found in the dilute acid hydrolysate of the residue following the dilute alkali extraction (Table V). This easily hydrolyzable fraction contained most of the hemicellulose. In grapefruit peel, the arabinose and galactose were lower compared to the similar components of the two oranges, but the xylose was slightly higher. The rag and pulp of the 'Valencia' orange were higher in arabinose and galactose than the other two varieties. Overlapping peaks of glucose and mannose were not easily separated, but they comprise less than 10% of the total sugars accounted for.

Glucose is the major monomer in the 72% $H_2SO_4$ soluble polysaccharide and comprises about 80 to 85% of the total monomers accounted for (Table VI). Only a trace of arabinose was detected in some peel samples but xylose was found to be in measurable quantities. Several peaks on the chromatograms were found in the various components. They were not identified positively. Peaks with retention time similar to some anomers of mannose not masked by glucose were found. Mannose has been previously noted by paper chromatography from citrus peel (5).

## Conclusion

With more than 4 million tons of processing waste each year from the citrus juice manufacturing industry in Florida, production of food grade products with high dietary fiber content can be profitable. Many food uses of the pulp component have already been proposed (9, 14). Studies of chemical composition of these waste components gave a better understanding of the nature of these materials and might lead to their better usage.

The fractionation scheme using dilute alkali, dilute acid and 12 M sulfuric acid divides the polysaccharides into the three definite parts. All of these waste components of both oranges and grapefruit vary only slightly in their

Table V. Carbohydrate monomers in the easily hydrolyzable fraction of the alcohol insoluble solids of citrus processing residue.

| Fruit and variety | Type of residue | Carbohydrate monomers (mg/g AIS) | | | | | % of Extracted material |
|---|---|---|---|---|---|---|---|
| | | Arab. | Xyl. | Gal. | Others | Total | |
| 'Pineapple' orange | peel | 67.0 | 16.8 | 40.4 | 13.6 | 137.8 | 57.4 |
| | rag | 56.4 | 18.0 | 41.6 | 8.6 | 124.4 | 69.1 |
| | pulp | 38.0 | 12.2 | 31.2 | 8.8 | 90.2 | 64.4 |
| 'Valencia' orange | peel | 78.8 | 20.0 | 73.6 | 22.2 | 94.8 | 77.0 |
| | rag | 71.6 | 16.6 | 51.6 | 19.6 | 159.4 | 63.8 |
| | pulp | 66.6 | 11.0 | 48.8 | 20.0 | 146.4 | 69.7 |
| 'Marsh' grapefruit | peel | 46.0 | 30.8 | 29.6 | 18.6 | 125.4 | 73.8 |
| | rag | 34.8 | 21.6 | 19.8 | 5.2 | 82.0 | 48.2 |
| | pulp | 31.4 | 16.0 | 20.8 | 6.6 | 75.4 | 44.4 |

Table VI. Monosaccharide in the "cellulose" fraction of the AIS of citrus processing wastes.

| Fruit and variety | Type of residue | Carbohydrate monomers | | | | | % of Extracted material |
|---|---|---|---|---|---|---|---|
| | | Arab. | Xyl. (mg/g AIS) | Gluc. | Others | Total | |
| 'Pineapple' orange | peel | trace | 10.5 | 106.0 | 17.0 | 134.5 | 46.5 |
| | rag | -- | 6.0 | 146.0 | 18.5 | 170.5 | 75.4 |
| | pulp | -- | 4.0 | 113.5 | 17.5 | 135.0 | 84.4 |
| 'Valencia' orange | peel | trace | 6.0 | 136.5 | 20.0 | 163.8 | 59.4 |
| | rag | -- | 4.0 | 120.0 | 15.0 | 139.0 | 57.6 |
| | pulp | trace | 3.0 | 111.5 | 21.5 | 137.0 | 66.5 |
| 'Marsh' grapefruit | peel | -- | 9.5 | 149.5 | 24.5 | 183.5 | 64.4 |
| | rag | -- | 7.0 | 141.0 | 16.0 | 164.0 | 63.0 |
| | pulp | -- | 5.0 | 106.0 | 24.0 | 135.0 | 89.4 |

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polysaccharide patterns. The AIS of the peel contained more cellulose than the rag or pulp. The dilute alkali extracts of all components contained mostly polyuronides and the dilute acid extracts the hemicellulose. The AIS, especially that of the pulp, contained some substances that are not solublized by the strong $H_2SO_4$. Lignin is usually considered to be in that fraction (5). The juice vesicle membrane which composes the main portion of the pulp AIS has been reported to contain cuticle wax, a lipoic compound (15), and lipids are also found in the juice sac contents (16). These compounds which are not removed by alcohol could be in the AIS of these materials.

Rhamnose was found in the dilute alkali fractions of the peel. The galacturonic acid is by far the major constituents in this extract of all three components. Arabinose is two or three times that of galactose and some xylose was also found.

More xylose was found in the dilute acid hydrolysate than in the other two fractions. Only about 55 to 70% of the AIS extracted by the dilute alkali can be accounted for by the sum of the sugars and the uronic acid. The recovery of the dilute acid extract as the monomers was also low, amounting to between 44 and 79%. There were some peaks on the chromatogram of this fraction, but the aggregated integrated area was only around 10% of the total. Peak with retention times close to glucose and mannose were present.

Xylose is also found in cellulose containing fractions, but glucose is the major sugar. Unidentified peaks occupied a noticeable amount of the total identified sugars on the chromatograms.

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# 16

# Cellulose, Xylan, Corn Bran, and Pectin in the Human Digestive Process

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Acid hydrolysis of human fecal samples collected after ingestion of different dietary fibers produced monosaccharides that were related to the fibers ingested. Dietary fibers studied in five normal male subjects were cellulose, xylan, corn bran and pectin. Increases in monosaccharides in recovered hydrolyzed feces from fiber diets compared to those from the fiber-free diet were: galactose for the pectin diet; xylose and glucose for the cellulose diet; xylose and mannose for the xylan diet; and arabinose, xylose, galactose and glucose for the corn bran diet. Comparison of the fecal results to those for the intact food fibers showed that fermentation of fiber components in the colon was different for each fiber.

Dietary fiber is often considered in terms of its physiological effects in man. It has been defined as that portion of plant foods resistant to hydrolysis by the alimentary enzymes of man (1) and as the indigestible material in the diet derived mainly from plant cell walls, including the sum of the polysaccharides and lignin in the diet that are not digested by the endogenous secretions of the lumen of the digestive tract (2). In addition to these physiological definitions a philosophical one has been proposed which states that dietary fiber includes all components of the diet which are imagined to pass through the small intestine substantially unchanged (3). It is also not a single substance, nor is it inert, indigestible material which simply passes through the human gut (4). Thus plant gums and mucilages are not cell wall components but are polysaccharides related in chemical structure and properties to cell wall constituents and not hydrolyzed by the human gastrointestinal enzymes. These materials are often in the diet as emul-

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sifiers, viscosity agents and stabilizers and not recognized as dietary fiber per se.

This broad understanding of dietary fiber makes it difficult to describe or characterize chemically and to elucidate its behavior in the gastrointestinal tract. Nevertheless with changing dietary habits and recommendations for food intake for good health and nutrition, it is important that the chemistry of this class of food components be more readily characterized and understood. Since much dietary fiber is of cell wall origin, some of the approaches that have been successfully used to define and explain the chemistry of plant cell walls (5, 6) should be applicable to research on the characterization and function of dietary fiber (7, 8, 9). To this end we have investigated several dietary fibers derived from plant cell walls both before and after complete passage through the human gastrointestinal tract.

Plant cell walls are chemically complex and many of the components we are primarily interested in are basically insoluble. These two facts have hampered structural studies on plant cell walls as well as studies designed to determine what these substances do in the gastrointestinal tract. Thus, for example, in order to study the chemistry of individual components of the cell wall they must be solubilized and purified, a task that is difficult to do without physically or chemically altering the components. In this study we have investigated those portions of several cell wall derived dietary fibers that are susceptible to hydrolysis in trifluoroacetic acid (TFA). Dietary fibers with significantly different compositions of acid hydrolyzable neutral monosaccharides were used in the study. They were a cellulose, a xylan, a corn bran and a pectin. Monosaccharides released by direct acid hydrolysis of the dietary fibers were determined before consumption by human subjects and compared to the monosaccharides released by direct acid hydrolysis of fecal material resulting from ingestion of each fiber and of fecal material from the subjects on a fiber free diet. Those portions of dietary fiber that did not survive the gastrointestinal tract were either digested and absorbed in the small intestine or were likely nutritional substrates for intestinal microflora growth and the production of microflora by-products. These include volatile fatty acids (VFA's) that can be absorbed and utilized by the host (10, 11) and the gases, carbon dioxide, hydrogen and sometimes methane (12, 13).

This study is part of a continuing investigation of the relationship between dietary fiber sources and bioavailability of nutrients. Specific objectives of this research were to investigate chemical differences of various sources of edible fiber and to determine the effects of the human gastrointestinal tract on these fiber sources.

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## Experimental

The design for the feeding portion of the experiment is described by Marthinsen and Fleming (14). Briefly, six normal healthy male volunteers, 21-35 years old with no histories of gastrointestinal disorders or food allergies, were selected for the study. Initially, subjects were provided with a nutritionally adequate blended-food diet on an outpatient basis for two weeks. Once subjects took up residence in the metabolic unit they undertook a strict daily regime. The 63-day metabolic study was divided into seven metabolic periods, each of nine days duration (Table I). All subjects received the basal, fiber-free diet during the first metabolic period. The subsequent test diet sequence was designed to minimize duplication of any previous diet effects (Table I). Diets included two bland formulations providing either 100% or 85% of the daily energy requirements for the individual. Four diets contained fiber sources including a cellulose (Alphacel from ICN Pharmaceuticals, Inc.), a pectin (from Sigma Chem. Co.), a xylan (from ICN Pharmaceuticals), and a raw corn bran (from Quaker Oats). The basal formulation provided 0.8g protein/kg body weight daily from egg albumin (Seymour Foods, Inc.). Fat (butterfat and cottonseed oil) provided 30% of the caloric requirements and the remainder was provided by cornstarch (CPC International), dextromaltose (American Maize Products Co.) and sucrose in a ratio of 5:5:1. Daily supplements of vitamins, minerals, and choline were taken.

During the test, dietary fiber was provided with each meal. Alphacel, xylan, and pectin were fed at 0.5g/kg body weight per day. Corn bran was considered to be approximately 80% dietary fiber and was fed at 0.6g/kg body weight. Each fiber was consumed as a pudding containing other dietary constituents. The remaining dietary constituents were consumed as a milkshake style drink.

Feces were collected daily, frozen, compiled into three-day pools, diluted with water and homogenized using an electric colloid mill. Homogenates were kept frozen prior to removing aliquots for analysis. Pooled collections from days 7-9 for each diet were used for the analyses except for subject #5 on the xylan diet where the collection from days 4-6 was used because of the loss of the 7-9 day sample. Dry weights were obtained by drying aliquots under vacuum at 70°C for 16 hours.

Hydrolyses were performed following the procedures of Albersheim et al (15) for the characterization of plant polysaccharides and described by us earlier for the hydrolysis of dietary fiber samples (16). For these fiber samples 2-6mg were hydrolyzed in 1ml of 2N TFA at 121°C for 1 hour. Fecal samples contained other components in addition to the polysaccharides or polysaccharide residues from the ingested food fiber so that aliquots for the hydrolyses were increased to 10-25mg

Table I. DIETARY SCHEME FOR SUBJECTS

| Metabolic Period | Diet | | | | | |
|---|---|---|---|---|---|---|
| | Subject | | | | | |
| | 1 | 2 | 3 | 4 | 5 | 6 |
| I | Basal | Basal | Basal | Basal | Basal | Basal |
| II | Cellulose | Xylan | Pectin | Corn bran | Basal | 85% Energy |
| III | Xylan | Basal | 85% Energy | Cellulose | Pectin | Corn bran |
| IV | 85% Energy | Cellulose | Xylan | Pectin | Corn bran | Basal |
| V | Corn bran | Pectin | Cellulose | Basal | 85% Energy | Xylan |
| VI | Pectin | Corn bran | Basal | 85% Energy | Xylan | Cellulose |
| VII | Basal | 85% Energy | Corn bran | Xylan | Cellulose | Pectin |

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(dry weight). TFA was evaporated and the sugars reduced with sodium borohydride. Excess sodium borohydride was destroyed with acetic acid and borate was removed with methanol. The alditols were converted to the corresponding acetates by adding 1ml of a 10:1 acetic anhydride/pyridine solution and heating at 121°C for 2 hours. The acetates were quantitatively separated by injecting 1µl of the reaction mixture onto a 1/8" X 6' stainless steel column packed with Supelco 2330 using 25 ml/min He as carrier gas and a FID detector. The oven was programmed from 200-224°C. The gas chromatograph unit was a Hewlett Packard 5880 Level 3. Mixtures of known sugars were reduced and acetylated to serve as standards. Samples were analyzed in triplicate and the results averaged.

## Results And Discussion

Dietary Fiber. Acid strength, temperature, time, and amount of sample per ml of acid solution all influence the amounts of monosaccharides produced on hydrolysis. From the results of preliminary studies conditions were selected for these hydrolyses which appeared to produce close to maximum amounts of each monosaccharide. All of the hydrolyses were conducted following the same procedure. We have previously shown that different dietary fibers are composed of significantly different complements of acid hydrolyzable neutral monsaccharides as determined by direct TFA hydrolysis (16). Not only did different dietary fibers give different results but different lots of the same fiber type gave different results.

TABLE II

COMPOSITION OF DIETARY FIBERS BY TFA HYDROLYSIS AS PERCENT OF ORIGINAL MATERIAL

| Fiber | Rham | Arab | Xyl | Mann | Gal | Glu |
|---|---|---|---|---|---|---|
| Alphacel | 0 | 0 | 4.6 | 0 | 0 | 6.3 |
| Xylan | 0 | 0.20 | 25 | 39 | 0 | 13 |
| Pectin | 1.0 | 1.2 | 0.1 | 0 | 2.0 | 0 |
| Corn bran | 0.1 | 13 | 24 | 0 | 3.4 | 20 |

The composition of dietary fibers fed in this study as determined by TFA hydrolysis are shown in Table II. The Alphacel contained 11% hydrolyzable sugars, the xylan 77% and the corn bran 60%. The small amount of xylose recovered from the Alphacel is most likely from hemicellulose present in the original wood

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from which the Alphacel was prepared. The xylan was reportedly from larchwood. The principle recovered sugars from the xylan were mannose, xylose and glucose in that order. The mannose to glucose ratio of 3 suggested the presence of a glucomannan, a hemicellulose found in wood. The free glucose content of the fiber was less than 0.1% (dry weight basis) as determined by water extraction and Statzyme analysis (Worthington Diagnostics) for glucose. Eighty percent of the glucose recovered from the corn bran was starch as determined by amyloglucosidase hydrolysis followed by analysis for glucose. Pectin contained small amounts of rhamnose, arabinose and galactose as expected.

Subject Responses. Early in the study subject #4 dropped out and was not replaced. In the following presentation and discussion of data results from subject #6 are sometimes quite different from those of the other subjects although in most cases directionally consistent. No explanation for this is offered at this time. Evaluation of the 100% and 85% energy diets will be made at a later time. Excretion of breath and flatus gases by the subjects has been reported (14).

Fecal Hydrolyses. The rhamnose, arabinose, xylose, mannose, galactose and glucose from TFA hydrolysis of aliquots of the 3-day fecal samples for each subject on each diet sequence are shown in Figures 1-5. For subject #1 (Figure 1) on the fiber free diet, small amounts of all of the sugars were produced on hydrolysis of the fecal sample. Fecal material from this subject on the Alphacel diet gave more glucose, on the xylan diet more xylose, on the corn bran diet more arabinose, xylose, galactose and glucose, and on the pectin diet more rhamnose and galactose. Similar results were observed for subjects 2, 3, 5, and 6 (Figures 2-5). For every subject the amounts of the sugars produced on acid hydrolysis of feces were different for each diet fed and were related to the specific fiber fed. Comparison of results from the several fiber-free diets run in each sequence showed directional increases in hydrolyzable monosaccharides in the feces which appear to be related to immediate diet history.

The amount of hydrolyzable glucose in feces following the Alphacel diets was more than could be accounted for if all of the Alphacel had been excreted, assuming the same amount of glucose was produced as that from the original Alphacel. Further examination of the samples showed that they contained both free glucose and starch. Free glucose was estimated by analyzing water extracts for glucose by both the Statzyme procedure (Worthington Diagnostics) and gas chromatography of the alditol acetates. Starch was estimated by treatment of autoclaved fecal samples with amyloglucosidase for 3 hours at 55°C followed by the same analyses for glucose. In feces from the Alphacel diets free glucose accounted for 5-15% and glucose from starch ranged from 15-25% of the total glucose recovered following TFA hydro-

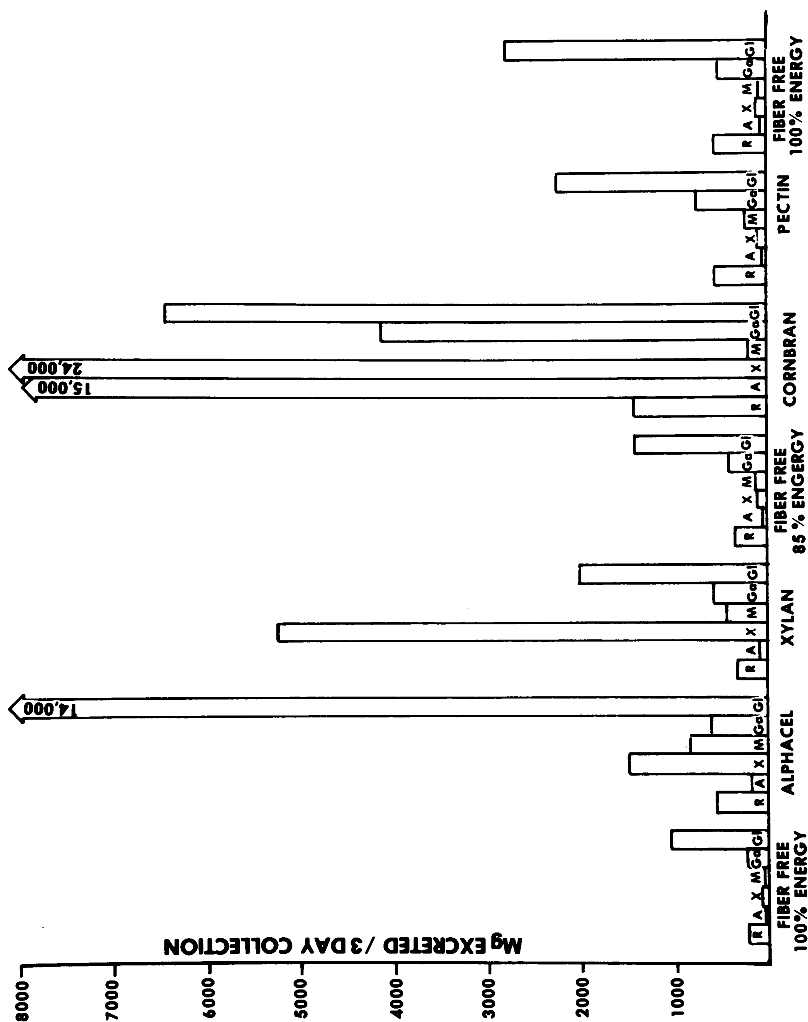


*Figure 1. Monosaccharides from the hydrolysis of aliquots of 3-day fecal collections for Subject 1 on the indicated diets. Key: R, rhamnose; A, arabinose; X, xylose; M, mannose; Ga, galactose; and Gl, glucose.*

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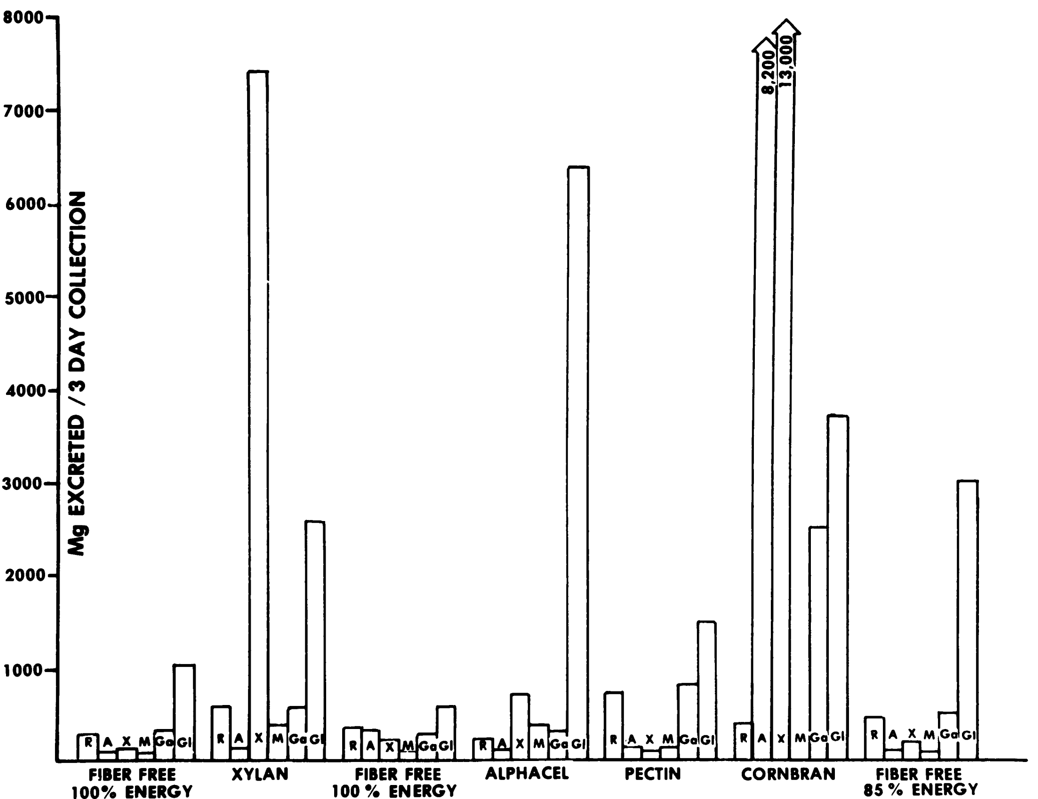


*Figure 2. Monosaccharides from the hydrolysis of aliquots of 3-day fecal collections for Subject 2 on the indicated diets. Key is the same as in Figure 1.*

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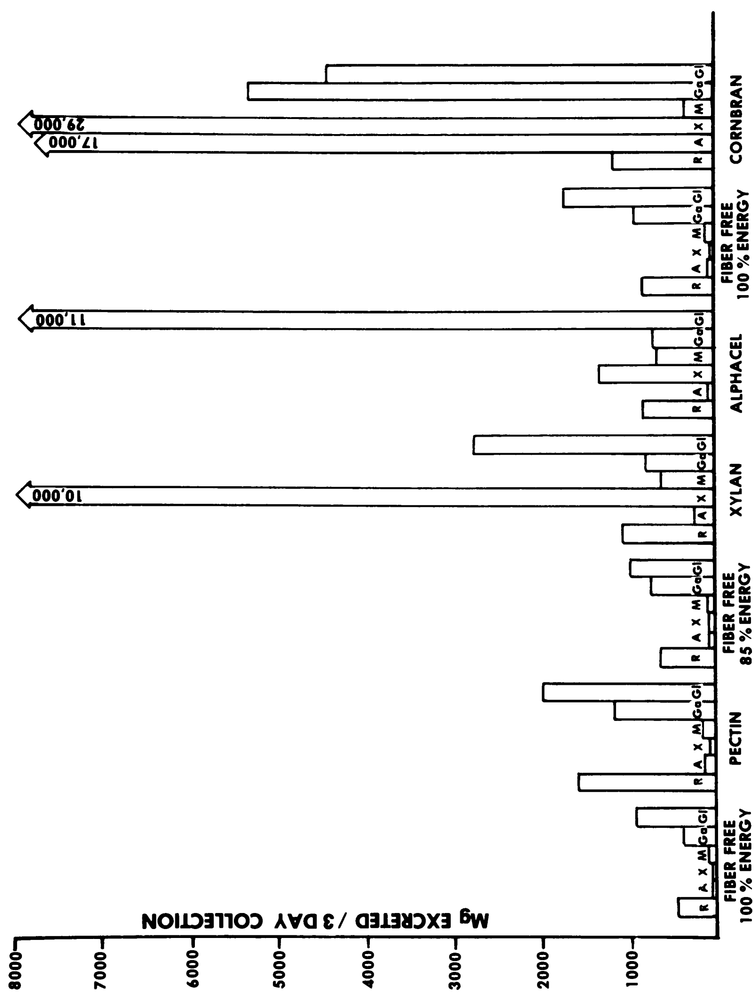


*Figure 3. Monosaccharides from the hydrolysis of aliquots of 3-day fecal collections for Subject 3 on the indicated diets. Key is the same as in Figure 1.*

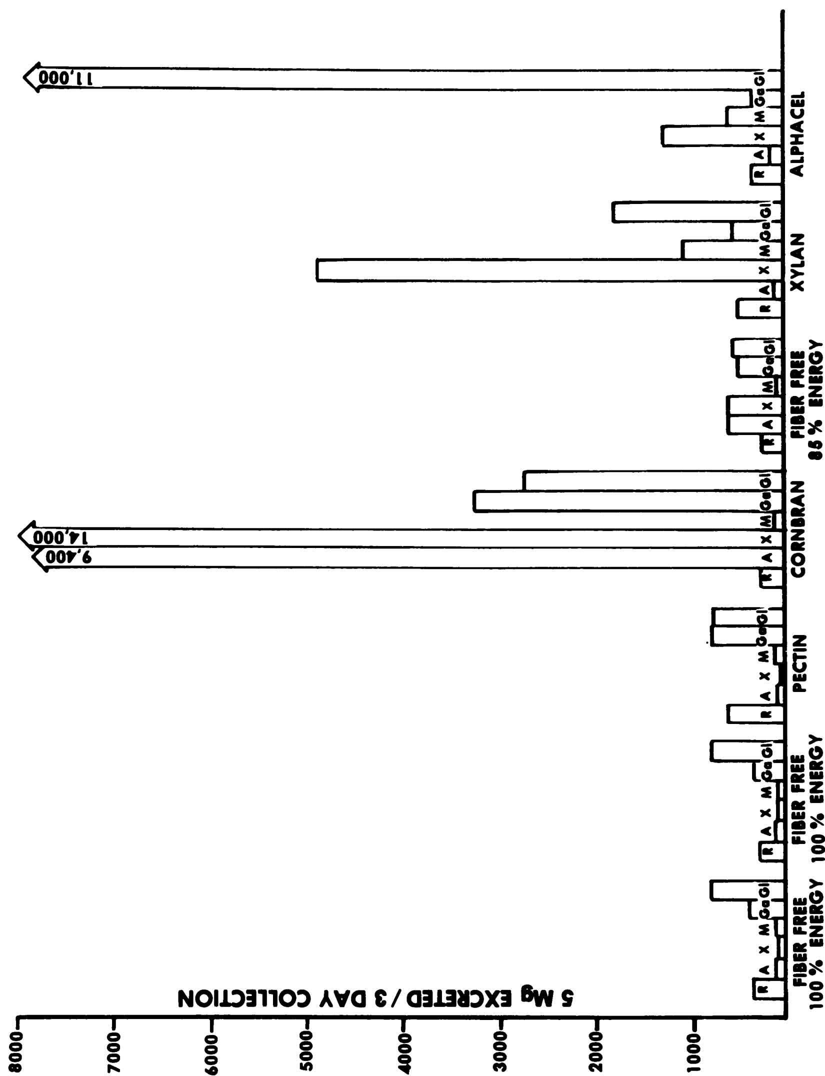


*Figure 4. Monosaccharides from the hydrolysis of aliquots of 3-day fecal collections for Subject 5 on the indicated diets. Key is the same as in Figure 1.*

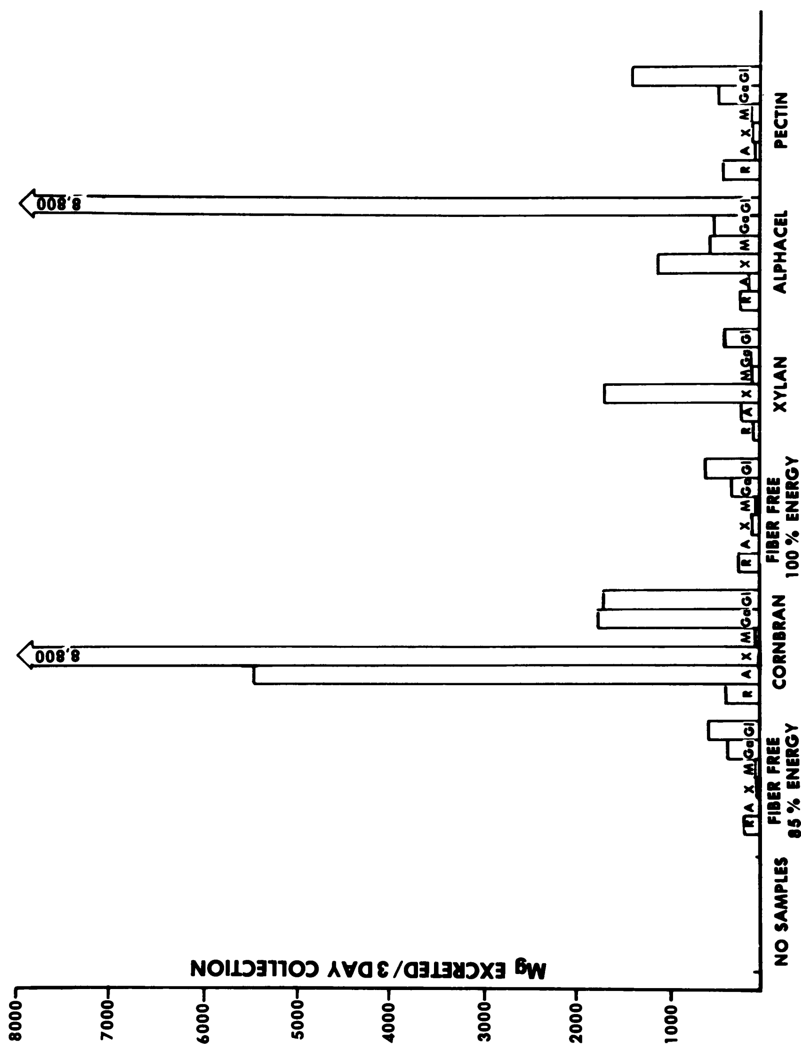


*Figure 5. Monosaccharides from the hydrolysis of aliquots of 3-day fecal collections for Subject 6 on the indicated diets. Key is the same as in Figure 1.*

lysis. This partially accounted for the large amount of glucose recovered from this diet. However, similar amounts of free glucose and starch were also found in fecal samples from the corn bran diet while smaller amounts were found in the fecal samples from the other diets including the fiber-free diets. While difficulties in these analyses prevented better quantitation, the results suggest that both Alphacel and corn bran may have inhibited starch digestion. This may be partially due to bulking effect and less effective substrate/enzyme interaction.

Small amounts of glucose were produced from cellulose preparations by hydrolysis with TFA as already noted (Table II) and reported by others (19). The amount produced probably depends on the physical form of the cellulose since different amounts were obtained from different preparations. Subtracting the amounts of free glucose and that derived from starch from the total amount obtained by direct TFA-hydrolysis of feces from some of the Alphacel diets still leaves more glucose than can be accounted for from unchanged cellulose itself. This suggests that passage through the gastrointestinal tract may have produced changes that made cellulose in recovered feces more susceptible to TFA hydrolysis than that which was fed.

Xylan and Corn Bran. The xylan and corn bran fibers were mainly hemicellulose and contained more complex mixtures of hydrolyzable monosaccharides than the other fiber sources. It was of some interest to consider the results from the hydrolysis of fecal samples from these diets in an alternate manner. The data were replotted to show monosaccharides recovered as percent of the amount fed for each subject. (Figures 6 and 7). For comparison the composition of the original fiber is shown on the extreme left as percent dry weight of the fiber.

As already noted the composition of the xylan was interesting because of its high mannose and glucose content, suggesting the presence of glucomannan in the xylan. The feces examined for hydrolyzable polysaccharides showed that the mannose (or mannan) was highly fermentable since only 0-3% of it survived to be excreted. Similarly less than 10% of the glucose originally present in the xylan ingested was recoverable by hydrolysis of fecal samples. Over 70% of the xylose in the xylan also disappeared. Thus, the original xylan may be composed of at least two readily fermentable polysaccharides, a xylan (β1-4 linked xylose units) and a glucomannan (β1-4 linked mannose and glucose in the ratio 3:1). Of the two polysaccharides the glucomannan was more readily fermentable since less of it survived the digestive process.

Corn bran has a large xylose to arabinose ratio of 1.8. Recovery of these sugars in the feces was the highest of any of the fibers fed. The xylose to arabinose ratio was also close to that in the bran fed, ranging from 1.5-1.7. Galactose, while in quite small amounts, survived in roughly the same

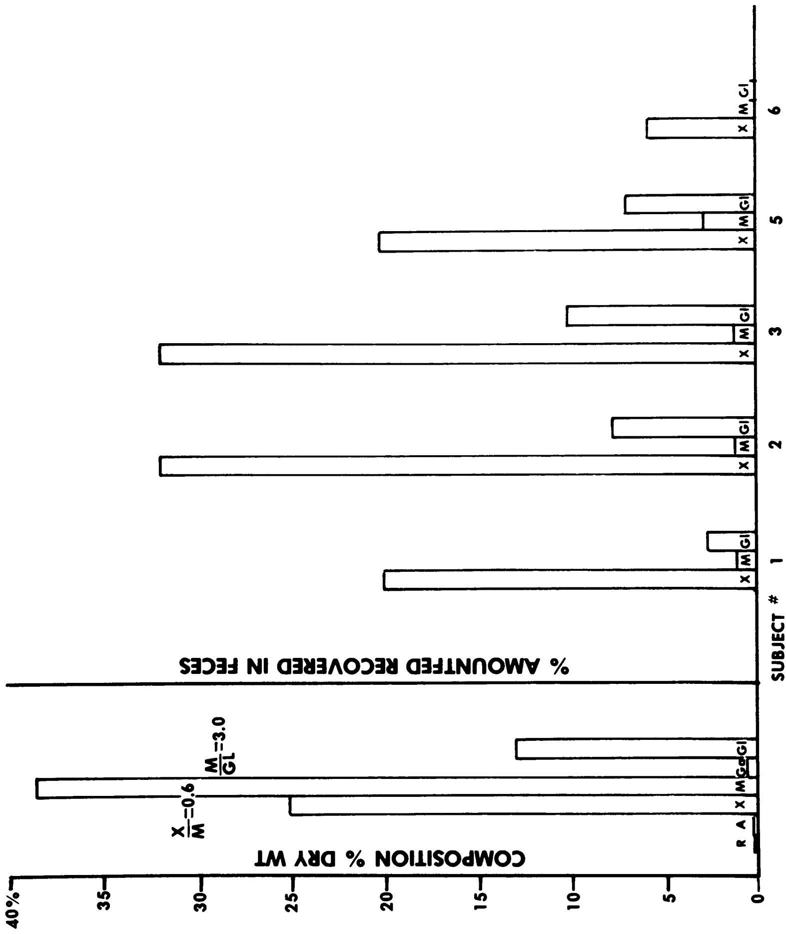


*Figure 6. Recovery of monosaccharides from xylan diets from each subject as percent of amount fed. Composition of fiber fed is shown at left.*

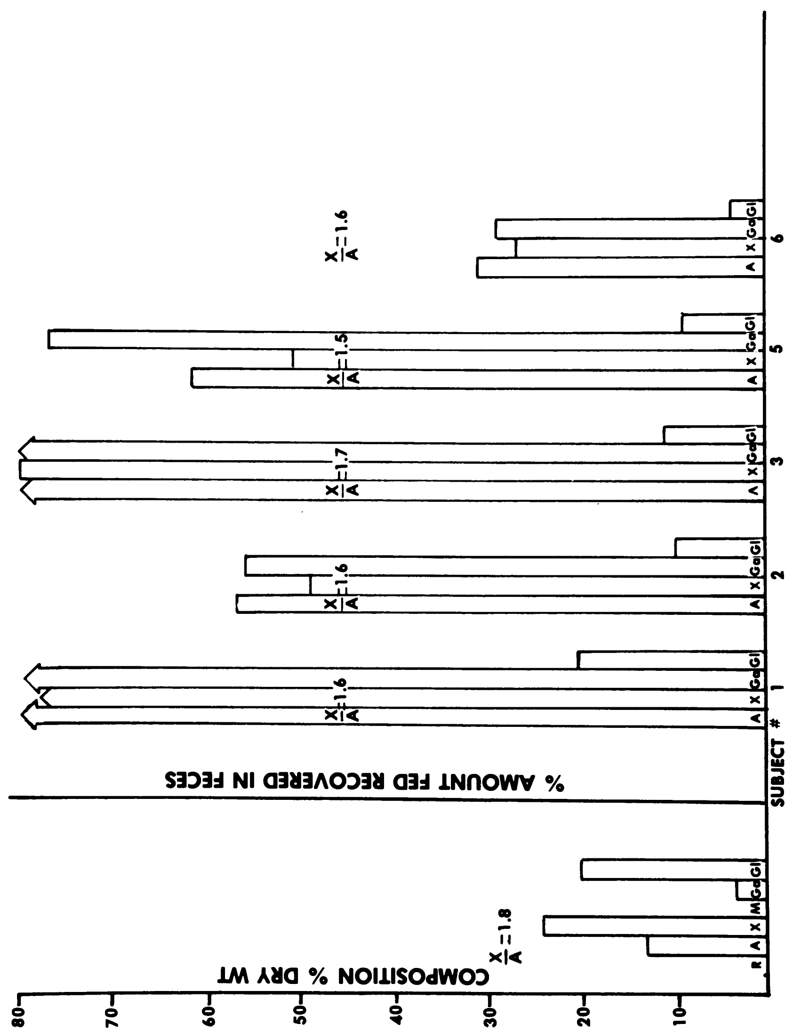


*Figure 7. Recovery of monosaccharides from corn bran diets from each subject as percent of amount fed. Composition of fiber fed is shown at left.*

proportions. The glucose value of 20% for the corn bran includes 16% starch and, by difference, 4% of the glucose is probably tied up in hemicellulose. Then a calculated value of hydrolyzable glucose in the feces based only on glucose in hemicellulose in the original fiber would be 4X that shown in Figure 7.

Comparison of these two sources of hemicellulose in the diets and their recoveries in the feces indicated that their behavior in the gastrointestinal tract may depend both on their composition and particle size. The xylan was a finely divided preparation while the corn bran was composed of much larger particles. Very little of the xylan was recovered in the feces compared to that recovered from the corn bran.

Fiber Input: Fecal Output. Fecal output on a fiber-free diet may include bacterial detritus, bile salts, mucus, intestinal wall cell debris, and perhaps fiber type material in the "fiber-free" diet that is as yet not recognized as such (e.g. material added as emulsifiers, etc). Fecal output from a fiber supplemented diet minus that from the fiber-free output should indicate the extent of the fiber itself that was excreted plus the increase in bacterial detritus, mucus and other material perhaps rendered less digestible due to the presence of the fiber. In Figure 8 the amount of each fiber consumed is shown compared to a corrected average (of the five subjects) total 3-day dry weight of fecal output and amounts of TFA hydrolyzable sugars present. The correction was made by subtracting the average fecal output and hydrolyzable sugars (shown in the extreme right in Figure 8) obtained from the fiber-free diets from the values obtained on the fiber diets.

Average fecal outputs for all of the fiber diets are larger than those from fiber-free diets. Most of the TFA-hydrolyzable sugars from the pectin and xylan fibers are not recovered in the feces (9, 17, 18). Both the Alphacel and corn bran diets produced large increases in fecal dry weights. For corn bran relatively similar proportions of xylose, arabinose and galactose found in the bran were found in the feces. The large bran particles and the chemical form of the polysaccharides probably limits the fermentability of these hemicelluloses (20, 21). It was possible to visually identify corn bran particles in fecal material.

Statistical Analysis. Means and 95% confidence intervals were calculated for the recovered monosaccharides (Table III). Data was treated by analysis of variance fitting diets and subjects. In some cases transformations were needed to stabilize the variances between diets. Significant differences in the composition of feces as a result of different diets are clearly indicated and are consistant with the previous discussion.

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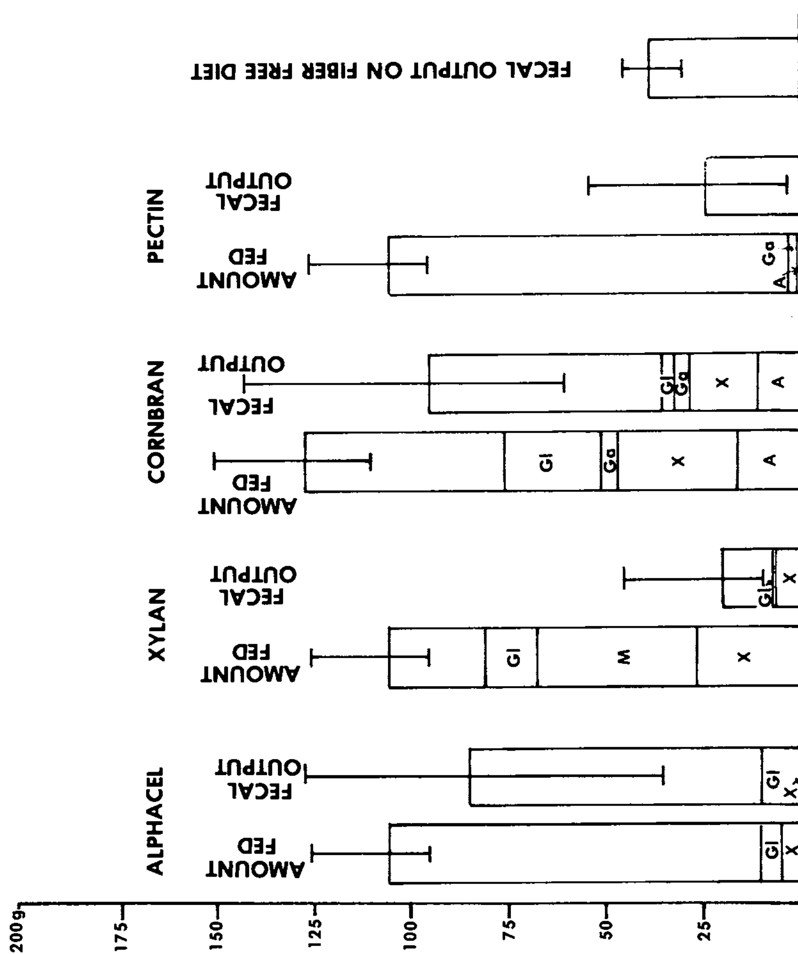


*Figure 8. Comparison of fiber input to fecal output (dry weight in grams) corrected for fecal output from fiber-free diet shown at extreme right. Average of five subjects for 3-day total fecal output.*

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Table III. FECAL HYDROLYSIS RESULTS: MEANS AND 95% CONFIDENCE INTERVALS*

| DIET | RHAMNOSE | | ARABINOSE | | XYLOSE | | MANNOSE | | GALACTOSE | | GLUCOSE | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean | 95% C.I | Mean | 95% C.I. | Mean | 95% C.I. | Mean | 95% C.I. | Mean | 95% C.I. | Mean | 95% C.I. |
| Fiber Free | 0.41 | 0.18<br>0.64 | 0.08 | 0.03<br>0.13 | 0.09 | 0.07<br>0.13 | 0.07 | 0.05<br>0.11 | 0.44 | 0.31<br>0.57 | 1.17 | 0.79<br>1.67 |
| Pectin | 0.77 | 0.54<br>1.00 | 0.09 | 0.04<br>0.14 | 0.06 | 0.05<br>0.09 | 0.13 | 0.08<br>0.19 | 0.80 | 0.67<br>0.93 | 1.50 | 1.04<br>2.10 |
| Alphacel | 0.43 | 0.20<br>0.66 | 0.12 | 0.07<br>0.17 | 1.13 | 0.81<br>1.57 | 0.61 | 0.40<br>0.92 | 0.49 | 0.36<br>0.62 | 10.1 | 8.06<br>12.5 |
| Xylan | 0.43 | 0.20<br>0.66 | 0.11 | 0.07<br>0.16 | 4.11 | 2.97<br>5.68 | 0.32 | 0.21<br>0.49 | 0.45 | 0.32<br>0.58 | 1.36 | 0.94<br>1.92 |
| Corn bran | 0.72 | 0.49<br>0.95 | 10.9 | 5.1<br>16.7 | 16.2 | 11.7<br>22.4 | 0.15 | 0.10<br>0.23 | 3.41 | 1.61<br>5.20 | 3.58 | 2.67<br>4.70 |

*For Grams Excreted/3 Day Pooled Collection; N = 5.

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## Summary And Conclusions

Marthinsen and Fleming (14) reported that methane excretion was greater while consuming the xylan and pectin diets than while consuming the other diets. Ingestion of these two fibers also caused higher flatus volume and hydrogen and carbon dioxide excretion. The Alphacel and corn bran diets resulted in breath and flatus gas excretion at levels equivalent to the fiber-free diets. These observations are consistent with the high recovery of fiber from the Alphacel and corn bran and the implied fermentation and hence low recovery of the xylan and the pectin.

The results from this work show that dietary fiber sources produce different patterns of monosaccharides on direct TFA hydrolysis. The results also show that it is possible to obtain significant information on the fate of dietary fiber sources by direct TFA hydrolysis of aliquots of recovered fecal material. Different dietary fiber sources give different amounts and patterns of monosaccharides that were relatable to the fiber sources ingested.

Additional experiments are needed to relate not only the monosaccharide composition of the fibers fed to fecal output but also the structures of those fibers and how they may have been modified in the digestive tract even if not fermented. The effect of fiber particle size and pretreatment should be studied. Free sugar, starch, cellulose and uronic acid measurements should be made in order to obtain a more complete picture of what survives and what is metabolized. The effect of dietary fibers on the digestion and utilization of other polysaccharides and other food components should be studied. This information, together with fermentation data, including gas and VFA production, will provide a better understanding of the role and value of different dietary fibers and their effects on nutrient bioavailability.

## Acknowledgment

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Reference to a company and/or product named by the U. S. Department of Agriculture is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

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# Properties of Wood Lignin

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To further the understanding of lignin as a component of the dietary fiber, studies of three aspects of the properties of enzymatically isolated wood lignin are discussed here: (1) the effect of cations and complexing ligands on adsorption of nitrosamines on lignin; (2) the binding of heavy metals on lignin; and (3) the adsorption of bile acids on lignin, wood and cellulose.

Lignin is a highly branched polymer composed of phenylpropane units that are believed to be chemically bound to the carbohydrates in wood. Either degradative processes, such as those used in producing kraft or Klason lignins, or extraction with organic solvents, such as those used in producing Brauns lignin, are used to isolate lignins from wood. Lignins isolated by these methods are unlikely to be useful for elucidating the role of lignin in dietary fiber because they are either chemically or structurally different from naturally occurring lignin (1). We have concentrated our efforts on lignins obtained from wood by enzymatic digestion (2). Lignins prepared by the enzymatic removal of the carbohydrates of plant materials bear a closer resemblance to the substances found in the digestive tract. To further the understanding of lignin as a component of dietary fiber, studies of three aspects of the properties of wood lignin are discussed here: (1) the effect of cations and complexing ligands on adsorption of nitrosamines on lignin; (2) the binding of heavy metals on lignin; and (3) the adsorption of bile acids on lignin, wood and cellulose.

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Effect of Cations and Complexing Ligands on the Adsorption of Nitrosamines on Lignin

The marked differences in the adsorption of N-nitrosodiethylamine on different lignin preparations enabled us (3) to correlate the adsorption of this nitrosamine with chemical studies of the various lignins. The adsorption of N-nitrosodiethylamine was also used as a diagnostic tool in defining optimum conditions for preparing the enzyme lignin. Two factors proved to be of particular importance: mechanical pretreatment of wood and the conditions of the enzymatic digestion process (4). The effect of these factors on the metal content in wood and lignin is discussed here.

The mechanical comminution process can introduce heavy metals, particularly iron, into the samples. Analysis by atomic absorption has shown (Table I) that the calcium, magnesium, and zinc content was not affected by the comminution method. Samples of wood pulverized by a Hurricane Pulverizer (a hammer mill), however, had an iron content one order of magnitude higher than those treated in a Wiley Mill (a rotating steel cutter). In both cases the samples were further ball-milled with ceramic balls. The enzymatic lignin prepared from the pulverized and ball-milled wood showed a high iron content (Table I). The content of metal ions in the wood samples was reduced by deionization by extraction with 1 M HCl and 1 M NaCl and subsequent dialysis. Similar treatment of the lignins showed a decrease in calcium, magnesium, and zinc content, with a relatively low degree of removal of iron. This indicates that lignin binds iron more strongly when isolated from the wood polysaccharides.

Another source of the metal ion content of lignin is from the enzyme solutions. Two types of enzymatic preparations were used: a mixture of the cellulases produced by cultures of *Schizophyllum commune* and *Trichoderma reesei* (5); and a commercial preparation (Cellucast, Novo Industry, Denmark) which is a concentrate of the cellulases produced by *Trichoderma viride*. Both types of enzyme preparation contain metal ions derived from the culture media.

N-nitrosodiethylamine is strongly adsorbed onto kraft lignin (6), Klason lignin (7), and onto freshly prepared or stored samples of enzyme lignin with low residual sugar prepared from Wiley-milled wood. By contrast all of the enzymatic lignin samples prepared from Hurricane-pulverized wood were poor adsorbers of nitrosamine. We believe that iron, introduced by the Hurricane pulverizer, forms a complex with lignin that alters its adsorptive properties. In one enzyme lignin sample freshly prepared from Hurricane-pulverized wood, the adsorption of nitrosamines was enhanced by washing

Table I

Minerals in Aspen Wood and Lignins (mg/kg)[a]

| | Ca | Mg | Fe | Zn |
|---|---|---|---|---|
| Wood, Hurricane-pulverized, ball-milled | 1600 | 220 | 350 | 50 |
| Wood, same, deionized[b] | 40 | 3 | 90 | 6 |
| Wood, Wiley-milled ball-milled | 1400 | 170 | 30 | 50 |
| Lignin, enzymatic[c] | 1200 | 75 | 370 | 8 |
| Lignin, same, deionized[b] | 90 | 9 | 280 | 7 |

[a]Determined by atomic absorption.

[b]Extracted with 1 M HCl and 1 M NaCl, dialyzed against distilled water.

[c]From Hurricane-pulverized, ball-milled wood.

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the lignin with a 1 molar solution of calcium, sodium or lithium chloride (Figure 1). The adsorption properties of lignin increase with increasing ionic radius of the cation. This indicates that probably an ionic strength effect is involved, affecting the composition of the layer of solvent (water) at the surface of lignin. However, when the sample of lignin was stored in a semi-wet state at 4°C for a month, this enhancement of nitrosamine adsorption by contact with concentrated salt solutions was lost.

Another sample of enzymatic lignin freshly prepared from Hurricane-pulverized wood was suspended in 0.1 M EDTA and stored at 4°C for one week. This treatment also enhanced the adsorption of nitrosamine (Figure 2). It therefore appears that ion exchange of heavy metals, notably iron, by complex formation with EDTA enhances the adsorption of nitrosamine on enzymatic lignin. Systematic control of the heavy metal ions in enzymatically prepared lignins is a necessary feature of future studies.

## Binding of Heavy Metals on Lignin

Adsorption of copper(II) and zinc(II) ions from unbuffered aqueous suspensions of lignin was followed by polarographic determination of the concentration of those ions remaining in solution, using varying concentrations of $Cu^{2+}$ and $Zn^{2+}$ ions equilibrated with lignin preparation for 20-24 h. The amount of the metal ion adsorbed by one gram of lignin (Figures 3, 4) was similar for both metals. The binding increases in the sequence: kraft lignin < enzymatic lignin < brown rot wood, for both copper and zinc ions, and the shapes of the adsorption isotherms were similar. The presence of two limiting regions, most clearly demonstrated for the adsorption of zinc ions on brown rot wood, indicates at least two different binding centers. The sequence of the binding ability of lignin for metal ions differs from that found for binding nitrosamine and indicates that binding sites for copper and zinc are distinct from those that interact with nitrosamine. Enzymatic lignins may contain residual cellulose and some amount of adsorbed proteins and/or peptides that adsorb copper and zinc. Elemental nitrogen and primary amino groups have been detected in our lignin preparations, but a systematic study of the effect of these residues on $Cu^{2+}$ and $Zn^{2+}$ binding is not yet available.

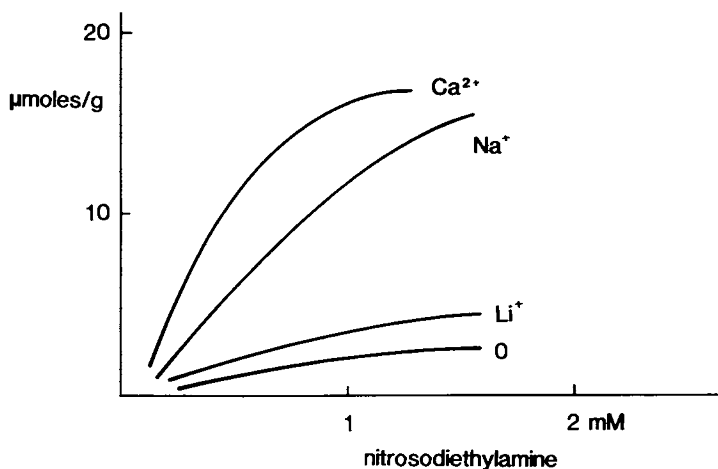


*Figure 1. Effect of high concentrations of solutions of strong electrolytes on the adsorption of* N*-nitrosodiethylamine on lignins. Dependence of the number of μmoles of the nitrosamine adsorbed on equilibrium concentration of free, unbound* N*-nitrosodiethylamine.*

*Lignin was produced by digestion of Hurricane-pulverized, ball-milled aspen wood with an 80:20 mixture of enzyme-containing supernatants from* Schizophyllum commune *and* Trichoderma reesei *cultures (final sugar content 4%). Samples were exposed for 30 min to 1* M *$CaCl_2$ ($Ca^{2+}$), NaCl ($Na^{+}$); LiCl ($Li^{+}$), or water (0). Suspension was centrifuged, supernatant discarded, lignin suspended in solutions containing varying concentrations of* N*-nitrosodiethylamine, equilibrated for 24 h, and remaining concentration of the nitrosamine determined in the supernatant.*

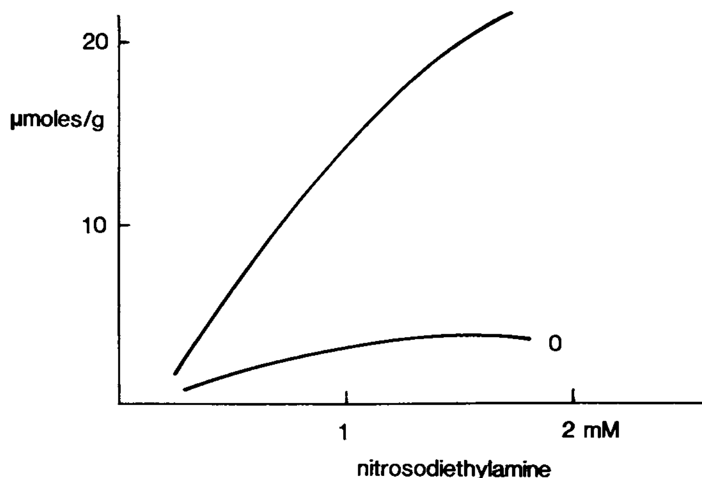


*Figure 2. Effect of treatment with EDTA on the ability of lignin to adsorb* N*-nitrosodiethylamine. Dependence of the number of μmoles of the nitrosamine adsorbed on equilibrium concentration of free N-nitrosodiethylamine.*

*Lignin was produced as in Figure 1 with a final sugar content of 16%. The lignin was suspended in 0.1* M *EDTA for 1 week. The suspension was then treated as in Figure 1.*

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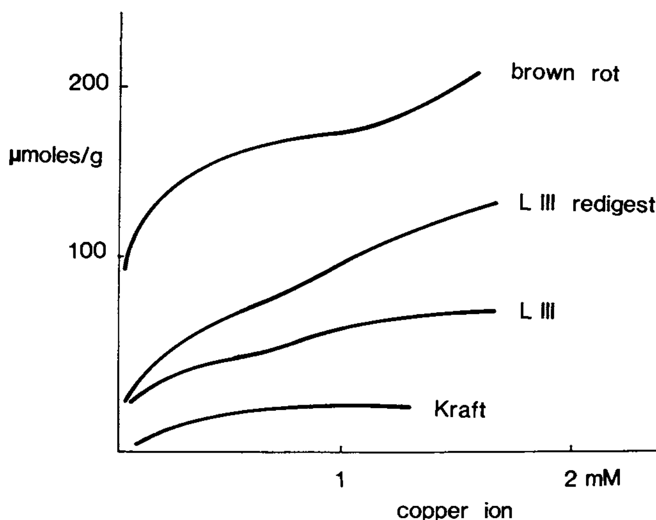


*Figure 3. Adsorption of cupric ions on lignins of various origin and similar materials. Dependence of the number of μmoles of $Cu^{2+}$ ions adsorbed on equilibrium concentration of the free $Cu^{2+}$ ions.*

*"Brown rot" refers to birch wood decayed by* Polyporus Betulinum *and digested with Celluclast (without milling pretreatment). One sample of lignin (L III redigest) was produced from Hurricane-pulverized, ball-milled aspen wood and digested with both Celluclast and the* Schizophyllum *and* Trichoderma *enzyme mixture (final sugar content 12%). Another sample of Lignin (L III) was produced from Hurricane-pulverized, ball-milled aspen wood and then digested with the* Schizophyllum *and* Trichoderma *mixture (final sugar content 4%); Kraft lignin was purchased from Westvaco. Samples of lignin were suspended in solutions containing varying concentrations of copper sulfate, equilibrated for 24 h, and remaining concentration determined in the supernatant.*

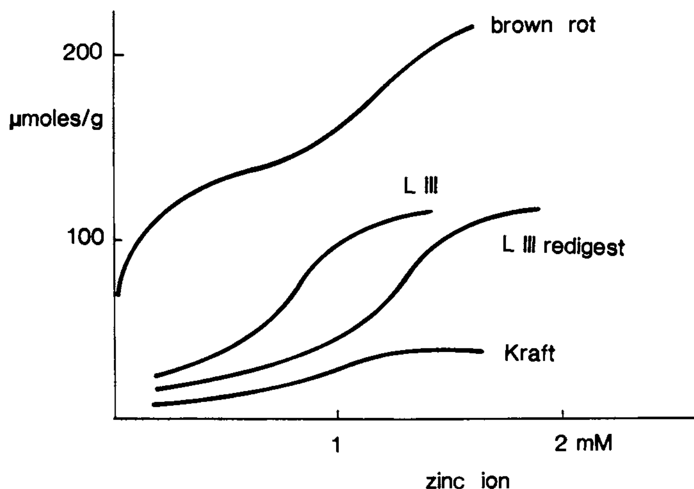


*Figure 4. Adsorption of zinc ion on various lignins and similar materials. The same samples of lignin as in Figure 3 were equilibrated with a zinc sulfate solution.*

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## The Adsorption of Bile Acids on Lignins

The adsorption of the bile acids from aqueous solutions of their salts was followed by measuring the concentration of the unadsorbed acid in the supernatant, using the measurement of the absorption spectra of polyenylic carbocations formed from bile acids in 72% sulfuric acid (8). For a given bile acid, e.g., cholic acid, much smaller differences in adsorption to a variety of lignins and wood related materials was found, as compared to adsorption of nitrosamines. Bile acids are thus less suitable for discriminating adsorptive properties of lignin preparations. Nevertheless, comparing the adsorptive capacities for a given mass of adsorbent follows a similar sequence for the adsorption of bile acids as was observed for nitrosamines. In both cases, cellulose and wood powders are very weak adsorbers. All of the lignins, both kraft and enzymatic, showed significantly greater absorptivity than wood (Figure 5). The sequence of adsorption properties of cholic, deoxycholic and taurocholic acids onto lignin (Figure 6) follows the same pattern as reported by Eastwood (9) and Kay (10), but it is doubtful if these differences are significant. The small differences in adsorptivity of these bile acids, as well as those of lithocholic and glycocholic, indicate that the side-chain and the number of the hydroxy groups play a minor role in the adsorptivity, probably due to a flat orientation of the rigid sterol molecule at the lignin surface.

The adsorption of bile salts to lignins is practically complete in about 5 h. When samples of lignin with adsorbed bile acid were separated from the supernatant, suspended in water and shaken for 3 h, 50% of the bile acid was desorbed.

## Experimental

Trembling aspen wood (*Populus tremuloides* Michx.) was reduced to a powder in a Hurricane Pulverizer. Samples were prepared from extracted pulverized wood by ball-milling for 10 days in a rotating ceramic mill equipped with an air-conditioning unit which prevented the temperature of the contents of the mill from rising above ambient. Digestion of the samples with cellulase was done with a mixture of *Schizophyllum commune* and *Trichoderma reesei* culture filtrates at 30°C for 3 days. One sample (Lignin III redigest) was digested a second time with a commercial enzyme preparation, Celluclast (Novo Industry, Denmark), at 50°C for 2 days. The isolated lignin suspension was stored at 4°C until analysis.

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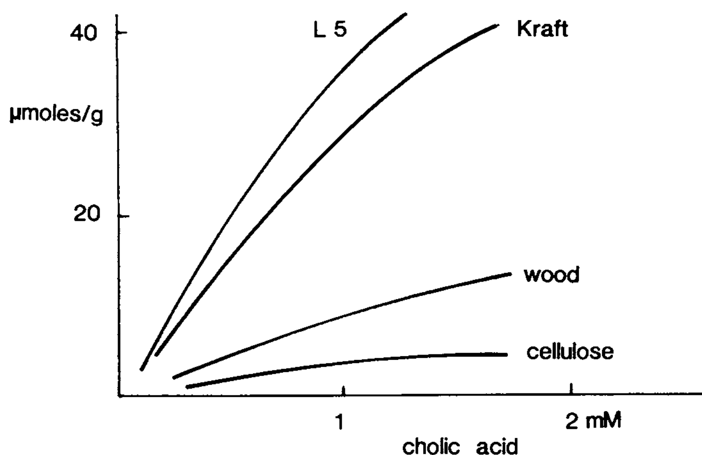


*Figure 5. Adsorption of cholic acid on cellulose, wood and kraft, and enzyme lignins. Dependence of the number of µmoles of cholic acid adsorbed on equilibrium concentration of the free cholic acid.*

*Lignin (L 5) was produced from Wiley-milled, ball-milled aspen wood and digested with the* Schizophyllum *and* Trichoderma *mixture (final sugar content 4%); Kraft lignin was purchased from Westvaco; microcrystalline cellulose (Avicel R) was obtained from the FMC Corporation (Newark). Samples of lignin, wood, or cellulose were suspended in unbuffered solutions of sodium cholate, equilibrated for 24 h, and remaining concentration determined in the supernatant.*

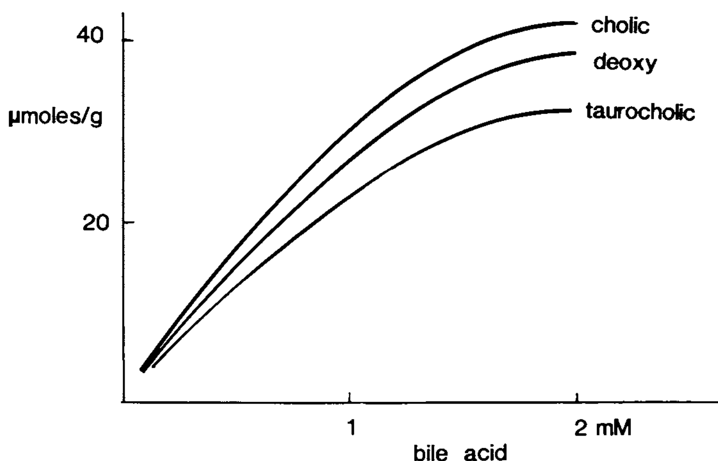


*Figure 6. Adsorption of cholic, deoxycholic, and taurocholic acid on enzyme lignin (L 5).*

*Lignin L 5 was obtained and treated as in Figure 5, solutions of sodium salts of cholic, deoxycholic, and taurocholic acids were equilibrated with lignin for 24 h, and remaining concentration in the supernatant determined.*

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One sample (L5) was produced from Wiley-milled, ball-milled wood which had been digested with the mixture of enzymes found in *S. commune* and *T. reesei* culture filtrates (5). This lignin sample was dried under vacuum at ambient temperature and then ground to a fine powder with a mortar and pestle.

Brown rot wood was prepared from birch wood which had been decayed by *Polyporus Betulinum* and digested with Celluclast without milling pretreatment.

Kraft lignin (lot no. RLX 4290:20B) was obtained from Westvaco, Charleston Research Center, North Charleston, SC. Microcrystalline cellulose (Avicel R) was obtained from FMC, Newark.

In the study of nitrosamine adsorption, lignin suspensions were stored at 4°C for less than 15 days. Samples were exposed to 1 *M* $CaCl_2$, NaCl, LiCl, or water for 30 min or to 0.1 *M* EDTA for 1 week. Suspensions were centrifuged and supernatants were discarded. Lignin samples were then suspended in unbuffered solutions containing varying concentrations of N-nitrosodiethylamine and equilibrated for 25 h. The remaining concentration of nitrosamine was determined in the supernatant by polarography (3).

Adsorption of copper(II) and zinc(II) ions was determined by suspending lignin samples in unbuffered solutions containing varying concentrations of copper sulfate or zinc sulfate. After equilibration for 20-24 h, the concentration remaining in the supernatant was determined by polarography.

Samples of lignin, wood, or cellulose were suspended in phosphate buffer (pH 7.0, $\mu$ = 0.5) containing varying concentrations of sodium cholate, sodium deoxycholate or sodium taurocholate. The concentration remaining in the supernatant after equilibration for 20-24 h was determined by measuring the absorption spectra of polyenylic carbocations formed from bile acids in 72% sulfuric acid (8).

Adsorption of selenium(IV) ions from unbuffered solutions of kraft lignin was examined in preliminary studies. Polarographic determination (11) of selenium(IV) remaining in solution after 25 h equilibration indicated that kraft lignin adsorbs a negligible quantity of selenium. A polarographically reducible organic compound which was extracted from the lignin prevented a more detailed analysis of the adsorption properties of selenium(IV).

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# 18

# Chemical and Physical Properties of Tobacco Fiber

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Fiber complexes of ten tobacco cultivars as by-products of the leaf protein extraction process contain levels of neutral and acid detergent fiber comparable to that of alfalfa fiber and in excess of that in wheat bran. Compositional analyses revealed lesser concentrations of cellulose, lignin, and lipid but a greater amount of hemicellulose in tobacco than in alfalfa. In reference to wheat bran, tobacco fiber has comparable amounts of Na, P, S, Mn, and Cu but at least 20-fold more Ca and several-fold more Fe. Among tobacco cultivars, dark green tobaccos accumulate more starch and protein than burley and Turkish types. Quantitative variation of cellulosic components and minerals is also evident among tobaccos. Average bulk fiber volume of tobacco cultivars is 14.2 mL/g as compared to 9.5 and 4.5 mL/g for alfalfa fiber and wheat bran, respectively. The water retention capacity of tobacco fiber (9 g water/g) is nearly double that of alfalfa fiber and eight times greater than wheat bran. Cation exchange capacity ranks the highest for tobacco, followed by alfalfa and then wheat bran. Sodium taurocholate binding capacity is comparable among the three fiber sources although the fiber complex of flue-cured tobaccos absorbs more than others. The above properties of tobacco fiber complex suggest its potential as a source of dietary fiber.

Epidemiological evidence in causative relationships between fiber-depleted diets and certain characteristically Western diseases has stimulated research interest in the physiological and physico-chemical properties of various dietary fibers in the past decade (1). Dietary fibers are composed of cellulose, lignin, and noncellulosic polysaccharides, all of which are the

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components of plant cell wall. Pure cellulose exerts little physiological function, whereas lignin and noncellulosic polysaccharides are capable of binding fatty acids, bile acids, and carcinogens (2, 3). Substances such as starch, protein, lipid, phytic acid, and minerals are usually associated with dietary fiber in forming a dietary fiber complex that alters physico-chemical properties of the fiber. Among the common foods of plant origin, breakfast cereals and wholemeal breads containing wheat bran are high dietary-fiber foods for both health conscious people and for the treatment and control of diabetes (4). Enrichment of foods with fiber from cereal hull, citrus pulp, guar gum and woody tissues is presently in the experimental stage. Although fibers in the natural foods of human diet are primarly derived from leafy vegetables, the purified dietary fibers of leaf tissue origin are not commercially available.

The recent achievement in extraction of leaf protein from young tobacco plants has advanced the possibilities for utilizing tobacco plant materials in human foods (5). Young tobacco plants grown under high density produced leaf biomass two to three times greater than stalk weight (6). Fibrous residue of young tobacco plants from the leaf protein extraction process can be a potential dietary fiber if the residue, after removal of plastic pigments, possesses desirable physico-chemical properties. The present study was therefore undertaken to evaluate the decolorized tobacco fibrous residues for chemical and physical properties and to compare them with wheat bran and alfalfa fibrous residue. Because of a broad genetic variation in morphology and chemical composition of plants within *Nicotiana tabacum*, the present study compared a number of tobacco types.

## Materials and Methods

A field experiment involving ten tobacco cultivars representing flue-cured, dark fire-cured, burley, Turkish and Maryland types was conducted in Lexington, Kentucky, in early spring 1980. About 110-120 plants per plot (1.5 x 1 m) per cultivar were established by direct-seeding and cultured according to conventional practices in the burley growing region. When plants reached 50 cm or taller in the middle of June, all plants within a 0.36 $m^2$ area were cut about 12 cm from the ground as one sample with three samples taken per plot. A total of 30 samples were separately processed to obtain deproteinized and decolorized fiber complex.

Deproteinization of fresh tobacco plants was accomplished by homogenization in a high capacity Waring blender with an equal weight of ice-cold 2% sodium metabisulfite solution. The homogenate was strained through three layers of cheese-cloth and one layer of Miracloth and, subsequently, the green fibrous

residue was washed with a sufficient volume of cold water to remove residual soluble protein. The deproteinized green fibrous residue was washed with several volumes of cold 80% isopropanol until cream colored. In addition, three samples of fresh alfalfa (before the flower bud stage) which were deproteinized and decolorized by the same process and wheat bran from a commercial mill were included for comparison. All samples were sieved through a 9-mesh screen and oven-dried (70 C) overnight before chemical and physical characterization.

All fiber complexes were analyzed for protein, starch, lipid, cellulose, lignin, and ash concentrations. Protein quantity was calculated from the difference in free and total amino acid contents before and after 6N HCl digestion. Ninhydrin reaction of amino acids was colorimetrically determined at 570 nm with known quantities of amino acids as standards (7). Starch was quantitated at 600 nm by the modified iodine stain method of Gaines and Meudt (8). The weight difference before and after overnight Soxhlet extraction of fiber complex with hexane measured lipid content. Cellulose and lignin quantitation on an ash-free basis was performed through gravimetric methods (9). Ash content was the residual weight of cellulose and lignin after charring in a muffle furnace at 550 C for 4 hr. The difference of the above six components from 100% was considered as apparent hemicellulose which includes hemicellulose,pectic substances, gums, and mucilages, if present. The same samples were analyzed for neutral and acid detergent fiber (NDF and ADF) content according to Van Soest's methods (10).

Mineral elements except phosphorus and molybdenum in the tobacco fiber were analyzed by use of Varian AA-6 Atomic Absorption Spectrophotometer. Samples were wet-ashed in nitric and perchloric acids (9:1, v/v) until completely digested. After evaporation to dryness, the ashes were solublized in 1N HCl for analyses. Quantitation of phosphorus was a colorimetric method using a Technicon Autoanalyzer and the color reaction of Fiske and Subbarow (11). Molybdenum was quantitated by the method of Eivazi et al (12) which determines, with good reproducibility, a quantity as low as 0.02 ppm in plant materials.

Physical properties measured in the present study included bulk volume, water retention capacity, and cation exchange capacity. Bulk volume measurement was the method of Montgomery and Baugardt (13). whereas water retention capacity was the amount of water retained by the fiber against a centrifugal force of 14,000 g for one hr after a 24 hr hydration period at 20 C (14). For determination of cation exchange capacity, the fiber complex was soaked in 2 N HCl for 24 hr followed by washing with deionized water. Hydrogen ions were dissociated from the fiber complex with 2N neutral NaCl solution which was then titrated against 0.05N NaOH (14).

Binding of sodium taurocholate by the fiber was the method of Kritchevsky and Story (15). A 50 or 100 mg sample of fiber

was incubated in 5 ml of HPLC-grade water containing 0.15 M NaCl and 8 mM (40 μ moles) or 40 mM (200 μ moles) sodium taurocholate at 37 C for one hr with gentle shaking. For the control, 5 mL of 0.15 M NaCl solution devoid of sodium taurocholate was added to the testing sample. Sodium taurocholate in the same NaCl solution with a concentration series of 40 mM, 20 mM, 10 mM, 5 mM and 2.5 mM was processed by the same procedure to establish a calibration curve. After incubation, the sample was filtered through a 0.2 μm microporous filter and the filtrate was ready for HPLC analysis. Varian Model 5000 Liquid Chromatograph equipped with a Vari-Chrom UV-Vis Detector was used. The HPLC conditions were: Micro Pak MCH-10 column (30 cm X 4 mm I.D.); eluent, 70% MeOH and 30% 0.15 M NaCl solution, isocratic; flow rate, 1 mL/min; detection at 210 nm. With a 10 μL injection and 15 min cycle, sodium taurocholate can be detected within 5 min. Control samples did not show any peaks with retention times similar to that of sodium taurocholate.

Nicotine contamination in tobacco fiber was assayed by the GLC equipped with an alkaline flame ionization detector. The extraction procedure and GLC conditions have been detailed in a recent paper (6). The sensitivity of detection is below 1 ppm.

All data were subjected to variance analyses. Whenever variation among fiber sources was statistically significant, the least significant difference (LSD) at the 5% and 1% level of probability was calculated for statistical inference of the results.

## Results

Chemical composition of tobacco and alfalfa deproteinized and decolorized fiber complexes and wheat bran is given in Table I. Dark green tobaccos including flue-cured (NC 95, NC 2326 and Coker 139), dark fire-cured (Ky 171 and Ky 151) and Maryland (MD 609) types had comparable amounts of protein with a mean of 15 g/100 g dry wt. The average value of the three burleys (Ky 16M, Burley 21 and Ky 14) was 9.46 g/100 g dry wt with Burley 21 showing significantly less than the other two. Turkish tobacco Xanthi nc resembled burley in protein content. Alfalfa fiber had a protein concentration similar to that of dark green tobacco, whereas wheat bran contained a significantly higher level among the fibrous samples under comparison. For starch content, a significant contrast existed between dark green tobacco and burley or Turkish type with the exception of MD 609 resembling burley in low starch concentration. Incidentally, Maryland and burley tobaccos are air-cured types. That starch content was low in alfalfa fiber and reached as high as 30% weight in wheat bran was not unexpected owing to the nature of the corresponding plant parts. There was little difference in lipid concentration among the fibrous residues of tobacco and alfalfa with a mean of 2.82 g/100 g dry wt. More than three

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Table I. Chemical Composition of Tobacco and Alfalfa Fibrous Residues and Wheat Bran.

| Fiber source | Protein | Starch | Lipids | Ash | Cellulose | Lignin | Apparent hemicellulose | NDF | ADF |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | g/100 g dry wt | | | | |
| Tobacco | | | | | | | | | |
| NC 95 | 13.54 | 4.30 | 2.44 | 7.14 | 31.38 | 5.68 | 35.52 | 53.46 | 41.51 |
| NC 2326 | 15.48 | 2.01 | 3.06 | 8.11 | 33.88 | 4.13 | 33.33 | 53.36 | 41.50 |
| Coker 139 | 15.61 | 2.22 | 2.78 | 12.55 | 32.21 | 8.25 | 26.08 | 55.91 | 47.25 |
| Ky 171 | 15.88 | 2.42 | 2.40 | 10.75 | 28.09 | 4.85 | 36.62 | 50.42 | 41.85 |
| Ky 151 | 14.27 | 1.38 | 3.38 | 9.31 | 33.83 | 5.10 | 32.75 | 55.36 | 42.15 |
| Ky 16M | 12.21 | 0.90 | 3.55 | 9.41 | 32.68 | 5.38 | 35.86 | 56.06 | 47.89 |
| Burley 21 | 6.02 | 0.33 | 2.67 | 12.79 | 32.71 | 5.77 | 39.71 | 53.84 | 45.15 |
| Ky 14 | 10.14 | 0.09 | 2.48 | 9.94 | 30.60 | 4.55 | 42.15 | 51.84 | 42.07 |
| Xanthi nc | 8.41 | 0.18 | 3.09 | 10.79 | 35.19 | 6.24 | 36.27 | 61.04 | 46.98 |
| MC 609 | 15.23 | 0.88 | 2.32 | 14.79 | 34.00 | 7.15 | 25.63 | 55.74 | 51.06 |
| Alfalfa | 13.12 | 0.60 | 2.80 | 4.20 | 44.23 | 9.91 | 25.05 | 61.84 | 50.71 |
| Wheat bran | 18.51 | 30.67 | 9.54 | 5.80 | 10.17 | 2.94 | 24.11 | 47.05 | 11.56 |
| LSD 0.05 | 2.44 | 1.20 | 0.99 | 1.07 | 2.53 | 1.09 | 3.62 | 5.26 | 2.28 |
| LSD 0.01 | 3.31 | 1.63 | 1.34 | 1.46 | 3.44 | 1.48 | 4.93 | 7.15 | 3.10 |

times this amount was found in wheat bran. Ash content varied widely among tobacco fibrous samples which had an average quantity of 10.56 g/100 g dry wt. The ash content in alfalfa fiber and wheat bran was about one-half or less than that in tobacco fiber.

On the same weight basis, wheat bran had only 1/3 to 1/4 the cellulose quantity present in tobacco and alfalfa fibers. Alfalfa fiber was also rich in lignin with a quantity three times greater than that in wheat bran. Lignin quantity in tobacco fiber was in-between those of alfalfa fiber and wheat bran; however, significant differences existed within and between tobacco types. Such a range of variation was also evident for the quantity of apparent hemicellulose in tobacco samples. Apparent hemicellulose content in alfalfa fiber and wheat bran was indifferent to the tobacco cultivars being on the low concentration end of the variation. Quantitative variation of NDF and ADF was present among tobacco cultivars but they lacked the same pattern of variation. The tobacco cultivar means for NDF and ADF quantity were 54.70 and 44.74 g/100 g dry wt, respectively. These values were significantly higher than those of wheat bran. Alfalfa fiber had the highest amounts of both detergent fibers although the amounts were not statistically different from those of the tobacco cultivars ranked high in detergent fiber content.

It has been shown that fiber-rich diets may impair the utilization of a number of essential minerals including calcium, iron, zinc, copper, and phosphorus (16). This can be corrected by dietary intake of minerals from a variety of foods, on the one hand, and the endogenous source of essential minerals in the dietary fiber, on the other. Eleven mineral elements were therefore quantitated in tobacco and alfalfa fibers and wheat bran (Table II). In reference to wheat bran, tobacco fiber contains comparable or slightly higher levels of sodium, phosphorus, sulfur, manganese and copper but at least 20-fold more calcium and several-fold more iron. Iron concentration varied nearly five-fold among tobaccos. Wheat bran was rich in potassium, magnesium, and molybdenum. Alfalfa fiber showed a close resemblance to tobacco in mineral content except for calcium, magnesium, and copper, which were significantly less.

Rasper (17) studied physical characteristics of dietary cereal fiber including those from wheat bran and reported that bran fiber had bulk volume around 3 to 5 mL/g depending upon preparation method. The wheat bran sample in the present experiment gave the bulk volume of 4.53 mL/g which is in good agreement with the values cited in literature. The bulk volume of alfalfa and tobacco fibers doubled and tripled the wheat bran value (Table III). Among tobaccos, Xanthi nc gave the largest bulk volume and NC 2326 the least. The variation seemed to be significant for flue-cured tobaccos but not for burleys. The average water retention capacity of tobacco fiber was 9 g water/g with a range of 7.40 to 10.86. Therefore, tobacco fiber held

Table II. Quantities of Mineral Elements in Tobacco and Alfalfa Fibrous Residues and Wheat Bran.

| Fiber source | Na | K | Ca | Mg | P | S | Fe | Mn | Zn | Cu | Mo |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | % | | | | | | ppm | | | | |
| Tobacco | | | | | | | | | | | |
| NC 95 | 0.49 | 0.28 | 1.98 | 0.12 | 0.18 | 0.22 | 542 | 115 | 58 | 22 | 0.43 |
| NC 2326 | 0.39 | 0.24 | 2.03 | 0.16 | 0.20 | 0.30 | 743 | 104 | 54 | 21 | 0.85 |
| Coker 139 | 0.44 | 0.90 | 2.53 | 0.20 | 0.22 | 0.43 | 1510 | 212 | 40 | 43 | 0.91 |
| Ky 171 | 0.45 | 0.49 | 2.71 | 0.18 | 0.26 | 0.30 | 701 | 103 | 30 | 21 | 0.65 |
| Ky 151 | 0.42 | 0.38 | 2.68 | 0.23 | 0.27 | 0.41 | 659 | 92 | 56 | 29 | 0.54 |
| Ky 16M | 0.53 | 0.69 | 2.68 | 0.19 | 0.31 | 0.26 | 293 | 99 | 37 | 12 | 0.23 |
| Burley 21 | 0.64 | 0.71 | 2.95 | 0.22 | 0.40 | 0.27 | 880 | 111 | 51 | 21 | 0.72 |
| Ky 14 | 0.48 | 0.51 | 3.08 | 0.32 | 0.38 | 0.34 | 738 | 99 | 116 | 38 | 0.49 |
| Xanthi nc | 0.51 | 0.55 | 2.92 | 0.27 | 0.35 | 0.31 | 861 | 118 | 59 | 32 | 0.57 |
| MD 609 | 0.52 | 0.96 | 3.12 | 0.25 | 0.32 | 0.36 | 1407 | 171 | 78 | 32 | 0.75 |
| Alfalfa | 0.35 | 0.11 | 1.22 | 0.16 | 0.13 | 0.14 | 357 | 21 | 36 | 7 | 0.50 |
| Wheat bran | 0.35 | 1.46 | 0.11 | 0.54 | 0.23 | 0.20 | 175 | 114 | 184 | 20 | 1.25 |
| LSD 0.05 | NS | 0.29 | 0.45 | 0.06 | 0.06 | 0.09 | 407 | 34 | 46 | 2 | 0.49 |
| LSD 0.01 | NS | 0.39 | 0.61 | 0.08 | 0.08 | 0.13 | 553 | 46 | 65 | 3 | 0.67 |

Table III. Physical Properties of Tobacco and Alfalfa Fibrous Residues and Wheat Bran.

| Fiber source | Bulk volume (mL/9) | Water retention capacity (g water/g fiber) | Cation exchange capacity (meq NaOH/g) X $10^{-2}$ |
|---|---|---|---|
| Tobacco | | | |
| NC 95 | 14.90 | 9.57 | 21.83 |
| NC 2326 | 11.33 | 8.40 | 17.50 |
| Coker 139 | 14.77 | 10.40 | 21.50 |
| Ky 171 | 14.87 | 7.40 | 20.00 |
| Ky 151 | 14.00 | 10.86 | 19.00 |
| Ky 16 M | 13.80 | 9.18 | 20.67 |
| Burley 21 | 13.97 | 8.47 | 21.50 |
| Ky 14 | 14.80 | 8.55 | 18.50 |
| Xanthi nc | 16.33 | 8.02 | 15.67 |
| MD 609 | 13.23 | 9.13 | 19.67 |
| Alfalfa | 9.47 | 4.92 | 15.33 |
| Wheat bran | 4.53 | 1.18 | 6.17 |
| LSD 0.05 | 0.93 | 0.89 | 2.47 |
| LSD 0.01 | 1.25 | 1.21 | 5.43 |

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twice the volume of water than its alfalfa counterpart and six-fold or more volume than wheat bran. The ranking of cation exchange capacity among the fiber sources followed the same order of the above physical properties with tobacco fiber being the highest and wheat bran the least. The average value of cation exchange capacity for tobacco cultivars was 19.58 X $10^{-2}$ meq NaOH/g. The difference between tobacco fiber and wheat bran was about three-fold. The cation exchange capacity of wheat bran was one-half the value of the alfalfa sample.

Results of sodium taurocholate binding from two experiments employing different combinations of sodium taurocholate and fibrous material was given in Table IV. There was a significant difference among tobacco types but not within a given type. Both experiments revealed that flue-cured tobaccos had higher sodium taurocholate binding capacity than the other tobacco types, and the binding amount was in proportion to the quantity of fiber. By averaging ten tobacco cultivars, the binding capacity was 2.47 μ moles/50 mg and 6.07 μ moles/100 mg for two experiments. These values were ranked the highest, followed by alfalfa; the lowest was wheat bran. The difference between the latter two was not statistically significant. The fiber of flue-cured tobacco was significantly higher in binding capacity than alfalfa fiber and wheat bran in most cases. However, the high binding capacity of flue-cured tobacco cannot be correlated with the quantity of any chemical constituents given in Table I.

Young tobacco plants usually contain about 0.5% of dry wt as nicotine although nicotine quantity varies widely among genotypes (18). Nicotine contamination in tobacco fibrous residue ranged from 180 to 340 ppm with a mean of $260 \pm 53$ ppm (data not shown). Samples of Ky 171 fiber which contains 256 ppm of nicotine were repeatedly washed with cold water (1:10, w/v) and the washed residues were analyzed for nicotine quantity by GLC. On the average, each washing removed two-thirds the nicotine in the residue; and after several washings, nicotine level approached 1 ppm (Figure 1). This points to the feasibility of lowering nicotine contamination in the fibrous residue to a negligible level. The washing process did not alter the concentration of protein and noncellulosic components.

## Discussion

Purified plant fibers for dietary purposes are combinations of cellulose, lignin, hemicellulose, gums and pectinaceous substances that exert chemical and physical actions in the gastrointestinal tract. Dietary fiber complexes such as wheat bran contain not only the fiber components but also other organic compounds that contribute to nutrition. In comparison to wheat bran, the deproteinized and decolorized fiber complexes of tobacco and alfalfa have favorable chemical composition as dietary fibers. Their low concentrations in starch and lipids

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Table IV. Binding In Vitro of Sodium Taurocholate to Tobacco and Alfalfa Fibrous Residues and Wheat Bran

| Fiber source | Experiment 1 μ moles/50 mg | Experiment 2 μ moles/100 mg |
|---|---|---|
| Tobacco | | |
| NC 95 | 3.65 | 7.89 |
| NC 2326 | 3.30 | 7.13 |
| Coker 139 | 2.78 | 7.88 |
| Ky 171 | 1.89 | 6.44 |
| Ky 151 | 1.91 | 4.78 |
| Ky 16M | 2.12 | 6.56 |
| Burley 21 | 1.86 | 4.37 |
| Ky 14 | 2.54 | 4.99 |
| Xanthi nc | 2.48 | 5.14 |
| MD 609 | 2.21 | 5.56 |
| Alfalfa | 2.16 | 4.93 |
| Wheat bran | 2.09 | 4.84 |
| LSD 0.05 | 0.88 | 1.83 |
| LSD 0.01 | 1.20 | 2.49 |

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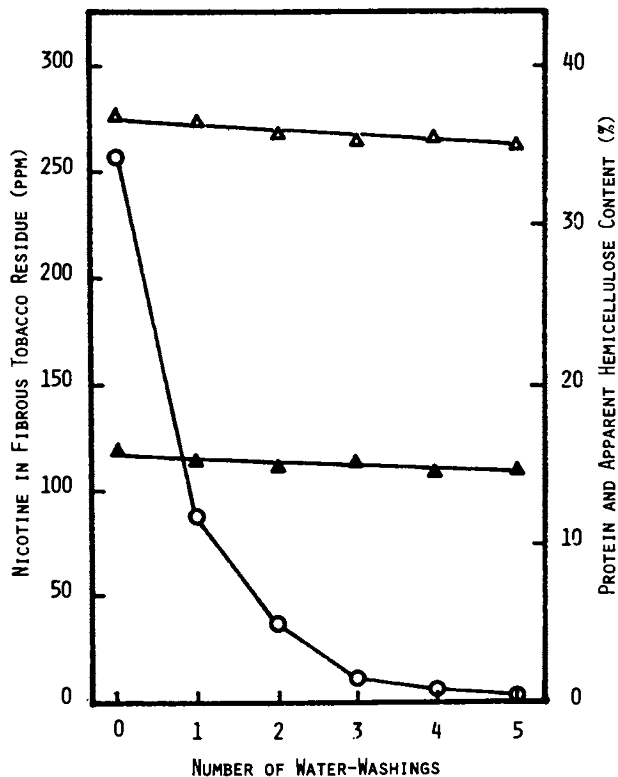


*Figure 1. Effects of water-washing on the quantity of nicotine (○), protein (▲), and apparent hemicellulose (△) in the fibrous residue of Ky 171 tobacco.*

and high amounts of cellulosic components seem to make them more suitable than wheat bran as low-calorie dietary fibers. This is further supported by the physical properties, especially for tobacco fiber, that a large bulk volume in accompaniment with high water retention and cation exchange capacity would be desirable. In addition, the absorption of bile salt (sodium taurocholate) by fobacco fiber is as effective as, or more so than wheat bran which suggests that tobacco fiber may also have the capability of regulating serum chloresterol levels. Nevertheless, the favorable chemical and physical properties of plant fiber complex measured in vitro still require confirmation from in vivo experimentation since the physiological performance and technological functionality of dietary fiber in fiber-enriched foods are influenced by many factors.

As suggested by Van Soest and Wine (10), the quantitative difference between NDF and ADF gives rise to a good estimation of hemicellulose quantity, and ADF is the sum of cellulose and lignin content. Taking wheat bran in the present study (Table I) as an example, the calculated values for hemicellulose and ADF are 35.49 and 13.12 g/100 g,which are in good agreement with Southgate's findings (19). However, the calculated value of hemicellulose is almost 50% greater than that of apparent hemicellulose. This is not surprising since wheat bran is rich in starch known to contaminate the NDF fraction. On the basis of NDF and ADF quantities, the calculated value of hemicellulose in the alfalfa sample is 11.13 g/100 g, while the average amount for tobacco fiber is 9.96 $\pm$ 2.82 g/100 g. The respective values of apparent hemicellulose are 225% and 345% greater. It has been reported that the ADF-reagent sometimes leaves substantial amounts of hemicellulose in the ADF-residue (20). Furthermore, Van Soest's detergent methods do not measure water-soluble components, whereas apparent hemicellulose includes these substances if they are not removed by 80% isopropanol washing. All these factors can contribute to the discrepancy between calculated and apparent hemicellulose values. This led to the suggestion that the pretreatment of fibrous material with lipid extraction and proteolytic and amylolytic enzymes would minimize erroneous results (19). One can also anticipate differences between tobacco and alfalfa and among tobacco cultivars in chemical composition and structure variation of cell wall as well as the nature of complex formation involving cell wall constituents and other substances. It would be highly desirable to compare the sugar constituents of tobacco fiber components with those of conventional dietary fibers.

The high concentration of iron and calcium in tobacco fiber would be desirable from a nutritional viewpoint. The bioavailability of mineral elements in dietary fibers has been discussed in the literature (16). Aside from fiber per se, phytic acid was found to be the major determinant of bivalent metal deficiencies (21). Phytate content is high in wheat bran and other

dietary fibers of seed origin since it is a source of glucuronate precursor for synthesis of cell wall polysaccharides during germination (22). Among the hundreds of compounds in tobacco leaf, phytate has never been mentioned in a large volume of tobacco literatures (18).

In analogy to phytate in wheat bran, tobacco plants accumulate 2 to 5% of dry weight as oxalic acid, predominately in the form of calcium salt, in mature leaves (18). Calcium oxalate is insoluble in water. Young plants of seven burley cultivars under high density growth had oxalate content in the range of 0.6 to 1.4% (23). The dietary intake of oxalate poses a concern for calcium and magnesium availability although it was documented that oxalate can be formed *in vivo* from diets containing ascorbate, glycine, glycolate, and glyoxylate (24). Oxalate content in tobacco fiber was not determined in the present study. However, the deproteinization and decolorization processes would remove soluble oxalate. Calcium oxalate may remain in the fibrous residue.and will not further deplete calcium in diets because oxalic acid is already bound to calcium. Many edible plants are rich in oxalic acid. For example, spinach contains 8 to 10% dry weight as oxalic acid (25). Five cups of tea per day in the English diet contributes to an intake of 75 mg oxalic acid (26). Oxalate content in tobacco fiber is probably in the same range as that of certain common foodstuffs.

Another concern of possible adverse effects of tobacco fiber complex as dietary fiber is nicotine contamination. Nicotine is regarded as highly toxic. Gasseline *et al* (27) reported that the oral $LD_{50}$ of an adult human has been estimated to be 30 to 60 mg (0.5 to 1.0 mg/kg body wt). They also stated that tobacco is much less toxic than expected from its nicotine content because intestinal absorption of nicotine as present in tobacco is so slow that metabolic inactivation sometimes keeps pace with absorption and the elimination of nicotine is completed within 16 hours. About 80 to 90% of the nicotine in human urine has already been detoxified. Since nicotine is very water-soluble, the washing of tobacco fibrous residue could lower nicotine to a negligible level as demonstrated in the present study. If the water-washed tobacco fiber contains 10 ppm of nicotine, a 10% incorporation in fiber-enriched formulation results in 1 ppm of nicotine in the formulated foods. The presence of nicotine in minute quantities was reported in tomato and other solanaceous vegetables (28). With the GLC method employing an alkaline flame ionization detector (6), a broad array of fresh and preserved foods was analyzed for nicotine contamination. Nicotine was detected at 2 to 15 ppm (on a dry wt basis) in tomato, potato, eggplant, and green pepper but not in alfalfa and wheat bran. Most interesting is the finding of nicotine in tea, expecially instant tea, at levels of 15-24 ppm. Nicotine quantity in tobacco can be drastically lowered by genetic means (29). Varietal difference in oxalate content in

tobacco leaf also indicates possible genetic regulation (23). If tobacco is grown as a food crop, the concentration of these undesirable leaf constituents can be lowered by means of breeding.

The potential of tobacco utilization as a source of soluble proteins and the deproteinized residue derived from the protein extraction process as animal feed and safer smoking material has been mentioned in recent years (5, 6, 30). The present study suggests that after decolorization tobacco fibrous residue may be a potential source of dietary fiber. Additional research is certainly needed to evaluate the nutritional and physiological significance of tobacco fiber in experimental animals. Some of these studies are presently being undertaken.

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# Products of Wheat Straw Biodegradation by *Cyathus stercoreus*

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The residual biodegradation products of wheat straw lignin attacked by Cyathus stercoreus for thirty days were identified. Low-molecualr-weight nonvolatile materials constituted less than 5% of the total $^{14}C$ released from labeled lignin. In this portion, syringic acid, vanillic acid, p-hydroxy benzoic acid, acetic acid, and 2-methoxy-succinic acid were found. The majority of the water-soluble biodegradation products (89%) are lignin-carbohydrate complexes with molecular weights greater than 1000. Analysis of the gases resulting from lignin breakdown and metabolism reveals primarily $CO_2$, with smaller amounts of ethanol, methanol, and acetone. The gas phase, flushed out with air and recovered, constitutes 50% of the total lignin breakdown products.

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In the field of biomass utilization, separating lignin from cellulose and hemicellulose by biological means has aroused a great deal of interest. Degradation of lignin during the process of separation is not necessarily detrimental if the lignin can be converted to useful chemicals. To fully understand the biological approach to modifying and solubilizing lignin, the biodegradation products of lignin must be identified and quantitated.

*Cyathus stercoreus* (Schw.) de Toni NRRL 6473 degrades lignin preferentially during a 62-day fermentation of wheat straw, but the biodegradation products have not been characterized (1). Other workers have identified residual phenolics in biodegradation broths of several woods and synthetic or isolated soluble lignins (2). More recent publications have identified functional group changes in degraded lignins rather than specific low molecular weight compounds (3). All essentially identify oxidized species as a result of fungal or bacterial degradation.

Current knowledge in lignin biodegradation supports a mechanism of random oxidation, generating aromatic compounds with various oxygen-containing side groups, aliphatic carboxylic acids from subsequent ring cleavage and ultimately, carbon dioxide (4-7).

The purpose of the present work was to identify and quantitate the residual products after a 30-day fermentation of *C. stercoreus* on wheat straw. The approach to this problem is limited by a few, significant considerations. Ideally, preliminary extraction of the straw eliminates interfering substances, such as fatty acids and other extractables present in large quantity, which overwhelm minute amounts of biodegradation products and thereby hinder their identification. However, the fungus does not grow well when all soluble nutrients are removed, as will be shown in later sections. Also, sterilization by autoclaving may generate soluble lignins that cannot be attributed to fungal action, and therefore controls must be run. More specifically, reproducibility of fermentation on a solid substrate inoculated with a 1 $cm^2$ plug of slow-growing *C. stercoreus* is difficult, especially because this fungus is sensitive to excess water and will not grow in submerged culture, although a high level of moisture is necessary for fungal growth.

After the fermentation is complete, extracted water-soluble degradation products lose much of their water solubility if taken to dryness in air or if heated. Freeze-drying overcomes this problem, but molecular weight distributions should be determined before freeze-drying because polymer chains can be fractured by growing solvent crystals (8).

## Materials and Methods

Sample Preparation. Three wheat straw samples were tested. One was a locally obtained, dried and baled wheat straw; the other two were Red River 68 and Butte HRS, grown in an environmental chamber. While monitoring the plant growth and lignin production in the straw as reported by Stone et al. (9), we grew the plants for 38 days (Butte HRS) or 48 days (Red River 68) and cut them for lignin labeling just prior to heading out of the grain.

In order to label the lignin, the roots were cut off and the freshly cut surface was immediately immersed in a 10-ml solution of uniformly labeled $^{14}C$-phenylalanine in a growth chamber until the solution was nearly all taken up. The cut surface was then immersed 1-2 cm in distilled water for 3 days, dried at 45° C to constant weight and ground to pass through a 1-$mm^2$ screen. The ground straw was washed with hexane at 35° C and dried, then soaked in water at 4° C for 16 h, washed, and finally treated with protease at room temperature for 3 h, water washed and freeze-dried. The protease used was Subtilopeptidase-A (Bacterial, Type VII) from Sigma Chemical Company in a pH 7 borate buffer at a concentration of 1 mg/ml. Total $^{14}C$ content, as decompositions per minute per mg (DPM/mg), was determined by pyrolysis of the straw in an oxygen atmosphere to $CO_2$ and trapping of the $CO_2$ in 10 ml of a scintillation cocktail consisting of 1,300 ml toluene, 1,200 ml methanol, 300 ml 2-aminoethanol, 0.0375 g 1,4-[2-(4-methyl-5-phenyl-1,3-oxazolyl) benzene and 30.0 g 2,5-diphenyloxazole.

Unlabeled wheat straw obtained locally was hexane washed, dried, soaked at 4° C in water that was decanted, and then either fermented directly or after further water washing. Also, at the end of the 4° soak the straw was broken up in a Waring Blendor to prevent trapping of water inside the hollow straw stem.

Fermentation was accomplished in air at 25° after inoculation with a 1-$cm^2$ plug of *C. stercoreus* that had been grown for 1 week on a potato dextrose agar plate.

Separation of Biodegradation Products. The scheme devised for separation and recovery of biodegradation products is illustrated in Figure 1. The ether phase extract, labeled C in Figure 1, would contain acidic materials such as vanillin, syringealdehyde and similar compounds if they were present. Some polyfunctional acidic compounds remain in the acidified water phase D. An alternate route using ion exchange resin is better suited for separation and identification of polyfunctional acidic compounds, such as hydroxybenzoic acid. These compounds will end up in the water extract labeled E in Figure 1. Unless other soluble compounds mask their presence, these acids can also be identified in the original extracted fermentation broth.

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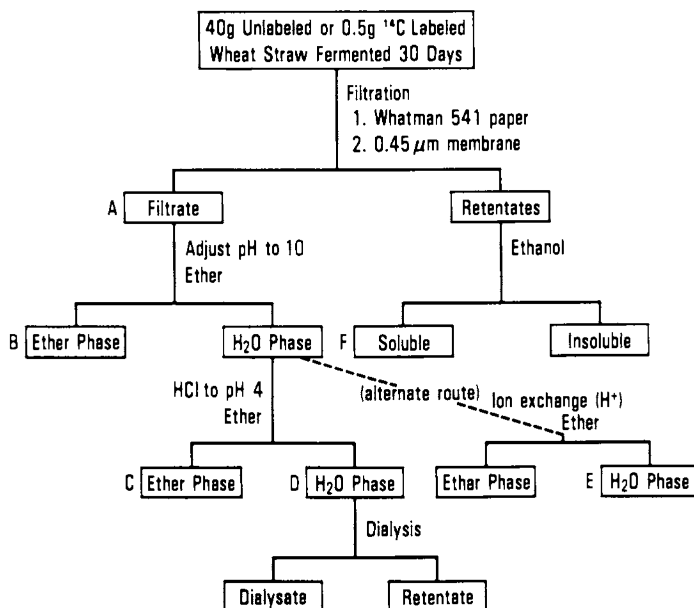


*Figure 1. Separation of biodegraded wheat straw.*

Characterization Methods. Labeled materials in soluble extracts were quantitated by counting 100 µl aliquots of the various solutions in the same 10 ml scintillation cocktail used to trap $CO_2$.

The quantity of $^{14}C$ in evolved gases was measured by daily 15-min flushes of the sealed fermentations with air into the same scintillation cocktail. The gas-phase components were identified by their retention times on a 5 ft X 1/8 inch Porapak Q column. To trap enough gas for identification, the evolved gases from 2 days of fermentation were flushed with helium into a 1 ft X 1/8 inch Porapak Q filled stainless-steel column filled with Porapak Q and immersed in liquid $N_2$. The column exit gas passed through a bubbler containing scintillation fluid. After the 15-min flush the column was capped on the entrance side and a new bubbler was attached. The column was then allowed to warm to room temperature. Trapped $CO_2$ rapidly eluted as the column warmed, and when gas evolution stopped the column was inserted into a Tracor Model 560 gas chromatograph (GC); the entrance side of the trap column was attached to the injection port and the exit side to a 4 ft by 1/8 inch Poropak Q column. The baseline had been established previously but residual $CO_2$ came off as helium carrier gas was turned on and the oven was programmed from 40° to 180° at 5°/min. Samples from the water phase were tested for volatiles other than $CO_2$. They were tested on the same Poropak Q column as gas-phase samples, but the column was programmed from 100 to 170° at 10°/min and held at 170° for 20 min.

To ensure that the volatiles other than $CO_2$ were from lignin degradation, the same liquid $N_2$ trapping procedure was followed. The column was attached at room temperature to a GC with a thermoconductivity (TC) detector leading to a flowing scintillation fluid trap for gases as they eluted. Results of the analysis of volatiles by GC were confirmed by high-pressure liquid column chromatography (HPLC) and infrared spectra of the major components.

Less volatile low-molecular-weight biodegradation products, were characterized by gas chromatography-mass spectroscopy (GC-MS). Higher molecular weight compounds were characterized by gel permeation chromatography (GPC), membrane filtration, dialysis and nuclear magnetic resonance (NMR).

The methylated ($CH_2N_2$) low-molecular-weight compounds were analyzed on a Kratos MS-30 equipped with a Perkin Elmer Sigma 3 gas chromatograph and a 3 ft X 1/8 inch OV-1 column. Retention times of the sample peaks identified by GC-MS were compared to those of known standards on a 6 ft X 1/8 inch OV-1 column on a Tracor model 560 GC for confirmation. Lignin content was measured by the UV method (10).

Control samples of wheat straw oxidized by the standard nitrobenzene method (11) and separated by the scheme illustrated in Figure 1 were also tested by the GC-MS method to confirm the validity of the separation and recovery techniques.

To characterize high-molecular-weight biodegradation products, an Amicon Model 202 Diafiltration Apparatus was used sequentially with XM300, PM10 and UM2 membranes at 10, 17 and 55 psi $N_2$ pressure, respectively. The samples tested were water-soluble biodegradation products that had never been freeze-dried. Concentrations of the various fractions were determined on aliquots so that GPC curves could be determined on samples that had always been in solution.

High-molecular-weight biodegradation products for NMR were isolated by dialysis and membrane filtration. Dialysis bags of regenerated cellulose were characterized with standard dextrans and raffinose. GPC curves of a dextran-raffinose mixture before and after separation by dialysis indicates a nominal molecular weight of 3,000 or greater was retained for NMR analysis. High-molecular-weight compounds isolated by this method were freeze-dried, and their $^{13}C$ NMR spectrum determined on a Brüker WH 90 in $D_2O$. Gel permeation chromatography (GPC) curves were determined with a Waters 244 HPLC on 2 columns of E-linear μ-Bondagel, 7.8 mm X 30 cm each. Water was the solvent at 1 ml/min and ambient temperature. Detection was by refractive index and UV absorbance at 254 mn.

## Results and Discussion

Recovery of the gas phase of fermented wheat straw and separation of the residue by the method illustrated in Figure 1 gave a material balance of the various fractions by weight $^{14}C$ content, as shown in Tables I and II. Although only 4.6% of the original 40 g of straw is extracted as water-soluble material after the fermentation and 9.3% of the radioactive straw by weight, a higher percentage of the original $^{14}C$ (13.3%) is in the recovered water-soluble material (Table I). Thus there is a higher concentration of lignin degradation products in the fermentation broth than the concentration of lignin in the wheat straw. Autoclaving unfermented straw yields 0.8% of the original radioactivity in the water phase. Careful drying to avoid loss of moderately volatile substances such as vanillin involved freeze-drying the water-soluble material from the 40-g straw fermentations. Additionally, $^{14}C$-labeled water solubles were counted by testing aliquots (100 μl) in scintillation fluid, both before and after freeze-drying for 54 h past apparent dryness. Less than 4.3% of the original $^{14}C$ was lost on freeze-drying and reconstitution, and no $^{14}C$ was detected in the -70° freeze-drying trap. Some loss of both weight and $^{14}C$ will occur from dissolved $CO_2$, methanol and ethanol. From the results in Tables I and II it can also be concluded that the gas phase contains approximately 13% of the original $^{14}C$. The total lignin is degraded 27% after 30 days, half in the gas phase and half in the water phase of the fermentation, before either phase is separated for identification. Identification of the components of the gas phase by retention time is illustrated in Figure 2.

A typical flame ionization detection (FID) curve is shown in Figure 2 (curve 2). Gases and water-phase volatiles identified by retention time are ethanol, methanol, acetone and acetic acid. A comparison of counts collected over 10° intervals in the programmed chromatograph to the retention times of peaks detected by both FID and TC indicates that all of the gases in addition to $CO_2$ have at least part of their origin in the $^{14}C$-labeled lignin (compare Figure 2, curve 3 to Figure 2, curves 1 and 2). Residual ethanol in the water phase was determined by HPLC to be 0.02% or 0.033 g in a 30-day, 40-g straw fermentation with free (filtered) atmospheric gas exchange. $CO_2$ and water were evident in the infrared spectra of fermentation gases, with no evidence of significant amounts of hydrocarbon gases.

As indicated by weight and $^{14}C$ analysis (Table II), few if any low-molecular-weight aldehydic phenols were isolated in ether phase C. Therefore, the analysis scheme (Figure 1) was tested by adding 0.05 g vanillin to a 30-day fermented wheat straw and carrying it through the extraction and freeze-drying procedure. The added vanillin was easily determined in ether extract C by GC. Only a trace was found in ethanol-soluble extract F, but even this trace, 0.1 mg, was detectable, in contrast to the extracts of biodegraded wheat straw that contained no aldehydic phenols. The absence of vanillin, syringealdehyde, and several other phenols in the water-soluble biodegradation products was confirmed by thin layer chomatography (TLC) in 95/5 benzene/methanol, using UV and radiocarbon detection. Of all the compounds examined, only carboxylated phenols do not migrate away from the origin in this solvent.

Ether extraction procedures have been used also to isolate aldehydic phenols from alkaline nitrobenzene oxidized lignin to characterize the lignin in various plants. $Fe(NH_4)_2(SO_4)_2$ washed ether should be used to prevent peroxide oxidation of the aldehyde groups (12). Using $Fe(NH_4)_2(SO_4)_2$ washed ether throughout, we demonstrated again that aldehydic phenols were not present in the ether extracts of biodegraded wheat straw in which over 2 g of lignin is degraded, but these phenols are easily found and quantitated when 2 g of wheat straw is oxidized by the standard nitrobenzene method and the phenols are recovered as illustrated in Figure 1. Table III lists the compounds found in nitrobenzene oxidized wheat straw, as well as similar results for extracts of the biodegradation products. The absence of low-molecular-weight biodegradation products in the ethanol extract is confirmed by the observation that the gas chromatographic curve for the ethanol solubles (silylated) of fermented wheat straw is identical to the curve for similar derivatized extracts of unfermented wheat straw carried through the same extraction procedure.

To test for polysubstituted phenols in the water soluble biodegradation broth, the methylated ($CH_2N_2$) derivative of freeze-dried extract A (Figure 1) was easily produced and yielded interpretable mass spectra. Several phenolic standards were

Table I. Quantitation of Biodegradation Products

| | Material Balance by Weight | | |
|---|---|---|---|
| | Total Sample[a] Basis | Lignin[b] Basis | Total Sample Basis[c] All Soluble Nutrients Removed |
| Starting material | 6.4% $H_2O$, 2.56 g 15.6% lignin, 6.24 g | 100%, 6.24 g | 6.4% $H_2O$, 2.56 g 15.6% lignin, 6.24 g |
| Hexane extract | 0.4%, 0.16 g | -0- | 0.4%, 0.16 g |
| 4° Water extract, decanted | 5.5%, 2.2 g | 4.8%, 0.3 g | |
| 4° Water extract and water wash | | | 5.6%, 2.25 g |
| Fermented filtered $H_2O$ solubles | 4.6%, 1.92 g | 7.1%, 0.44 g | 2.6%, 1.02 g |
| 0.45 um filtered $H_2O$ solubles | | | 2.3%, 0.939 g |
| EtOH solubles after fermentation | 1.3%, 0.53 g | 6.4%, 0.3$\pm$0.1 g | |
| Fermented[e] extracted dry straw | 69.5%, 27.8 g | 65.2%, 4.07 g | 78.8%, 31.52 g |
| Lost or in gas phase | 12.5%, 5.0 g | 16.5%, 1.03 g | 6.3%, 2.5 g |
| Trapped gases | | | |

[a]Average of 5 determinations on 40 g wheat straw

[b]Lignin determinations by UV method (10)
[c]Average of 2 determinations.

[d]Decomposition per minute.

[e]Not corrected for fungal mass.

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| $^{14}C$ Balance DPM[d] X10$^{-6}$, Percent | | | |
|---|---|---|---|
| Sample 1 | Sample 2 | Sample 3 | Sample 4 |
| Butte HR | Butte HR | RR 68 | RR 68 |
| 38.03 | 25.04 | 43.88 | 35.68 |
| | | | 5.0, 14.0% |
| 5.47, 14.4% | 3.33, 13.3% | 5.27, 12.1% | 4.72, 13.2% |
| 0.13, 0.3% | | | 0.25, 0.7% |
| 25.0, 65.8% | 16.9, 67.3% | 24.8, 56.5 | 12.8, 35.8% |
| 6.2% | 12.3% | 17.6% | -6.4% |
| 5.04, 13.3% | 1.27, 5.1% | 5.90, 13.5% | 20.2, 56.7% |

TABLE II. Separation of Water-Soluble Biodegradation Products by Ether Extraction (Figure 1)

| | Percent of Water-Soluble Products in | |
|---|---|---|
| | Ether Extract[a] of Basic $H_2O$ | Ether Extract[b] of Acidic $H_2O$ |
| By weight | 0.41 | 0.96 |
| By $^{14}C$ label | 0.03 | 0.4 |

[a]As illustrated by the extract labeled B in Figure 1.

[b]As illustrated by the extract labeled C in Figure 1.

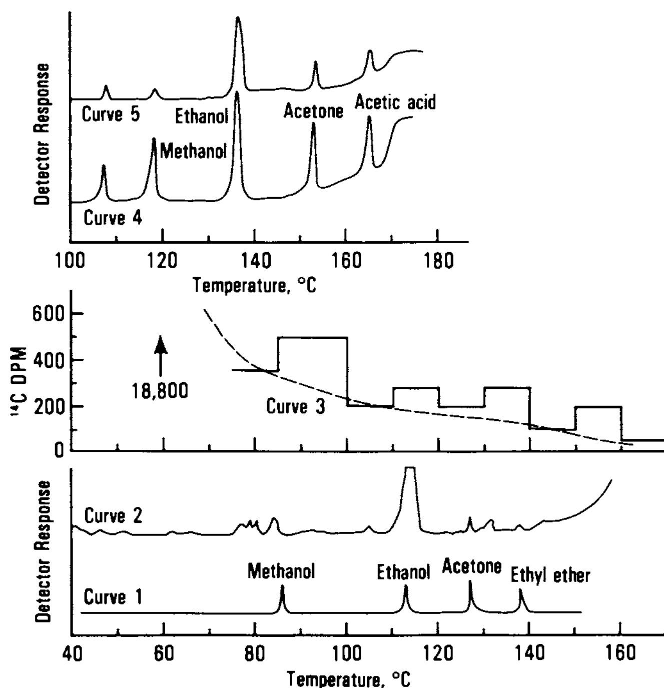


*Figure 2. Gas chromatographic curves for volatiles. Key: Curve 1, standard mix in water of less than 1% each of methanol, ethanol, diethyl ether, and acetone flushed with He, trapped and tested on Poropak Q as were the fermentation gases (FID); Curve 2, gas phase of eight-day fermentation trapped and tested as described in text (FID); Curve 3, collected and counted GC effluents (*see *text); Curve 4, standard mix of 0.1% each methanol, ethanol, acetone, and acetic acid; and Curve 5, water phase of fermentation.*

Table III. GC-MS Identified TMS Derivatives of Biodegraded and Oxidized Wheat Straw[a]

| Extracts of Biodegradable Straw | | Extract[c] of Nitrobenzene Oxidized Wheat Straw |
|---|---|---|
| EtOH Extract[b] | Extract[c] | |
| Palmitic acid·TMS<br>Linoleic acid·TMS<br>Pentose·TMS<br>A high molecular weight (422) hydrocarbon, not TMS derivatized which dominates the GC curve in size. Probably a triterpene. | No monomeric phenols<br>Tetramer and pentamer polydimethyl siloxane artifacts. The same 422 molecular weight compound as in column 1. | Phenol·TMS<br>Lactic acid·TMS<br>Glycolic acid·TMS<br>p-Hydroxybenzaldehyde·TMS<br>Vanillin·TMS<br>Syringealdehyde·TMS<br>2 isomers of Nitrobenzene |

[a]Kratos MS-30 equipped with a Perkin Elmer Sigma 3 gas chromatograph. An OV-1 surface-coated open tubular capillary column was held at 70° for 3 min, then programmed at 4°/min to 250° after sample injection.

[b]Isolated as illustrated in Figure 1.

[c]Isolated as illustrated by extract C in Figure 1.

methylated for comparison to the unknown. Since methylated extract A had not been separated into various components before methylation, the gas chromatograph curve shows many peaks (Figure 3, curve 1). The smaller peaks in the area of methylated polyfunctional phenols are therefore difficult to identify from their mass spectrum alone. A technique called mass chromatography was used wherein distribution of several major masses that appear in a standard spectrum is plotted underneath the gas chromatograph curve as in Figure 3. In this way, coincidence of several masses that are present in a standard mass spectrum under a single peak in the gas chromatograph curve identifies the location of that standard. This, in conjunction with retention time, confirms the presence of a compound in the unknown, whereas the absence of such coincidence at a given retention time confirms the absence of a compound to very low levels of detectability in the original sample. Using this technique, methylated vanillic acid, p-hydroxybenzoic acid, 2-methoxy succinnic acid and syringic acid were found to be present. The palmitic acid, linoleic acid and triterpenes found earlier were the dominant materials in the water solubles examined by this technique, as can be seen in Figure 3. The magnitude of these three materials prevents putting a larger sample on the GC-MS instrument to enhance the interpretation of small peaks. Larger samples possibly could burn out filaments in the mass detection system. For this reason, pre-extraction of the triterpenes and fatty acids as much as possible was attempted with hexane. The fatty acids and triterpenes constitute the interfering substances referred to in the introduction.

Since separation of these lower molecular weight compounds is not easily achieved by ether extraction, they are not quantitated by the separation scheme of Figure 1. Separation of higher molecular weight materials for characterization was also necessary. Both of these goals were achieved by membrane filtration, dialysis and GPC characterization.

Membrane filtration results in Table IV gave us considerable insight into the molecular weight distribution of residual biodegradation products. We concluded from these results that few of the residual biodegradation products are present as low-molecular-weight species. Compounds of less than 1,000 nominal molecular weight constitute 11% of the water-soluble biodegradation products, with monomeric aromatics only a portion of this. GPC curves of the various membrane-separated fractions confirm that the high-molecular-weight fractions contain the majority of ultraviolet absorbing species (Figures 4 and 5).

Figure 6 shows the $^{13}C$ NMR spectrum of high-molecular-weight biodegradation products from membrane filtration and dialysis and the NMR spectrum of a lignin-carbohydrate complex published by Himmelsbach and Barton (13). The spectrum of the biodegradation products shows predominantly carbohydrate polymers. The combination of weight and $^{14}C$-label distribution among sequential membrane filters, the UV absorbance of these species,

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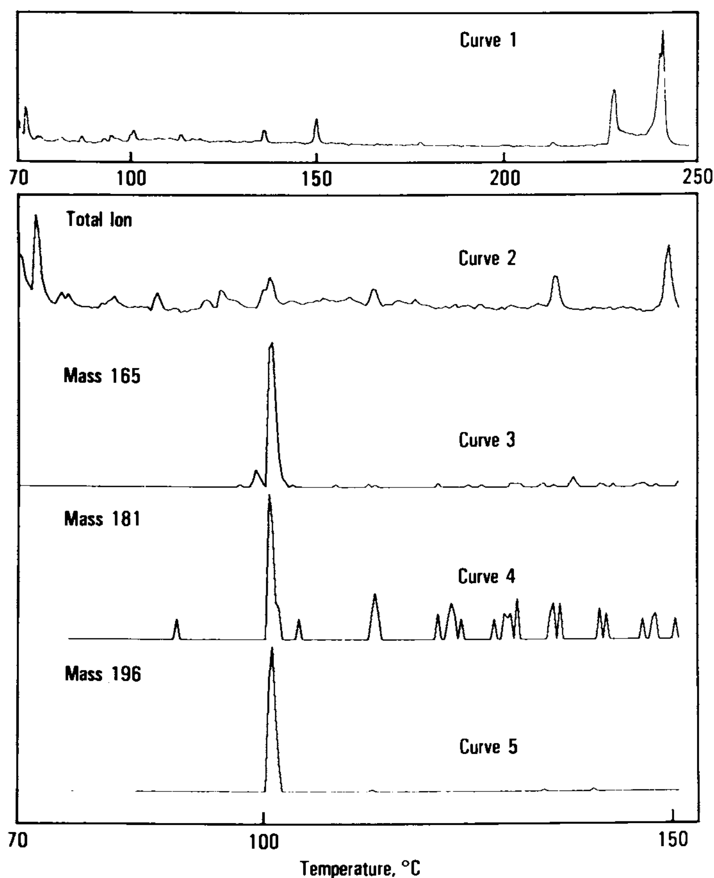


*Figure 3. GC-mass spectra curves. Key: Curve 1, total ionization vs. temperature curve for methylated water-soluble biodegradation products; Curve 2, a portion of Curve 1 for searching; Curve 3, plot of mass 165 ions received while Curve 2 was being collected; Curve 4, plot of mass 181 ions received while Curve 2 was being collected; and Curve 5, plot of mass 196 ions received while Curve 2 was being collected.*

Table IV. Membrane Filtration of Biodegradation Products

| Retained by[a] Membrane | Percent of $H_2O$ Solubles Weight for the 40 g Wheat Straw Fermentation | Percent of $H_2O$ Solubles for the $^{14}C$ Labeled Lignin Fermentation |
|---|---|---|
| X M 300 | 13.2 | 22.8 |
| PM 10 | 10.9 | 23.9 |
| UM 2 | 48.8 | 42.0 |
| Through UM 2 | 21 | 11.3 |
| Recovery | 93.9 | 95.5 |

[a]Nominal molecular weights retained by the XM300 membrane is >300,000, by the PM10 membrane is 10,000 to 300,000, and by the UM2 membrane is 1,000 to 10,000.

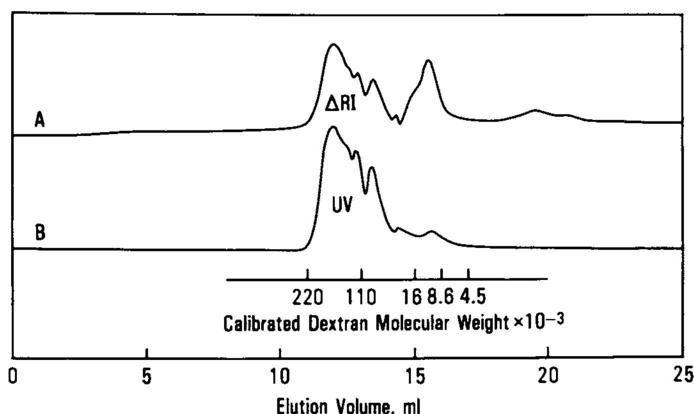


*Figure 4. Gel permeation chromatograms of the total water-soluble biodegradation product. Detection for Chromatogram A is by refractive index change, and Chromatogram B is by UV absorbance at 254 nm.*

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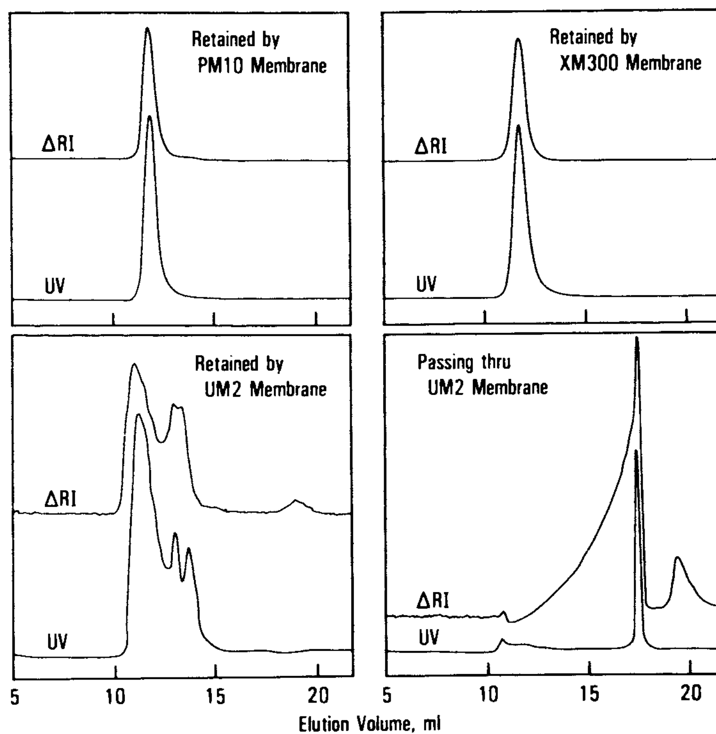


*Figure 5. Gel permeation chromatograms of the membrane filtration fractions using the same conditions as for the unseparated sample described in Figure 4.*

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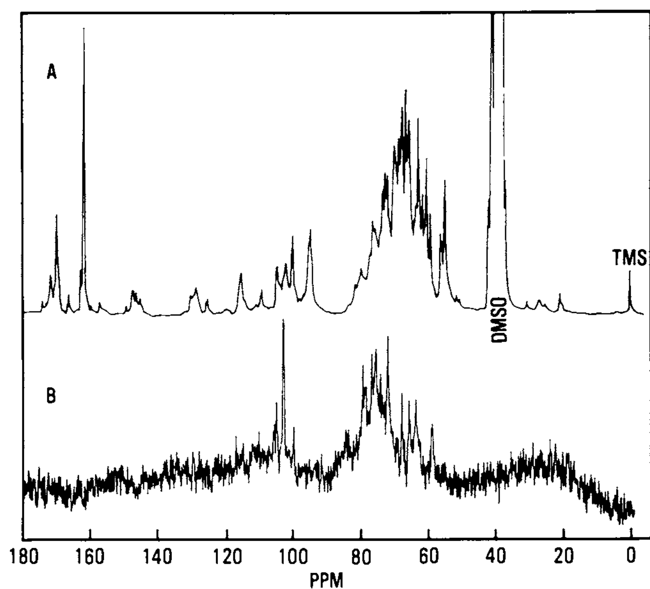


*Figure 6.* $^{13}$*C-NMR spectra of a lignin-carbohydrate complex A (*13*), and the high molecular weight water-soluble biodegradation products isolated by dialysis (B).*

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and the NMR spectrum in Figure 6 lead to the conclusion that the overwhelming majority of residual biodegradation products are lignin-carbohydrate complexes.

## Conclusions

Although some low-molecular-weight oxidized aromatic species can be found in biodegradation products of *Cyathus stercoreus* on wheat straw, the predominant residual biodegradation species are lignin-carbohydrate complexes. The other major product of degraded lignin is carbon dioxide. Lesser amounts of ethanol, methanol, acetone, acetic acid, 2-methoxy succinnic acid, vanillic acid, syringic acid and p-hydroxy benzoic acid were also found. These findings are consistent with current thinking on mode of attack of fungi on lignocellulosics as described earlier. If random oxidation cleaves high molecular weight water-soluble fractions from lignocellulosics, one would expect that they cannot be metabolized; however, as these are broken down to low-molecular-weight materials by continued oxygenation, these fractions are rapidly metabolized to $CO_2$. Thus, the residual fermentation broth would contain predominantly indigestible high-molecular-weight species and a few low-molecular-weight species and a significant amount of $CO_2$ would have been generated. We find such a composition.

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# 20

# Biphenyl Hydroxylase, Arylhydrocarbon Hydroxylase, and Epoxide Hydrolase

## Activities in Intestinal and Liver Microsomes of Rats Fed Selected Types of Dietary Fibers

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"Dietary fiber" has been shown to be protective against induction of colon cancer by some chemical carcinogens. One possible mechanism for this protective action may be altered xenobiotic metabolism. The affects of diets containing different types of purified fiber on intestinal and liver microsomal metabolism of biphenyl, benzo(a)pyrene and epoxides were assessed. Adult, male Wistar rats were individually fed chemically defined, nutrient density balanced rations containing either no dietary fiber, or 15% pectin, Metamucil, lignin or cellulose for 30 days;a similar group was fed commercial laboratory chow diet as reference. The microsomal hydroxylations of biphenyl and benzo(a)pyrene (BaP) were compared following incubation with appropriate preparations of liver and intestinal mucosa. The hydroxybiphenyl products were determined by fluorimetric HPLC, hydroxylated BaP was determined by fluorimetry, and epoxide hydrolase activity was determined using 3-(p-nitrophenoxy)-1,2-propene oxide as a substrate. Activities were higher with liver microsomes than the intestinal microsomal preparations for each assay. Fiber-fed rats showed significantly higher hepatic but not intestinal epoxide hydrolase levels than did control rats. Hepatic arylhydrocarbon hydroxylase levels were higher in synthetic diet-fed rats than lab chow-fed animals. Variations in intestinal biphenyl hydroxylase activities were not significant with respect to fiber type regardless of the specific metabolite measured. Hepatic biphenyl hydroxylase activities were significantly higher with synthetic diet-fed rats.

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Some current hypotheses about the etiologies of colon cancer and cholesteremic disease impute a special function to dietary fiber and to some of its components; that function is generally associated with disease prevention. Evidence for the protective function of dietary fiber has been obtained both from epidemiological studies (1-8) and from experiments with animals.

Animal experiments, in particular, have been useful in testing various hypotheses about the relative effectiveness of different kinds of dietary fiber and about possible mechanisms of fiber effects. Thus, studies of bulking action (9-10), of selective secondary bile acid binding (11-12), of increased intestinal transit times (13), of altered bacterial activities and secondary bile acid production (14-15), and selective binding of carcinogens (16-19) have been carried out in laboratory animals. Several related studies have been conducted in humans as well. Among the latter are investigations of the relationship between the bulk fiber content of diets and fecal bile acids or other steroids (4,20-21).

Because of the possible interplay of many factors and processes that might occur among the different chemicals constituting dietary fiber itself and carcinogens or promoters, we have been conducting a systematic study of such interactions using a model experimental design with the rat. Our model consists of feeding purified diets containing different purified fibers, in hydrated form, for a 30 day period to allow for thorough bacterial adaptation(22). At that time each rat was treated _per os_ with a single dose of a xenobiotic chemical selected to exemplify a specific aspect of xenobiotic metabolism. Toxicokinetic analyses were then utilized to quantify the critical parameters, such as absorption and elimination rate constants for the parent compounds, their bacterial metabolites, and metabolites undergoing enterohepatic circulation. By repetitive study with different xenobiotics within the fixed dietary design, a comprehensive understanding of the fiber:animal:carcinogen interactions is being developed (22-24).

In order to fully interpret the toxicokinetic experiments, additional basal data are required from this diet model; it is particulary necessary to establish parameters for the hepatic and intestinal xenobiotic metabolism in rats on the standard fiber diets. This paper presents our findings of the effects of different dietary fiber types on hepatic and intestinal metabolism of reference xenobiotics.

## Previous Work Relating to Selection of Substrates

**Characteristics of Intestinal vs Hepatic Xenobiotic Metabolism.** Intestinal mucosa has been shown both to metabolically activate and to deactivate xenobiotics. The mixed function oxidase (MFO)

of intestinal mucosa responds to inducers and inhibitors differently than hepatic MFO. Intestinal MFO also differs from hepatic MFO in its constituative levels, metabolic patterns, and substrate specificity (25-41).

Biphenyl Hydroxylation. Factors affecting 2-,3-, and 4-biphenyl hydroxylase enzymes are: age, sex, species, nutritional status, genetic background, xenobiotic preexposure, and tissue type. The 2- and 4-hydroxy biphenyl hydroxylases have been demonstrated to be different on the basis of selective induction, conducted both in vivo and in vitro, and on different species specificities(42-51). Toftgard et al.(51) reported that changes in different forms of cytochrome P-450 give rise to altered proportions of biphenyl metabolites. It is now clear that biphenyl 2-hydroxylase activity is conferred only by cytochrome P-448-type induction. Rat hepatic biphenyl 4-hydroxylase is maintained at higher constituative levels than 2-hydroxylase, and is conferred by both cytochromes P-450 and P-448 (52). Wiebkin et al.(53-54) summarized advantages for use of biphenyl as substrate in that its metabolic profile provides an indicator to the toxicity of substrate and products, it highlights further metabolism of primary metabolites, and indicates the relationships among different MFO inducers; for these reasons we chose to use biphenyl as an indicator substrate to assess overall MFO metabolism, as suggested by Billings and McMahon (55).

Intestinal microsomal metabolism can be monitored via biphenyl hydroxylation as well. Biphenyl hydroxylase assays are more sensitive than some other hydroxylase assays which has permitted measurements of the low constituative enzyme levels in intestinal mucosa and some other extrahepatic tissues (56-59).

Arylhydrocarbon Hydroxylase. Benzo(a)pyrene (BaP) is considered an ideal model compound for monitoring arylhydrocarbon hydroxylase (AAH) MFO levels in rats. Its metabolism has been widely investigated in both rat liver and extrahepatic tissues (40-41,61-70). Arylhydrocarbon hydroxylase has been compared to biphenyl hydroxylase in the intestines of rats (25,28). Intestinal AAH has been shown to be induced up to 30-fold with cytochrome P-448-type inducers, while being insensitive to P-450-type induction. Therefore, we have used intestinal AAH in conjunction with biphenyl hydroxylation, to evaluate the effects of dietary fibers on intestinal metabolism.

Epoxide Hydrolase. Epoxide hydrolase (EH) activity has been studied in numerous tissues with many different substrates (71-72). Hepatic microsomal EH has been shown to be induced by polycyclic aromatic hydrocarbons, but is insensitive to cytochrome P-450 type inducers (73). Epoxide hydrolase has been related to the activation of polycyclic aromatic hydrocarbons into reactive carcinogens (70). Epoxide hydrolase activity has been determined

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in the stomach as well as the intestine and other extrahepatic tissues (74-75). Indications of the effects of specific diet fibers on epoxide hydrolase activity could be directly applied to substantiate or modify fiber:carcinogenesis hypotheses.

## Methods and Materials

Dietary Fibers. The cellulose used in these experiments was SolkaFlok, KS-1016, a cellulose derived from sulfite processing of wood (birch, beech, and maple) having a particle size of 290 microns and containing 0.15-0.2% lignin and 8-9% hemicellulose (Brown Co.,Berlin, N.H.). The lignin used was Indulin AT, which is a Kraft lignin described as being 99% lignin and 1.0% dry ash, free of simple sugars, insoluble in water and soluble in alkali (Westvaco, North Charleston, S.C.). The lignin was Soxhlet extracted for 30 hours using methylene chloride which was evaporated from the lignin in a fume hood. The extracted, dried lignin was then suspended in deionized water and filtered through a Buchner funnel with Whatman 1 filter paper. This washing process was repeated until the filtrate was light orange in color. The hemicellulose used was Metamucil (Searle Laboratories, Chicago,Ill.), a pharmaceutical preparation of Ispaghula husk from *Plantago ovata*(Forsk) which has been characterized (76). The pectin used was an NF citrus pectin, finely ground to a powder with particle size of 200 microns (Hercules,Inc., Wilmington,DL).

Diet Preparations. The American Insitute of Nutrition (AIN) synthetic diet formulation was used along with gelatin to form hydrated gelled diets. The ingredient composition in grams/100 grams diet was: dextrose 64.6, casein 16.2, dl-methionine 0.2, AIN salt mixture 3.5, AIN vitamin mixture 1.5, gelatin 6.0, and lard 8.0 for the control diet; fiber diets contained 15% less of each ingredient plus 15g fiber. Dry ingredients were premixed and the hydrated gelled diets were prepared weekly by adding water (190ml/100g) and lard to the appropriate amount of premix, blending for 1 minute, letting the diets gel for 2 hours at 5°C, and reblending the semigelled diets to evenly distribute insoluble ingredients. The prepared diets were stored at 5°C in tubs with plastic film laid directly on the surface of the diets to minimize evaporative loss.

Animal Treatment. Six groups of 11, thirty-day old, male Wistar rats (100-120g), were randomly divided into individual cages. After five days acclimation the groups were fed: Purina Lab Chow *ad libitum*, control (fiber-free gelled diet) and the gelled diets which contained 15% pectin, Metamucil, lignin, and cellulose, respectively. Every second day each rat was weighed, unused food was weighed and fresh diet placed in feeders secured at the front of each cage. At the end of 28 days, two rats from each group were sacrificed each day for 5 consecutive days.

Enzyme Preparation. Each rat was weighed, killed by cervical dislocation, the liver removed and 2g homogenized in 10 ml 0.05M Tris., 0.15M KCL, pH 7.8 buffer. Intestinal microsomes were prepared according to Stohs et al.(28). The entire small intestine of each rat was removed, washed with buffer, measured for length, split longitudinally with scissors, laid mucosal side up on a glass plate and scraped with a glass slide. The mucosal cells were weighed and homogenized in 10ml buffer with 20% glycerol, 25mg trypsin inhibitor and 2 units heparin. Hepatic and intestinal homogenates were centrifuged at 600g for 2 minutes and then at 13000g for 20 minutes, with the microsomes sedimented at 105,000g for 60 minutes.

Assays. Protein was determined according to Lowry et al(77)). Biphenyl hydroxylase activity was determined with 2 mg microsomal protein, 400 micromoles NADP, 4 micromoles glucose-6-phosphate, 3 micromoles magnesium chloride, and 0.5 units glucose-6-phosphate dehydrogenase in 1 ml total volume. After a 2 minute preincubation, 1 mM biphenyl in 10 microliters of acetone was added to start the 15 minute incubation. The reactions were stopped with 0.5 ml 6N HCL, extracted with isooctane and chromatographed by HPLC on Micropac $NH_2$-C-10 (Varian) with isooctane:acetonitrile:isoamyl alcohol (100:4:4)(59). Detection of the 2-, 3-, and 4-hydroxybiphenyl products was by fluorescence detection with excitation at 275nm and emission at 38nm. Quantitation of 2-, 3-, and 4-hydroxybiphenyls was accomplished with peak height comparisons to 1.25, 2.50, and 3.75 micrograms of each standard; standards were incubated and extracted with each set of determinations. Epoxide hydrolase activity was determined by the method of Giuliano et al(75) using 3-(p-nitrophenoxy)-1,2-propene oxide as substrate with 0.5mg microsomal protein. The diol was separated using ODS reverse phase HPLC with 40% acetonitrile in water. Detection was accomplished using UV absorbance at 315nm. Arylhydrocarbon hydroxylase activity was determined by the assay procedure of Dehnen et al.(78) on an AmincoBowman spectrofluorometer after incubations with 0.5mg microsomal protein and NADPH generating system. One unit activity is defined as picomoles/mg/minute as based on the fluorescence of 20 ng 3-hydroxybenzo(a)pyrene.

## Results and Discussion

Results of body, liver and liver/body weight ratios after 30 days on specified diets are listed in Table I. Each of the treatment groups showed an initial weight loss upon introducing the purified diets. However, all but the lignin-fed animals quickly recovered to steady weight gains throughout the rest of the experiment. Lab Chow-fed rats were significantly heavier than the purified diet-fed rats. Body weights in pectin-and Metamucil-fed rats were significantly (0.05 level) higher than control-,

Table I. Body and Liver Weights and Liver to Body Weight Ratios of Fiber-Fed Rats.[a]

| Diet Type | Body Weight | | Liver Weight | | Liver Wt./Body Wt. | |
|---|---|---|---|---|---|---|
| | g | | g | | g | |
| Control (No Fiber) | 182 ± | 12 | 6.90 ± | 1.38 | 0.037 ± | 0.006 |
| Lab Chow | 300[b] | 28 | 11.14[b] | 1.63 | .037 | .004 |
| 15% Pectin | 220[c] | 18 | 7.73 | 1.19 | .034 | .003 |
| 15% Metamucil | 224[c] | 12 | 7.83 | 1.19 | .034 | .006 |
| 15% Lignin | 153 | 15 | 5.61 | 0.64 | .036 | .003 |
| 15% Cellulose | 172 | 10 | 6.10 | 0.50 | .035 | .002 |

[a] Values are means ± S.D. for groups of 11 rats.

[b] Significantly different from all other groups (P=.05).

[c] Significantly different from the other groups fed purified diets (P=.05).

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lignin- or cellulose-fed animals. Lignin-fed animals could be considered to be under weight. Lignin- and control-fed animals ate significantly less purified diet than other treatment groups. Liver weights in Lab Chow-fed rats were also significantly higher than in rats fed purified diets. The liver weights of pectin- and Metamucil-fed rats were slightly higher (not significantly) than control-, lignin- or cellulose-fed rats. Liver:to:body ratios were not significantly different in any of the treatment groups. The influence of specific dietary fibers on small intestine length, on the amount of mucosal material scraped from the intestine, and on the ratios of grams mucosa/cm or cm/g mucosa is indicated by the data in Table II, while Table III lists the intestinal microsomal protein (expressed as total or mg/cm small intestine). Lab Chow and diets containing pectin or Metamucil produced intestine lengths that were significantly longer than in the rats fed the control, lignin, or cellulose diets. These results support similar findings by deBethizy (23-24) and Rotenberg and Jakobsen (79). The stable gels formed by Metamucil and pectin fibers caused significant intestinal tract enlargement, even though fed in hydrated form. The rats fed Lab Chow and the pectin diet had the highest mucosal weights, which were significantly higher than in control-,lignin-,or cellulose-fed animals. Lignin-fed animals yielded the least amount of mucosal material, which coincides with their having the shortest small intestines and lowest body weights (Table IV). Total intestinal microsomal protein did not differ significantly (0.05 level) among treatment groups, although pectin diet and Lab Chow-fed rats had relatively higher total microsomal protein values. Both liver and intestinal mucosa microsomal protein values are listed in Table IV. There were no significant differences between treatment groups for either tissue.

Listed in Table V are the values of 2-,3-,4-biphenyl hydroxylase activities in liver microsomes of fiber-fed rats. Values are expressed as nanomoles (2-, 3-,or 4-hydroxybiphenyl)/mg/15 minute incubation; the least detectable amount was 0.05 moles/mg. The hepatic microsomal levels of 4-biphenyl hydroxylase were on the order of 40-fold higher than the 2-or 3-biphenyl hydroxylase activities. This difference is slightly greater than the higher constituative 4-biphenyl hydroxylase levels reported by others (46,50,80). The yield of 2-hydroxybiphenyl was significantly higher with hepatic microsomes from lignin-fed animals than with microsomes from any of the other treatment groups. The slightly higher biphenyl hydroxylase values for the purified diet-fed rats is notable. Lignin-fed rats also showed the highest 3-hydroxybiphenyl yields although this effect was not significant. The levels of hepatic microsomal 4-hydroxybiphenyl hydroxylase were significantly lower in Lab Chow-fed animals than lignin-fed animals. There were no significant differences among the purified diet treatment groups, however, lignin-fed rats again had slightly higher levels of activity.

Table II. Small Intestine Length, Mucosal Weight and their Ratio in Fiber-Fed Rats.[a]

| Diet Type | Mucosa | Small Intestine | Mucosal Wt./cm | cm/g Mucosa |
|---|---|---|---|---|
| | g | cm | g | cm |
| Control (No Fiber) | 2.65 ± 0.47 | 94 ± 4 | 0.028 ± 0.005 | 36.79 ± 6.92 |
| Lab Chow | 3.52[b] .42 | 111[c] 8 | .032 .004 | 31.84 2.88 |
| 15% Pectin | 3.15[b] .39 | 108[c] 6 | .029 .004 | 34.70 4.95 |
| 15% Metamucil | 2.78 .51 | 108[c] 6 | .025 .004 | 39.86 6.39 |
| 15% Lignin | 1.98 .35 | 93 6 | .021 .003 | 48.01 6.74 |
| 15% Cellulose | 2.30 .35 | 96 4 | .026 .004 | 41.06 6.48 |

[a] Means ± S.D. for groups of 11 rats.

[b] Mucosal weight significantly greater (P=.05).

[c] Intestine significantly longer (P=.05).

Table III. Microsomal Protein in Small Intestine Mucosa of Fiber-Fed Rats.[a]

| Type | Total mg | | mg/cm Length | |
|---|---|---|---|---|
| Control (No Fiber) | 13.77 ± | 5.80 | 0.14 ± | 0.06 |
| Lab Chow | 14.81 | 7.88 | .13 | .07 |
| 15% Pectin | 15.23 | 5.45 | .14 | .05 |
| 15% Metamucil | 10.50 | 5.20 | .19 | .05 |
| 15% Lignin | 10.09 | 6.74 | .11 | .08 |
| 15% Cellulose | 11.27 | 6.42 | .12 | .07 |

[a] Mean microsomal protein ± S.D. for groups of 11 rats; differences were not significant (P=.05).

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Table IV. Microsomal Protein in Liver and Intestinal Preparations from Fiber-Fed Rats.[a]

| Diet Type | mg Protein/g Liver | mg Protein/g Mucosa |
|---|---|---|
| Control (No Fiber) | 11.8 ± 6.0 | 5.59 ± 3.48 |
| Lab Chow | 14.5 4.6 | 4.26 2.46 |
| 15% Pectin | 12.4 5.4 | 4.97 1.87 |
| 15% Metamucil | 9.4 3.7 | 3.71 1.94 |
| 15% Lignin | 12.1 7.4 | 5.11 3.83 |
| 15% Cellulose | 15.1 4.3 | 4.91 2.30 |

[a] Values are mean ± S.D. for groups of 11 rats. Differences among treatments were not significant (P=.05).

Table V. Biphenyl Hydroxylase Activities in Liver Microsomes of Fiber-Fed Rats.[a]

| Diet Type | Nanomoles/mg/15 min 2-OH | | 3-OH | | 4-OH | |
|---|---|---|---|---|---|---|
| Control (No Fiber) | 0.43 ± | 0.10 | 0.40 ± | 0.13 | 15.98 ± | 5.75 |
| Lab Chow | .30[b] | .06 | .36[b] | .07 | 9.77[b] | 1.94 |
| 15% Pectin | .44 | .15 | .32 | .09 | 12.46 | 3.04 |
| 15% Metamucil | .41 | .08 | .29 | .07 | 13.82 | 4.00 |
| 15% Lignin | .81[c] | .35 | .62 | .18 | 20.03 | 5.65 |
| 15% Cellulose | .30 | .06 | .28 | .07 | 11.43 | 2.57 |

[a] Values are mean hydroxylase activity ± SE (N=10) in nanomoles/mg protein/15 min.; min. detectable amount was 0.05 nmole.

[b] Difference from lignin-treated animals significant (P=.05).

[c] Difference from control and all other groups significant (P=.05).

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Intestinal microsomal biphenyl hydroxylation for fiber-fed rats is listed in Table VI. Due to the variability of the data there were no significant (0.05 level) differences among the groups of fiber-fed rats. Lignin feeding led to the highest intestinal 2-biphenyl hydroxylase activities, while all the rats fed purified diets had higher levels than the Lab Chow group. Among the groups fed purified diets, the pectin-fed rats had the lowest 2-biphenylhydroxylase levels. Pectin feeding also gave the highest intestinal 3-biphenylhydroxylase although that difference only approached significance at the 0.05 level. Lab Chow-fed rats had the lowest 3-biphenyl hydroxylase level in intestinal microsomes. Intestinal microsomes from fiber-fed rats did not differ significantly (0.05 level) in 4-biphenyl hydroxylase activity. Cellulose-fed animals had the lowest level, with the controls and lignin-fed rats showing the highest intestinal microsomal 4-biphenylhydroxylase values. Pectin- and Metamucil-fed rats had relatively low levels of 4-biphenyl hydroxylase activities.

The results of a preliminary intestinal microsomal biphenyl hydroxylase trial are listed in Table VII. These rats were on the same hydrated purified diets containing 15% fiber for 32 days before the microsomal enzyme determinations. One difference between these animals and those in the primary experiment reported herein is that they were started on the diets at 45 days of age and weighed about 200g at the start. The biphenyl hydroxylation incubation was done with 5 mg intestinal microsomal protein as compared to 2 mg protein used in the subsequent incubations. The biphenyl hydroxylation values were not significantly different (0.05) in any of the treatment groups although Lab Chow-fed rats had slightly higher 4-hydroxy biphenylhydroxylase values.

Benzo(a)pyrene hydroxylase activities in hepatic and intestinal microsomes of fiber-fed rats are presented in Table VIII. There were no significant differences (0.05 level) among any of the purified fiber treatment groups in either liver or intestinal microsomes. Lab Chow-fed rats had the lowest liver microsomal benzo(a)pyrene hydroxylase (AHH) level. The indication of enhanced intestinal P-448-dependent hydroxylation of biphenyl in the pectin-fed rats (seen as elevated 3-hydroxybiphenyl formation in microsomes, Table VI) was not supported by these preliminary data.

Microsomal epoxide hydrolase activities are listed in Table IX. There was significantly higher (0.05 level) hepatic microsomal epoxide hydrolase activity in pectin-,Metamucil-, and cellulose-treated rats, as compared to control-, lignin- and Lab Chow-fed animals. There was no significant difference in intestinal microsomal epoxide hydrolase activities among any of the treatment groups.

Studies directly probing the interaction of dietary fibers on foreign compound metabolism are limited. Brown,*et al.*(81) reported that rats fed basal chow diets had no liver metabolism differences compared to rats on diets supplemented with pectin or

Table VI. Biphenyl Hydroxylase Activity in Intestinal Microsomes of Fiber-Fed Rats.[a]

| Diet Type | Nanomoles/mg/15 min | | |
|---|---|---|---|
| | 2-OH | 3-OH | 4-OH |
| Control (No Fiber) | 0.38 ± 0.16 | 0.79 ± 0.35 | 1.13 ± 0.74 |
| Lab Chow | .30 .09 | .46 .14 | .79 .24 |
| 15% Pectin | .19 .05 | 2.08 .74 | .35 .09 |
| 15% Metamucil | .50 .01 | .88 .13 | .92 .45 |
| 15% Lignin | .68 .32 | 1.14 .26 | 1.32 1.06 |
| 15% Cellulose | .47 .07 | .46 .13 | .22 .08 |

[a] Values are mean hydroxylase activity ± SE for groups of 7 rats; differences among treatments were not significant (P=.05).

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Table VII. Small Intestine Parameters and Biphenyl Hydroxylase Activities in Intestinal Microsomes of Fiber-Fed Rats.[a]

| Fiber Type | Intestine x) Length (cm) | Mucosal x) Weight (g) | Microsomal x) Protein (mg/g) | Biphenyl Hydroxylase[b] 2-OH | 3-OH | 4-OH |
|---|---|---|---|---|---|---|
| Control (No Fiber) | 100 ± 3 | 2.30 ± 0.45 | 7.28 ± 0.90 | 0.95 ± 0.22 | 0.98 ± 0.09 | 0.77 ± 0.11 |
| Lab Chow | 108 6 | 2.51 0.70 | 4.41 1.40 | .41 .12 | .72 .09 | 1.19 .56 |
| 15% Pectin | 115 6 | 3.78 0.59 | 3.30[c] 1.20 | .69 .08 | .58 .14 | .23 .05 |
| 15% Metamucil | 119 8 | 2.55 0.27 | 6.39 0.64 | .83 .15 | .85 .11 | .33 .07 |
| 15% Lignin | 101 7 | 2.56 0.25 | 3.22 0.50 | .97 .42 | .44 .19 | .45 .07 |
| 15% Cellulose | 103 5 | 2.86 0.62 | 5.73 1.24 | .97 .21 | .73 .12 | .73 .09 |

[a] Values are means ± S.D. of 3 rats, except hydroxylase values where n=2.

[b] Nanomoles/mg protein in mucosal microsomes/15 min.

[c] Significant difference from control, P $\geq$.05.

Table VIII. Benzo(a)pyrene Hydroxylase Activities in Hepatic and Intestinal Mucosa Microsomes of Fiber-Fed Rats.[a]

| Diet Type | Picomoles/mg/min Liver | Picomoles/mg/min Small Intestine |
|---|---|---|
| Control (No Fiber) | 216 ± 87.9 | 14.5 ± 1.6 |
| Lab Chow | 92[b] 7.1 | 19.9 6.8 |
| 15% Pectin | 193 81.1 | 17.9 4.7 |
| 15% Metamucil | 263 98.1 | 18.7 5.3 |
| 15% Lignin | 255 86.4 | 19.3 3.4 |
| 15% Cellulose | 245 98.1 | 19.6 4.8 |

[a] Values are means ± SE for groups of 7. Quantitation was based on the fluorescence of 20ng 3-hydroxy-benzo(a)pyrene and scaled to mg microsomal protein.

[b] Significant difference from controls, $P \geq .05$.

Table IX. Epoxide Hydrolase Activities in Liver and Intestinal Mucosa Microsomes of Fiber-Fed Rats.[a]

| Diet Type | Nanomoles/mg/5 min Liver | Small Intestine |
|---|---|---|
| Control (No Fiber) | 38.0 ± 8.13 | 14.9 ± 2.94 |
| Lab Chow | 45.0 7.24 | 13.1 3.28 |
| 15% Pectin | 64.3[b] 11.81 | 12.6 3.05 |
| 15% Metamucil | 62.0[b] 13.32 | 16.3 3.24 |
| 15% Lignin | 44.9 4.94 | 14.3 4.33 |
| 15% Cellulose | 64.4[b] 22.1 | 18.6 7.01 |

[a] Values are means ± SE for groups of seven scaled to mg microsomal protein for 3-(p-nitrophenoxy)-1,2-propene oxide as substrate.

[b] Significantly different from controls, P $\geq$.05.

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wheat bran as judged by antipyrine and acetaminophen metabolism. Chadwick *et al.*(82), however, demonstrated significant enhancement of lindane metabolism in pectin-fed rats. Proia *et al.*(83) recently reported that pectin feeding lowered hepatic cytochrome P-450 levels while at the same time increasing intestinal benzo(a)pyrene hydroxylase levels. It remains to be evaluated to what extent the differences observed in drug or other foreign compound *in vivo* metabolism in rats fed pectin or other purified fibers can be explained by differences in constituative levels of the relevent enzymes in liver and intestinal mucosa. Our data suggest that MFO hydroxylating activities in both tissues are not particularly sensitive to effects of feeding purified fibers at high levels (the diet model used in our work represents extremes in fiber intake). The significant effect on hepatic epoxide hydrolase seen with pectin-, Metamucil-, and cellulose-fed rats (Table IX) may point toward a more important response in relation to chemical carcinogenesis. Studies are in progress in our laboratory to compare GSH-transferase activities and the tissue glutathione status in specific fiber-fed rats. These studies may enhance our appreciation of the contributions of the non-oxidative enzymes in foreign compound metabolism to the relationships between nutritional state and chemical carcinogenesis.

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