The purpose of the study was to set up an *in vitro* model of the colon which would permit the analysis of cellulose fermentation by human colonic microflora. Studies of the degradation of polysaccharides by colonic bacteria may help to explain the observed physiological consequences of consuming dietary fiber common in foods. This study resulted in the use of a simple anaerobic batch fermentation system. It is assumed that the bacteria in fresh feces are representative of colonic bacteria. This batch culture system consists of the culture medium, the food fiber and the fecal inoculum. The fecal inoculum is prepared from freshly voided feces from a single individual. The food fiber is prepared from the vegetable/fruit starting material by repeated extraction with 90% ethanol, resulting in an alcohol insoluble residue (AIR). Extents of cellulose fermentation were measured after 4, 8, 12 and 24 hour fermentation periods at 37°C. The cellulose content of the samples before and after fermentation was determined by measuring the glucose yield (glucose oxidase assay) from an acid hydrolysate of the residue remaining after repeated acid detergent extractions. The extent of cellulose fermentation was then estimated by difference. The susceptibility to intestinal fermentation of the cellulose component of acorn squash and red beets was investigated using this model system. The cellulose
content of squash and beet AIR was 26.71\%±0.95\% and 23.22\%±0.89\%, respectively. The extent of cellulose of fermentation of squash cellulose after 4, 8, 12 and 24 hrs incubation was 6.04\%±0.69\%, 10.58\%±2.10\%, 17.11\%±6.37\% and 96.18\%±1.36\%, respectively. The extent of fermentation of beet cellulose after 4, 8, 12 and 24 hrs incubation was 17.52\%±1.83\%, 23.52\%±1.44\%, 30.53\%±4.12\% and 96.06\%±0.39\%, respectively. The results indicate that the cellulose component of both vegetables is susceptible to considerable degradation within the human intestinal tract.
In Vitro Fermentation of Dietary Cellulose
by Human Fecal Microorganisms
by
Hung-Pi Chang

A THESIS
submitted to
Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Completed April 10, 1991
Commencement June 1991
APPROVED:

Professor of Food Science and Technology in charge of major

Head of Department of Food Science and Technology

Dean of Graduate School

Date thesis is presented April 10, 1991

Typed by Hung-Pi Chang
ACKNOWLEDGEMENTS

I wish to express my sincere gratitude to Dr. Mike H. Penner for his guidance and encouragement during my study and research.

I thank Dr. Susan Vendeland for skilled technical assistance.

Special thanks go to my graduate committee: Dr. Anna Harding, Dr. Lorraine Miller and Dr. Ronald Wrolstad.

Special thanks also go to Dr. Juinn-Chin Hsu and Xiaolin Huang for their helpfulness in the study.

Finally, I wish to express my sincere gratitude to my parents, family and my boy friend Dr. Sung-Ben Huang for their concern and support during my study.
# TABLE OF CONTENTS

## INTRODUCTION

1

## LITERATURE REVIEW

5

- DIETARY FIBER AND HEALTH
- HUMAN CELLULOSE DIGESTION AND HEALTH
- *IN VITRO* METHODS FOR MEASURING CELLULOSE DIGESTIBILITY IN HUMANS

12

5

18

## MATERIALS AND METHODS

26

- SUBSTRATE PREPARATION
  - Alcohol Insoluble Residue (AIR)
  - H$_2$O/AIR
  - Cummings AIR

26

26

28

29

- Updegraff Method
- Modified Updegraff Method
- Acid-Detergent Fiber/Glucose-Oxidase Method

29

30

35

## IN VITRO FERMENTATION METHOD

38

- Selvendran Method
- Van Soest Method

38

45

## SQUASH, BEETS AND REFINED CELLULOSE FERMENTATION

48

- AIR Preparation for squash and beets
- Van Soest Method, *In Vitro* Fermentation
- ADF/Glucose-Oxidase Method for Determination of Cellulose Content

48

50

52

## RESULTS AND DISCUSSION

54

## METHODS OF CELLULOSE DETERMINATION

54

- *IN VITRO* FERMENTATION SUBSTRATES

59
IN VITRO FERMENTATION METHODS 60
FERMENTATION OF SQUASH, BEETS AND AVICEL CELLULOSE 67
RECOMMENDATION FOR FUTURE RESEARCH 71
BIBLIOGRAPHY 72
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Structure of cellulose.</td>
<td>13</td>
</tr>
<tr>
<td>2.</td>
<td>Preparation of cabbage, carrot and apple AIR (Alcohol Insoluble Residue) and H2O/AIR.</td>
<td>27</td>
</tr>
<tr>
<td>3.</td>
<td>Cummings method for preparation of AIR (Alcohol Insoluble Residue).</td>
<td>28</td>
</tr>
<tr>
<td>4.</td>
<td>Updegraff and modified Updegraff method for cellulose determination.</td>
<td>31</td>
</tr>
<tr>
<td>5.</td>
<td>Glucose standard curve of modified Updegraff method.</td>
<td>32</td>
</tr>
<tr>
<td>6.</td>
<td>Avicel standard curve of modified Updegraff method.</td>
<td>33</td>
</tr>
<tr>
<td>7.</td>
<td>Acid Detergent Fiber/Glucose oxidase method for cellulose determination.</td>
<td>36</td>
</tr>
<tr>
<td>8.</td>
<td>Glucose standard curve of ADF/glucose oxidase method.</td>
<td>37</td>
</tr>
<tr>
<td>9.</td>
<td>Medium preparation for Selvendran in vitro fermentation system.</td>
<td>39</td>
</tr>
<tr>
<td>10.</td>
<td>Anaerobic dilution solution preparation for the collection and dilution of fecal inoculum: Bryant Method.</td>
<td>42</td>
</tr>
<tr>
<td>11.</td>
<td>Anaerobic dilution solution preparation for the collection and dilution of fecal inoculum: VIP Anaerobe Method.</td>
<td>43</td>
</tr>
<tr>
<td>12.</td>
<td>Inoculation and fermentation, Selvendran in vitro fermentation system.</td>
<td>44</td>
</tr>
<tr>
<td>13.</td>
<td>Medium and anaerobic dilution solution preparation, Van Soest in vitro fermentation system.</td>
<td>46</td>
</tr>
</tbody>
</table>
15. AIR (Alcohol Insoluble Residue) Preparation, of Cummings method. 49

16. Substrate hydration, Van Soest in vitro fermentation system. 50

17. Van Soest and Acid Detergent Fiber/Glucose oxidase oxidase method for squash, beets and Avicel cellulose digestibility. 53

18. Time course of digestion and recovery of the cellulose component of squash. 69

19. Time course of digestion and recovery of the cellulose component of beet roots. 69

20. Time course of digestion and recovery of the cellulose component of microcrystalline cellulose. 70

21. Time course of digestion and recovery of the cellulose component of squash, beet roots and microcrystalline cellulose. 70
LIST OF TABLES

Table                                                                 page
1. Cellulose content of cabbage, apple, carrot, and microcrystalline   56
   cellulose fiber preparations determined by the modified
   Updegraff method.

2. Cellulose content of squash and beet fiber preparations determined  57
   by the ADF/glucose oxidase method.

3. Comparison of methods of cellulose analysis.                        59

4. Relative digestibility of carrot and Avicel as measured by the     61
   Selvandran in vitro fermentation system.

5. pH changes during squash fermentation in Van Soest fermentation    62
   system.

6. Digestibility of AIR cellulose from squash.                         64

7. Digestibility of AIR cellulose from beet roots.                    65

8. Digestibility of microcrystalline cellulose (Avicel).               66

9. Comparison of Selvendran and Van Soest in vitro fermentation methods. 67
IN VITRO FERMENTATION OF DIETARY CELLULOSE BY HUMAN FECAL MICROORGANISMS

INTRODUCTION

The complex role of dietary fiber and its relationship to the promotion of either good health or disease has been the subject of considerable speculation. An increased amount scientific and clinical evidence has been presented which demonstrates physiological effects that may be important to understanding the relationship between human diet and disease or, in a more positive sense, between diet and good health. It has been generally assumed that fiber is an important component of the human diet. However, no mechanism has been accounted the beneficial effects of fiber experimentally demonstrated.

One reason for the inability to connect diet fiber to a specific mechanism is that fiber is a complex mixture of macromolecules. However, it has been determined that metabolism within the intestinal tract is a factor which is likely to influence physiological response to dietary fiber. Fiber fermentation results in a range of end-products, including short-chain fatty acids, gases and secondary metabolites. Volatile fatty acid production increases when carbohydrate input is increased, and it decreases when cultures are deprived of carbohydrates(1). Thus, it is possible to maintain actively fermenting viable cultures in vitro for prolonged periods, a technique which may prove useful to the investigation of the colonic metabolic function(2).

It has been suggested that diets high in fiber may be associated with a low incidence of colon cancer, diabetes and heart disease. Furthermore, considerable interest
has been expressed in the postulate that a deficiency of dietary fiber will influence the etiology of a number of diseases that are common in Western societies. The implicated diseases can be divided into two main groups: (1) the metabolic disorders, including colon cancer, cholelithiasis and diabetes mellitus, and (2) the pressure diseases, such as large bowel diverticular disease and spastic bowel syndrome. The role of dietary fiber in offering protection against large bowel cancer cannot be related to any single effect on the colonic metabolism, and may rather be due to a combination of the dilution of colonic contents, shortened transit time, altered bacterial metabolism, or other properties such as the adsorption of potentially harmful materials. Nonetheless, the consumption of high-fiber diets has been publicly encouraged for its important role in the regulation of colonic function, even though the significance of these fermentation products in relation to chronic diseases remain unknown. To better understand the role of fiber in human health, it is necessary to understand the factors influencing the microbial metabolism of fibers within the intestinal tract.

The cell walls of plants are not digestible by human digestive enzymes, and in most foods they provide the bulk of the food residue which enters the colon. To utilize cell walls as a carbon source, the saccharolytic bacteria which inhabit the colon must have the capability to attack a wide variety of glucosidic linkages, but the extent of the colonic degradation of many polysaccharides is not known. Some polysaccharides may not be broken down to any extent, while some may be partially degraded, and others may be completely fermented into volatile fatty acids and gases. Cellulose is a major component of dietary fiber in all plant species, and is likely the predominant non-starch polysaccharide found in most human diets. The metabolism of this important fiber component is largely unknown, but from dietary balance studies it is clear that in most diets cellulose is to some extent digested. However, there is considerable range in the digestibility of cellulose in different foods. This range has been rationalized by the
assumption of differences in cellulose physical properties, and by the acknowledgment that cellulose digestibility may be influenced by the presence of other fiber components, such as lignin. These explanations, however reasonable they appear to be, have not been experimentally verified. The emphasis of the current study is placed on cellulose since of all the complex carbohydrates, it is the most difficult to degrade. Thus, the objective of this study is to determine whether or not cellulose can be broken down by intestinal microorganisms using in vitro methods.

In vitro fermentation systems offer several benefits which include reduced costs, relative freedom from animal experimentation, the ability to simulate reproducible digestion conditions for better comparative studies and, more importantly, the fact that the duration and conditions of fermentation can be controlled. To understand the degradation of dietary fiber in the human colon, the principal object of this study is to develop a feasible method for the measurement of intestinal cellulose digestion. To this end, in vitro fermentation utilizing a human fecal inoculum provides an appropriate technique for the study of the digestibility of fiber by human intestinal microorganisms. Human fecal inoculum is assumed to be representative of the colonic microflora. However, the results obtained from this type of study are best interpreted in relative terms. One aspect of this technique that has received relatively little attention is the question of how to best obtain an appropriate dietary fiber preparation.

Previous studies of the cellulose metabolism within the intestinal tract have indicated that commonly encountered methods used for the preparation of plant cell wall material (i.e., dietary fiber) may influence the digestibility of cellulose within the preparation. Dietary fiber preparations are often the insoluble alcohol residues of target foods, a procedure for preparing dietary fiber which is known to coprecipitate intracellular components with the cell wall material. This coprecipitation is likely to
result in the formation of complexes with physical properties distinct from the original fiber.

Thus, the objective of the current study was to develop a procedure using \textit{in vitro} carbohydrate fermentation to determine the digestibility of food fiber cellulose. This study is designed to evaluate the role of alcohol insoluble residue (AIR) fiber on the large intestinal microflora in the human. The basic system components include the sample to be digested, a culture medium, reducing solution and inoculum.
LITERATURE REVIEW

1. Dietary Fiber and Health

1.1 Definition of Dietary Fiber

Dietary fiber is an important food component consisting of plant materials which are resistant to hydrolysis by the endogenous enzymes of the mammalian digestive tract. The most abundant components of dietary fiber are found in or are associated with the cell walls of plants(3,4,5). Most of the polysaccharides are not degraded appreciably or absorbed as they pass through the stomach and small intestine, thus reaching the colon relatively intact. In contrast, simple sugars and disaccharides are not likely to be available as substrates in the colon. Since they are efficiently absorbed in the small intestine and do not reach the colon(6,7).

Samples high in starch generally contain the greatest amount of dietary fiber and the least amount of water on a fresh plant basis(8). The simplest and most precise definition of the major fiber fraction is non-starch polysaccharide (NSP), which for purposes of analysis can be divided into cellulosic and non-cellulosic polysaccharides (NCP)(4,9). The latter includes such polysaccharides as hemicellulose, pectin, guar and the plant gums. As methods are developed it may be possible to classify NCP into water-soluble and water-insoluble components as well. Water-soluble NCP may be among the most digestible components of dietary fiber. Since water-soluble polysaccharides including pectin and ispaghula, are almost totally degraded in the gut(10).
1.2 Recommended Dietary Fiber Intake Values

Plant cell walls are the main source of dietary fiber (DF), and most of the human DF intake is derived from the cell walls in such foods as fruits, vegetables, and cereal products(4). The average DF intake in the United Kingdom is about 20 g/person/day, about one-third of which is derived from cereal sources. There is no official recommendation on a desirable level of intake, but about 30 g/person/day is frequently recommended. The British DF intake is small in comparison to a rural African diet, which can contain as much as 100-170 g DF/day(11). The reason why certain groups of rural Africans rarely suffer noninfectious colonic diseases has been attributed to a natural African diet which is high in fiber content(12).

For carbohydrate intake, recent estimates are that at least 60 g of carbohydrates must enter the colon each day to provide sufficient nutrients to support the daily bacteria yield. When this carbohydrate amount is not metabolized, not only is it a valuable source of energy lost, but the osmotic activity of the unabsorbed sugars serve to retain water in the colonic lumen, an effect which can result in severe osmotic diarrhea(2).

1.3 Effects of Dietary Fiber on Health

The consumption of high-fiber diets is presently being encouraged(13,14) because of the reported beneficial effects of DF on health, particularly with respect to heart disease(15) and cancer(16-19). These beneficial effects have been attributed to the stimulatory effect of DF on colonic production and the consequent absorption of short-chain fatty acids(SCFA)(20), which have been implicated in irritable bowel syndrome(21) and colorectal carcinogenesis(22), as well as for their effect upon glucose tolerance(23), cholesterol metabolism(24) and energy metabolism(25,26). Despite the acknowledged physiological importance of SCFA, very little is known about the effects
of purified DF supplements on the quantities and types of SCFA produced in the colon(20).

The colon serves to salvage carbohydrates that have not been absorbed in the small intestine. These carbohydrates are converted by anaerobic colonic bacteria to volatile fatty acids (VFA), which are then rapidly absorbed(2). VFA stimulate the absorption of salt and water and is therefore unlikely to cause diarrhea(27). In addition, the VFA have an antibacterial effect, preventing the establishment of pathogenic bacteria, such as the *salmonella* species, and affecting colonic blood flow(27). The human large intestine contains a luxuriant mixed culture of bacteria, 99% of which are anaerobes(28). Most of these organisms are saccharolytic, deriving their energy primarily from carbohydrates and their derivatives(29). The principal functions of the large intestine are to conserve water and electrolytes within the gut during digestion, to provide a controllable route for the excretion of metabolic and toxic substance waste products, and to safely contain the microorganisms that in the fermentation process conclude the digestive procedure(27).

The elucidation of the role of DF in large-bowel diseases requires an understanding of the substrate composition, the rate and extent of fermentability, water-holding capacity, and of the mass of microbes proliferating in the gut(30). Large bowel cancer is thought to be due to the presence of an excess of bile acids in the colon that either directly affect the mucosa or, more likely, promote the effect of carcinogenic substances present in the bowel lumen(31). Dietary fiber may protect the colon from bile acids and carcinogens by absorbing them and promoting rapid elimination(9). Another effect is that DF increases fecal weight, SCFA production and alters mouthtoanus transit time, lumen pH, mucosal morphology and colonic functions(32,33). Studies of DF and their effect upon bowel functions have indicated various changes in stool output and composition, not all of which are solely attributable to water-binding properties.
measurable in vitro(34). It is commonly believed that a major component of human feces is undigested plant material or fiber, and when stool output is increased by the addition of fiber to the diet, the increase is due to water held in the colon by this material(35).

The ingestion of plant cell wall polysaccharides increases fecal bulk because of the increase in solid mass, as well as the increased bacterial mass and water retained by these components(36). This increase in solid matter could be due to extra unavailable fiber or extra available nutrients, such as proteins, fats or minerals(37). The bacterial mass is the major component in all regions of the large intestine and accounts for more than 61% of the dry weight and more than 81% of the wet weight of intestinal contents(38). Bacteria are normally about 80% water(39) and, since they comprise the major fraction of fecal solids, are an important water-holding component in the gut(40). If, as has been suggested(28), colonic disease is more common in people who have small stool output and slow transit, the control of colonic microflora will be important in the determination of disease susceptibility in different individuals. The proposed mechanisms of increasing fecal weight include: (1.) increasing the water content of digesta, with resultant reduced intracolonic pressure and increased ease of defecation(12), (2.) increasing digesta mass, with increased rate of passage and reduced contact with toxic substances, and (3.) the fermentation of increased fiber-producing microbial mass, which can act as a sink for ammonia and other toxic substances(41,42).

Thus, it has been suggested that a lack of DF can lead to the development of a variety of important diseases in humans. These diseases are largely chronic in nature and are probably multifactorial in origin(4). The polysaccharides pectin and gum have little effect on fecal bulk. Therefore, they may owe their action to events in the small intestine and appear to be extensively degraded in the large intestine(43).
1.4 Dietary Fiber and Fermentation

Carbohydrate breakdown by the anaerobic flora of the gut is an important component of digestive physiology and metabolism in ruminants and is known as fermentation(44). Fermentation is an important but scarcely recognized part of human digestive physiology. It has a direct bearing on salt and water absorption from the colon, on bowel habits, on the excretion of toxic substances, and on nitrogen and sterol metabolisms. It may also influence the intermediary metabolisms of the colonic epithelium, liver, and peripheral tissues(27).

There are three important fermentation products: VFA (acetate, propionate and butyrate), gas (carbon dioxide, hydrogen and methane) and energy. The quantification of fermentation in the human colon is difficult, but minimum estimates based on substrate availability and fecal excretions have suggested that 20-40 g of carbohydrates pass through this pathway with the production of from 200-400 mmol of VFA(45). Acetate, propionate and butyrate are the major volatile fatty acids which occur in the human large intestine. These VFAs are physiologically active in the colon and are absorbed through gut wall, thus providing an additional source of energy for the host. Absorbed butyrate is an important substrate for colonic epithelial-cell metabolism in animals, and in isolated human colonic cells it is metabolized in preference to glucose. Absorbed butyrate and propionate are cleared by the liver, and little appears in the peripheral blood. It is likely that acetate passes into the circulatory system and is taken up by the tissues(27). All three acids may influence intermediary metabolisms. In the liver, propionate inhibits gluconeogenesis(46). Cancer cell biological studies have shown that butyrate has antineoplastic properties(47).
The equation for fermentation in the human colon is:

\[ 34.5 \text{C}_6\text{H}_{12}\text{O}_6 \rightarrow 48 \text{CH}_3\text{COOH} + 11 \text{CH}_3\text{CH}_2\text{COOH} + 5 \text{CH}_3(\text{CH}_2)_2\text{COOH} + 23.75 \text{CH}_4 + 35.25 \text{CO}_2 + 10.5 \text{H}_2\text{O} \]

Gaseous products are either expelled via the rectum or are absorbed and exhaled. Measurement of the breath excretion of methane and hydrogen has been widely used in clinical and physiological studies of humans(27), which have demonstrated that hydrogen is also produced in large quantities during fermentation(4). Hydrogen produced by colonic bacteria and excreted in breath is a useful index of carbohydrate malabsorption. Since colonic contents are often acidic in individuals with carbohydrate malabsorption and in normal newborns, it has been determined that colonic acidification has an effect on H2 production. In turn, the consumption of carbohydrate substrates and H2 production by human colonic bacteria can be directly related to luminal pH(48).

Important intermediates in the fermentation process include ethanol, methanol, formate, lactate and succinate, which are rarely found in human feces since they are further metabolized to become end-products. Fermentation increases colonic bacterial activity, leading to changes in bile acid, nitrogen metabolism and the synthesis of vitamins. It is possible that the protective effect of dietary fiber against large-bowel cancer may be related to the metabolic activity of the intestinal flora. Fermentation also provides the means by which the microflora of the large intestine obtain energy for the maintenance of cellular function and growth(49). The extent of fiber fermentation is influenced by a number of factors including water-solubility, the chemical structure of the fiber, lignification, particle size and other components of diet, as well as the amounts ingested.

Variance in the processing of dietary fiber may also affect its degradation. Van Soest et al.(50), in a series of comparative studies of the fermentation of dietary fiber in
humans and other animals, observed that fermentation is largely dependent on body size. In the colon, polysaccharides are fermented by the bacterial flora, which compose approximately 40 to 55% of the volume of the contents of the human colon(27) and are then excreted in the feces(51,52). In a controlled diet, fecal solids were found to be composed of 55% bacteria, 17% undigested fiber and 24% water-soluble material(40). Human feces collected on different occasions have been found to be sufficiently uniform to yield similar in vitro fermentation findings(53). Therefore, it has been concluded that human fecal material is a practical source of microorganisms for the evaluation of the fermentation properties of substrates. The inoculum of fresh human feces provides a mixed flora of the type found in the colon(54).

Nearly all of the bacteria found in the human colon are obligate anaerobes, and most require a fermentable carbohydrate(55-57). The most common fiber fermentation bacteria in intestinal tract ecosystems are *Bacteroides* spp., *Clostridium clostridiiforme* and *Bifidobacterium* species(51). These bacteria are nonsporing anaerobes which obtain their energy from the fermentation of carbohydrates. Their fermentation products, which accumulate in the intestinal tract, include the volatile fatty acids and essential nutrients for herbivores(58). The species composition of the colonic flora is not significantly affected by diet(59-61). A bacterium isolated from human feces belonging to the genus *Bacteroids* is capable of degrading on intact and insoluble plant cell walls(62). This species accounts for approximately 20% of the normal flora of the human colon(56,63). Moreover, strains of *Bacteroides ruminicola* and *Bacteroides succinogenes*, which degrade cellulose, hemicellulose and pectin, have been isolated from the rumen of cattle(64-66).
2. Human Cellulose Digestion and Health

2.1 Cellulose Definition

It was nearly 150 years ago that Anselme Payen(67) discovered the substance that was subsequently given the name cellulose by isolating it from green plants. This material has been under continuous investigation since that time. At present, vegetable fiber constitutes the sole source of industrial cellulose, and billions of tons are produced annually by the processes of photosynthesis(68). However, the production of cellulose is not confined to the vegetable kingdom. Some marine organisms of the Tunicata class are generally regarded as a form of cellulose and bacterial cellulose has been known for a long time. In nature, cellulose is never found in the pure state. In wood, plant stalks, leaves and the like, cellulose is more commonly associated in considerable quantities with other substances, including lignin and hemicellulose. Thus, according to the species, on a dry basis wood contains between 40 to 55% cellulose, 15 to 35% lignin, and 25 to 40% hemicellulose(69).

Cellulose differs from most of the other polysaccharides found in plants in that it consists of very long molecular chains with only one repeating hexose residue. Cellulose has a highly ordered, fibrous morphology and occurs naturally in a crystalline state, exhibiting a crystalline polymorphism in which the degree of crystallinity is dependent upon the source of the cellulose(67). Cellulose is one of the most important commercial raw materials and is the parent substance for a large variety of chemical derivatives. Cellulose consists of macromolecules of at least several hundred to several thousand anhydroglucose units, and is the carbohydrate part of plant cell walls, formed only of the glucose molecules(70).

It is generally accepted that cellulose is a linear condensation polymer consisting of D-anhydroglucopyranose units joined together by β-1,4 glucosidic bonds; thus, it is a 1,4-
β-D-glucan. The structure of cellulose is shown as Figure 1. The chain molecules in natural cellulose are of unequal lengths and there is variance in the number of glucose units in different chains. Cellulose has three reactive hydroxyl groups per anhydroglucose repeating unit. When the cellulose molecule is fully extended it takes the form of a flat ribbon with hydroxyl groups protruding laterally and is capable of forming both inter- and intra-molecular hydrogen bonds. The surface of the ribbon consists mainly of hydrogen atoms linked directly to carbon and is therefore hydrophobic. It strongly influences the chemical reactivity and solubility of cellulose. As a result, cellulose is virtually insoluble in most common solvents. Its supramolecular structure determines many of its chemical and physical properties.

![Cellulose Structure](image)

**Figure 1.** Structure of cellulose.

### 2.2 Cellulose Digestibility

Cellulose, the main structural component of plant cell walls is the most abundant of the fiber components. Compared to the non-cellulosic fiber polysaccharides, it is relatively resistant to intestinal microbial fermentation. Since human digestive juices do
not contain cellulase(71), the digestion of cellulose is presumably the result of bacterial action, particularly in the colon. The cellulose that reaches the large bowel is a probable influence upon structure, and may act both as a chemically inert bulking agent and as a molecular sieve, affecting the access of nutrients to bacteria. The fermentation of cellulose, which requires a complex interaction of micro-organisms, eventually produces short chain fatty acids, such as Bacteroides are grown in media containing glucose or other monosaccharides since the cellulose carbon source has extremely low levels of polysaccharide-degrading activity. However, when these bacteria are exposed to a fermentable polysaccharide, large increases in the level of polysaccharide-degrading activity may be observed(55). In addition, animal studies have shown that cellulose is acted upon by bacteria to form absorbable volatile fatty acids(72,73).

Cellulose digestion in the human has been found to reflect extreme variability in apparent digestibility(74). This has been attributed to differences in microbial flora(37), in gastrointestinal rates among individuals, in the source of the cellulose, in chemical composition, degrees of cellulose purification, in cellulose structural properties, and to the application of inadequate methodologies(74,75). Cellulose in consumed foods and purified forms of cellulose may have very different physical properties, thus contributing to conflicting views of the role of cellulose in the gut. For example, average cellulose digestibility has been measured at 74% in a mixed diet, 75% in cabbage, and about 53% in bran, but only at 25% in the Solka Floc in Van Soest's group studies(42). The digestive capacity of colonic microorganisms has also been tested in vitro, and it was determined that the purified cellulose was virtually indigestible, while that from cabbage was extensively degradable. The human digestibility of fiber also reflects an extremely wide range of variation in individual subjects. Thus, it is not surprising that results from different laboratories have shown wide variation since individual subjects have demonstrated widely different results, even when maintained on a uniform diet and
based upon the application of standardized analytical techniques(76). For example, in 1970 Southgate(77) reported that cellulose digestion in young men and women averaged approximately 26% (approximating the results obtained by Kelleher in 1984(74)) in contrast to a study in 1979 which reported a mean digestion of 72% for a similar group of subjects(37). In 1943, Macy(76) reported that the average daily excretion of cellulose in the feces of a group of children ranged from 2.6 to 98.5% of their intake. Evidently, there are no definite relationships between the excretion or digestibility of cellulose and sex, account of intake or age. However, the absolute values for the digestibility of cellulose tend to be greater for older children and elderly males have been found to digest cellulose to a much greater extent than males than a younger age group. This wide range of individual variation may be due to differences in the fineness of the particles exposed to digestion, to peristaltic action, or to other physiologic characteristics.

Refined cellulose is chemically processed hence it differs markedly from natural cellulose in both its physical and biological properties(78). Solka Floc is comprised of about 85% wood cellulose and 15% nonglucose hemicelluloses. In one experiment 16 g/day of Solka Floc was ingested in a semi-purified liquid diet and only 8% of the cellulose was digested(79). Substantial quantities of unprocessed food are digested during transit through the human gastrointestinal tract whereas little or no digestion of Solka Floc occurs(79). Thus, the digestion of Solka Floc is much less than that of fiber found in foods. More than half of the fiber in a diet containing fruits and vegetables can be degraded, while the apparent digestibility of purified cellulose is minimal(79). Cellulose digestibility in the human is proportional to the "laxation rate"(75), and is more complete when the transit time is long. The time food residues spend in the human colon, normally between 18 to 66 hrs, may be precisely the variance time during which cellulose degradation occurs(4). It is possible that residual cellulose exerts a water-
holding effect and thus alters stool transit time and weight, but this would not provide an explanation for changes in stool pH.

2.3 Cellulose Digestibility and Health

Cellulose is found in abundance in nature in virtually all plant tissues and the most common components of the human diet. It can change human gut functions, especially those of the large intestine. The cellulose metabolism of humans has long been focused upon the intestines, but progress has been slow due to the lack of accurate chemical methods for its measurement. The mechanisms for the effect of cellulose remain unknown, but it has been speculated that they may be untreated from increased excretion of bile acids in the feces. For the greater part, evidence has suggested that carcinogens or co-carcinogens are a bacterially metabolized bile acids(80). Some colonic bacteria have been used to degrade bile acids into recognizable carcinogens and degraded bile acids have been identified as co-carcinogens in animal models. Cellulose serves to increase fecal bile salt excretions(81), this action is important to the lowering of serum cholesterol, an effect which often follows the administration of this type of substance(82). In 1959, Portman and Stare(83) reported that the level of indigestible residues in diets may affect lipid metabolism. However, it was subsequently reported by Prather(84) and Graned(85) that there was no significant effect on the serum cholesterol, total lipids, and the phospholipid levels of young women and middle-aged men who received cellulose diets. When cellulose was confined to 20% of the diet, there was a significant reduction in serum cholesterol, total lipids and phospholipids, accompanied by increased bile acid excretion among children(86). Butkeys et al. (85) reported that dietary cellulose exerted no beneficial effect upon lowering human cholesterol levels. However, at higher levels cellulose exerted a beneficial effect in lowering serum cholesterol levels among children.
A desirable dietary fiber should have hydrophilic properties in order to increase stool bulk, not only through added fiber but also by increasing water-holding ability(87). Cellulose consumption significantly increased mean daily wet and dry stool weights and the frequency of defecation at effectively shortened transit times. Dietary fiber protects against colorectal cancer by speeding colonic transit (thus reducing the time a carcinogen is in contact with the bowel mucosa) and by increasing stool weights (i.e., by diluting carcinogenic concentrations)(88). In another experiment, fecal excretions of calcium and magnesium were significantly increased when diets containing cellulose were fed(89). Although the mean magnesium and calcium balances were lowered as the cellulose was consumed, the plasma levels of magnesium and calcium remained within normal limits. In 1981, Bhat(90) reported that addition of cellulose to the diet significantly increased the fecal excretion of calcium, phosphorus, and iron as well as the serum calcium, and that the inorganic phosphorus and iron levels decreased significantly among adolescent girls. It is possible that cellulose binds calcium, phosphorus, and iron and increases their excretion, thus adversely affecting serum calcium, phosphorus, and iron levels. Therefore, high cellulose intakes may not be advisable, or mineral intakes should be increased, when high fiber diets are consumed by human adolescents.

Cellulose is also an important nutritional component of the human diet. Volatile fatty acids are a major product of the microbial degradation of cellulose, and the major portion of VFAs may be absorbed and used as an energy source. Cellulose ingestion has been associated with a reduction of colonic neoplasia among both animals and humans, as well as a reduction in the total number of colonic tumors. This protective effect of cellulose appears to be time-dependent and has been associated with a shift in tumor distribution from the proximal colon to more distal sites(91). However, no significant differences were detected in relation to tumor size or histopathology.
Cellulose has a different effect upon the human colon. The measures that characterize optimal bowel function are presumably those that prevent prolonged residue residences within the colon and promote ease of defecation, while at the same time permitting adequate small bowel digestion and absorption and large bowel fluid and electrolyte reabsorption. Purified cellulose given in reasonable quantities does not lower human serum cholesterol concentrations, and only minimally increases fecal bile acid excretions. In contrast, pure cellulose seems to impair the absorption of minerals from the gut. When cellulose feeding levels are set too high (e.g. 30 to 40 g), the feces tends to harden because of the lowered moisture content(87). Type, source, degree of purification, grind and degree of hydrogenation have been documented as influential factors in the ultimate physiological response of humans to cellulose. Various forms of cellulose are currently available as commercially marketed products. Most of these products are solid or semi-solid in consistency and are generally consumed in relatively modest amounts(87).

3. *In Vitro* Methods for Measuring Cellulose Digestibility in Humans

3.1 *In Vitro* Methodology

*In vitro* digestibility measures have been used widely to estimate *in vivo* digestion. It is extremely difficult to investigate the fermentative functions of the proximal colon *in vivo* because of its inaccessibility. Moreover, the intubation and infusion of solutions may disturb the stagnant hypoxic conditions necessary for the growth and activity of anaerobic bacteria(92). Development of conditions which could achieve a closer approximation of the *in vivo* environment are desirable. An alternative approach would be to study the metabolic functions of colonic bacteria under steady state conditions *in vitro*. The application of such a technique would enable the
completion of studies to define the conditions under which optimal fermentation of carbohydrates take place.

Since biochemical mechanisms are difficult to assess *in vivo*, an *in vitro* fecal incubation system has been adapted for the current study in an attempt to clarify the differences in available substrates within the human colon. The principal goal of this study is to develop an *in vitro* fermentation system which can provide a reasonable facsimile of fermentation within the human large intestine. The research assumption is that such an *in vitro* system will be suitable for a variety of studies of microbial activities within the large intestine(93).

The application of *in vitro* techniques for the study of human colonic metabolism will provide the advantage of a controlled and well-defined environment, and it is the intent of the current *in vitro* study that it simulate *in vivo* experimental conditions as closely as possible. For the current study, an *in vitro* mixed microbial batch culture technique was developed to culture human fecal microorganisms. The basic components of this system include a culture medium, a reducing solution, the substrate to be digested and human fecal inoculum(42). The *in vitro* system also provides the additional advantage that single, relatively pure substrates can be used, thus facilitating chemical analysis. In contrast, *in vivo* systems suffer from interference from other components in the diet(94). Wolin et al.(95,96) examined rumen fermentation *in vitro* in semicontinuous cultures. These cultures were started with rumen contents and were provided with substrates approximating normal ruminent diets. Analysis of the products as well as the enumeration and isolation of bacteria in an *in vitro* semicontinuous culture system indicated that these fermentations were similar to those of the human large intestine(94). At the same time, a fermentation equation derived from the concentration of volatile acids found in human feces also suggests that the fermentation in the large intestine is similar to rumen fermentation(58).
The proposed system was used to study the features of overall fermentation including the influence of diet, pH and the effect of inhibitors of methanogenesis\(^{(97,98)}\). In this respect, it was important that methodological developments should be linked to studies of cellulose metabolism. The procedure, when initiating an \textit{in vitro} fermentation, is to add the medium to the substrate overnight prior to inoculation. Since it has been observed that many substrates cannot be easily soaked in the solution. Thus the substrates were soaked in the medium at \(4^\circ\text{C}\) overnight prior to inoculation to ensure the complete hydration of the fiber matrix. This allowed the cellulolytic anaerobes in the liquid phase of the inocula to have immediate access to their insoluble substrate. By this mean, VFAs also removed important influences upon bacterial growth; VFAs are rapidly absorbed \textit{in vivo}, but were accumulated in the proposed in vitro system, thus inhibiting fermentation reaction\(^{(1)}\).

3.1.1 Medium

Since the pH level affects the microbial population, viable counts, and the types of the organisms, this visual check was used to describe fermentation changes induced by changes in the pH of the medium\(^{(97)}\). The fermentor, maintained at a pH of 6.7, contained the types of bacteria often found in high concentrations\(^{(99)}\) and maintained a fermentation pattern similar to that of an \textit{in vivo} condition between 6.0 and 7.1. There is a marked change in the fermentation pattern when the pH drops below 6.0. For the current study, a relatively large volume of buffer solution was added in an amount adequate to maintain the pH level within the usual limits for digestion, while assuring that the final acid concentration will not exceed that found in humans. It was considered possible that bicarbonate would be a more natural constituent in the inorganic medium than phosphate\(^{(100)}\). Thus, the pH is maintained at 7.0 by a controlled infusion of sodium bicarbonate\(^{(2)}\), and all pH measurements are conducted with a pH electrode. It
is important that pH be carefully controlled and the inoculum maintained as near as possible between 37-40°C since the temperature of the medium can also affect the pH level.

When preparing the medium, it was kept oxygen-free by flushing the medium with oxygen-free gas and storing it in tightly-stoppered flasks containing oxygen-free gas. Anaerobiosis was maintained throughout the collecting, processing, and inoculating periods(101). In addition, Na₂S·9H₂O was added to each tube just prior to inoculation to absorb all oxygen, and N₂ was employed as the headspace gas to avoid the pH effects encountered with the use of CO₂(62).

3.1.2 Reducing Solution

Oxygen-free CO₂ or other appropriate gas was bubbled through the medium to introduce CO₂ and to keep out air. The gas was passed into the vessels by means of a needle attached by rubber tubing to the gas sources, and the subsequent entrance of oxygen was prevented by closing the vessels with rubber-stoppers as the needle was removed. At the same time, the medium also contained the resazurin Eh indicator, which changed from pink to colorless. The reducing agent was then added to the medium to obtain low oxidation-reduction potential and to use resazurin as an indicator of anaerobiosis. The redox potential of all cultures remained below -300mv, which is suitable to the growth of anaerobes(2). The redox potential was unaffected by carbohydrate input. The anaerobic reducing solution was used to dilute the fecal contents prior to inoculating the medium.

3.1.3 Inoculum

The fermentation of dietary fiber by anaerobic microorganisms in the colon is important to the human body. It has been demonstrated that fiber sources differ in
The extent and rate of fermentation (102, 103), but it is difficult and expensive to measure the fermentation in vivo (54). The most accessible method to obtain a population of microorganisms is to utilize fresh human feces. Total bacterial counts and the numbers of species and their distribution have not been found to change significantly within the healthy colons of subjects administered high-fiber diets in comparison to low-fiber control diets. Consequently, human fecal microorganisms have been used in numerous in vitro studies of colonic fermentation. A batch-culture technique utilizing human fecal microflora was used to determine the desirable digestive time of the in vitro fermentability of the substrates and to obtain fermentation residues. Human feces collected on different occasions were sufficiently uniform to yield similar in vitro fermentation findings among a variety of collections. Thus, human fecal material is a practical source of microorganisms for the evaluation of the fermentation properties of substrates (54).

How closely the in vitro population approximates real populations for the same substrate is not well known. In spite of some degree of difference, the bacterial populations in vitro and in vivo were quite similar (98). This suggests that human colon bacteria can be maintained in mixed populations over the long-term in in vitro experiments which closely resemble the bacterial populations of humans fed the same diet. The inoculum of fresh human feces provided a mixed flora of the type found in the colon.

3.1.4 Substrate Preparation

Most of the methods available for the fractionation and isolation of the various cell wall constituents from plant tissue use the alcohol insoluble residue (AIR) as the starting material (60). Dietary fibers are prepared as AIR, containing the total plant cell wall (6). The most important factor in the use of AIR for the quantitation of the plant cell wall...
wall is because low molecular weight sugars and oligosaccharides can thus be solubilized. In addition, water-soluble polymers, such as the pectin substrates which are extensively degraded during their passage through the large intestine(36), remained with the residue. Water is the most commonly used extracting pectin, and the entire analysis of the dietary fiber can be accomplished using this preparation. AIR is easy to prepare in gram quantities, but still offers some disadvantages. For instance, it contains about 2% starch, which can be used initially as an alternative carbon source by the bacteria. Moreover, it contains appreciable amounts of precipitated proteins and other intracellular compounds which must be removed before attempting any detailed studies on the cell wall polymers constituting the dietary fiber(105). To achieve complete removal of proteins and starch it is necessary to ensure complete disruption of tissue structure.

SCFA excretion in human feces is influenced by the presence of the fiber source in the diet. Moreover, the proportions of acetate, propionate and butyrate can be influenced by diet. Most frequently, fecal microflora have been shown to be stable despite dietary changes, although diet does appear to influence what is available as substrate and it also appears to have an effect upon the enzymatic activities of the microbes. For the current study, AIR was selected for cellulose digestion experiments because the low molecular weight sugars, oligosaccharides, lipids and pigments are removed. The particle size of the substrate preparations was also controlled since it is believed to affect the response of the fecal microflora to fiber. For example, grinding of the sample resulted in significantly reduced fecal output because of reduced fecal water(106).

3.2 Measurement of Cellulose Content

Quantitative determination of cellulose in biological materials is tedious and time-consuming when using established methods(107). These methods require that a rather
large sample be repeatedly extracted with a series of solvents to successively remove lipids, proteins, and pigments (108). Each desired fraction is collected by the same means, then dried and weighed. The accuracy of these methods is dependent upon the efficiency with which extraction removes unwanted materials from the appropriate fraction. However, cellulose content is much easier to determine than those of other cell wall polysaccharides. Cellulose digestion was determined by observing a visible loss of the cellulose from flasks of the human fecal inoculum-cellulose medium after determination of the incubation digestive time. When combined with a colorimetric assay for hexose, this method can be used to measure the cellulose content of bacterial cultures (109). Otherwise, Avicel, a microcrystalline cellulose produced controlled acid hydrolysis, can be used to establish the efficiency of the extraction procedure for the recovery of cellulose. The average carbohydrate yield was in excess of 99%, while that of the unextracted sample averaged 101% (107). Slightly lower yields for the extracted sample can be attributed to a small loss of cellulose during removal of the extraction reagent and wash solvent. The total glucan value can be used as an accurate measure of cellulose content (110).

Cellulose residues were isolated from AIR with acid detergent, as described by Van Soest (1975). The use of the acid detergent provides an accurate means to measure cellulose plus lignin in food stuffs (7). The residue were then hydrolyzed with 12M of sulfuric acid for 1 hr, the acid was diluted to 1M, and then subjected to continuous hydrolysis in an autoclave for 1 hr. Thus, cellulose in the dietary fiber was completely hydrolyzed (111). Cellulose can be hydrolyzed only at a slow rate when heated with 1M sulfuric acid, unless previously treated with 12M sulfuric acid. Cellulose content was measured by colorimetric assay of the glucose released when the insoluble residue from the 1M sulfuric acid hydrolysis was incubated with 12M sulfuric acid. Then, an aliquot
of the hydrolysate was analyzed for glucose, using the glucose oxidase method described by Dahlqvist(112). The basic principle of this method is:

\[
\text{Glucose} + \text{H}_2\text{O} \xrightarrow{\text{Gluc. oxidase}} 2\text{H}_2\text{O}_2 + \text{gluconic acid}
\]

\[
2\text{H}_2\text{O}_2 + \text{o-dianisidine} \xrightarrow{\text{peroxidase}} 2\text{H}_2\text{O} + \text{o-dianisidone (red)} \xrightarrow{\text{at 420 nm}} \text{o-dianisidone (brown)}
\]

The glucose liberated is assayed with a Tris-buffered glucose oxidase reagent. Tris was used in order to inhibit disaccharidases, which are present as contaminants in commercially obtained glucose oxidase preparations(112). The cellulose values were corrected for the degradation of glucose, as determined by acid hydrolysis of the Avicel cellulose.
MATERIALS AND METHODS

1. Substrate Preparation

1.1 Alcohol Insoluble Residue (AIR)

A dietary fiber sample was prepared as an alcohol insoluble residue (AIR). Fresh sample (cabbage, apple and carrot) was weighed, washed and chopped. The sample was then cut into fine pieces with a food processor and refluxed for 2 hrs in a 95% aqueous ethanol solution. Accounting for the water content of the products, the equilibrated concentration of ethanol in this first reflux was 47.79%, 53.78% and 45.57%, for cabbage, apple and carrot respectively. After 2 hrs, the mixture was filtered through a Buchner funnel under negative pressure. The residue remaining in the funnel was extracted with 90% ethanol under refluxing conditions for an additional two hours. The resulting suspension was filtered and the residue was subjected to acetone extraction under refluxing conditions for an additional hour. Following filtration, the residue was air dried overnight. The dried material was ground into a fine powder to provide the AIR. The flow stages are shown in Figure 2.

1.2 H₂O/AIR

A fresh sample (apple and carrot) was weighed, washed, chopped and cut into fine pieces with a food processor. Prior to extraction with 95% ethanol, the sample was blended with water and filtered at room temperature to remove the water soluble fiber. The residue was then extracted by refluxing in 95% ethanol, 90% ethanol and acetone as described above for the AIR preparation. The resulting residue was dried at room temperature. The flow stages are also shown in Figure 2.
Figure 2. Preparation of cabbage, carrot and apple AIR (Alcohol Insoluble Residue) and H₂O/AIR.
1.3 Cummings AIR

A fresh sample was washed, chopped and weighed, and then blended with an amount of 95% ethanol appropriate to achieve a final concentration of 80% (v/v) ethanol. The resulting suspension was then filtered, using a 36 μm nylon mesh over a Buchner funnel. The residue was transferred to a round-bottom flask and refluxed with 80% ethanol for 2 hrs and refiltered as above. The residue was then extracted with refluxing acetone for 1 hr, refiltered, stirred with acetone for 30 min, and again refiltered. The last cycle was repeated twice, to give a total of 3 room-temperature acetone washes, and the resulting residue air dried overnight. Figure 3 illustrates the Cummings (32) procedure for the preparation of AIR.

Figure 3. Cummings method for preparation of AIR (Alcohol Insoluble Residue).
2. Determination of Cellulose Content

2.1 Updegraff Method

A sample in amount of 50.0 mg was placed in a centrifuge tube, to which was added 3.0 mL of nitric/acetic acid reagent, as described by Updegraff(109). The nitric/acetic reagent was prepared by mixing 150.0 mL of 80% acetic acid and 15.0 mL of concentrated nitric acid. To suspend the 50 mg sample in the added nitric/acetic reagent, an initial 1.0 mL of reagent is added followed by mixing with a vortex mixer, then addition of the remaining 2.0 mL reagent and mixing again. The resulting suspension is then immersed in a boiling water bath. The sample is left in the boiling water bath for 30 min to removed the hemicellulose and xylosans. The resulting sample is then centrifuged for 5 min at 1,200 rpm. The supernatant is discarded and the remaining residue washed with the nitric/acetic acid mixture (3.0 mL x 2) and acetone (3.0 mL x 4) by repeated resuspension and centrifugation. The resulting residue is then dried by evaporation overnight. In some cases the sample appeared to still be damp after 24 hr evaporation, indicating the presence of some moisture or solvent. Ten mL of 67% H₂SO₄ was added to the final dried residue and the sample allowed to stand for one hour at room temperature in order to dissolve the cellulose. One mL of the resulting solution was then diluted to 100.0 mL with distilled water. One mL of the diluted cellulose solution was then placed in a 150 x 18 mm screw-cap culture tube to which was then added 4.0 mL of distilled water and then 10.0 mL of cold anthrone reagent. The anthrone reagent consisted of 0.2 g of anthrone in 100.0 mL of concentrated H₂SO₄, the reagent being chilled in a refrigerator prior to use. The solution containing the anthrone reagent was mixed well with a Vortex mixer and the tubes placed in a boiling water bath for 15 min. After the 15 min incubation the tubes were allowed to cool at room temperature for 15 min. The absorbance of the resulting solution was then measured at a
wavelength of 620 nm against a reagent blank. Figure 4 shows the flow stages for this procedure.

2.2 Modified Updegraff Method

A cellulose determination similar to that described above was done with the exception that the dried residue remaining after acetic/nitric and acetone washes was added to 1.0 mL of 12M H₂SO₄. This cellulose/H₂SO₄ mixture was incubated for 1 hr. at 35°C, for cellulose dissolution. The resulting cellulose solution was then diluted with 11.0 mL of distilled water and heated in a 100°C water bath for 2 hrs, as described by Hsu et al. (75). The resulting cellulose hydrolysate solution was then diluted with distilled water to 100.0 mL. Ten mL of cold anthrone reagent was added to 1.0 mL of the diluted cellulose hydrolysate and the resulting solution was heated in a boiling water bath for 15 min (113). After cooling, the absorbance at 620 nm of the resulting solution was measured as described above. The anthrone reagent was prepared by adding 10.0 g thiourea and 0.5 g anthrone to 660.0 mL concentrated H₂SO₄, then diluting with distilled water to 1L and allowing the reagent to cool at 4°C prior to use. The flow stages for this procedure are also shown in Figure 4.
50 mg sample

- add 3 mL nitric/acetic acid reagent*
- heat in 100°C for 30 min
- centrifugate at 1200 rpm for 5 min
- wash with nitric/acetic acid (3.0 mLx2)
- wash with acetone (3.0 mLx4)
- evaporate overnight

- add 10 mL of 67% H₂SO₄ for 1 hr
- dilute 1 mL to 100 mL
- add 4 mL H₂O to 1 mL of diluting solution
- add 10 mL anthrone reagent
- heat at 100°C for 15 min
- read O.D. value at 620nm

- add 1 mL of 12M H₂SO₄
- heat at 35°C for 1 hr
- dilute to 1M H₂SO₄
- heat at 100°C for 2 hr
- dilute to 100 mL
- add 10 mL anthrone/thiourea reagent to 1 mL of diluting solution

* nitric/acetic acid reagent: mixture of 150 mL 80% acetic acid and 15 mL conc. nitric acid.

Figure 4. Updegraff and modified Updegraff method for cellulose determination.
2.2.1 Standard Curves

The cellulose content of Avicel, marketed as a pure cellulose preparation, was estimated by assaying Avicel against a standard curve derived from assays of pure glucose. Glucose was selected for this purpose since cellulose is composed of glucose. Glucose and Avicel assays followed the procedure described in section 2.2. The calibration curve for the initial assay was based on known standard glucose solutions. The standard solution for glucose was prepared as follows. Pure glucose 0.09 g was dissolved in 1.0 mL of 12M H₂SO₄ and heated at 35°C for one hour. The resulting mixture was diluted with 11 mL of distilled water, heated in a 100°C water bath for 2 hrs then diluted with distilled water to 1L to provide the standard glucose solution. Serial dilutions of this standard glucose solution were then assayed by the anthrone method (section 2.2) resulting in a standard curve, calibration curve, based on glucose. A representative calibration curve is shown on Figure 5.

![Figure 5. Glucose standard curve of modified Updegraff method.](image-url)
Avicel was used as the primary reference for analysis of the cellulose content in food samples. Avicel was taken as 99% cellulose based on assays against glucose standards. A calibration curve, based on Avicel, was developed as in section 2.2 except that the Avicel solution was diluted to 500.0 mL with water (rather than 100 mL) prior to the addition of the anthrone reagent. Then 1.0, 0.8, 0.6, 0.4 and 0.2 mL of the resulting diluted solution were introduced, respectively, to five separate tubes, followed by the addition of an appropriate quantity of distilled water to achieve a final volume of 1.0 mL in each tube. The anthrone assay was then done on each tube. The calibration curve was obtained by plotting the resulting absorbance versus mg cellulose (Avicel) per mL. A representative calibration curve is shown as Figure 6.

![Figure 6. Avicel standard curve of modified Updegraff method.](image-url)
2.2.2 Calculation of Cellulose Content

Cellulose is a linear polymer consisting of recurring glucose units joined together by β-1,4 glucosidic bonds. Therefore, when using a calibration curve based on glucose a conversion factor must be used to account for the fact that one water molecule will be removed for every glucosidic bond formed. The converting factor is 0.9. Therefore, the cellulose content of Avicel was calculated as follows:

\[
\% \text{ cellulose} = \frac{(K)(\text{dilution factor})(.9)}{\text{Sample weight}} \times 100\%
\]

where K is the number of μg glucose per mL as obtained from the glucose based calibration curve.

A calibration curve based on Avicel was used to determine the cellulose content of food fibers. Hence, on these calculations the glucose conversion factor was not used. The cellulose content of Avicel is 99.1%. The cellulose content of the food fiber was calculated by the following formula:

\[
\% \text{ cellulose content} = \frac{(a)(100)^* (.991)^**}{\text{Sample weight}} \times 100\%
\]

a: μg/mL cellulose content against Avicel standard curve
* the sample was diluted 100 fold during the assay
** the cellulose content of Avicel is 99.1%
2.3 Acid-Detergent Fiber/Glucose-Oxidase Method

The acid-detergent fiber (ADF) content of the sample was determined following the method described by Goering and Van Soest (117). The sample was placed in a 600 mL tall-form beaker, without a spout, containing 100 mL acid detergent and 2 mL of decahydronaphthalene and the suspension refluxed for one hour. The acid-detergent solution consisted of cetyltrimethylammonium bromide in sulfuric acid, 20 g/L 1N H₂SO₄. Following refluxing, the solution was filtered through a sintered glass crucible (C), washed with acetone, and dried overnight in a hood. The crucibles containing the fiber residue were then placed in a 50 mL beakers, to which 8.33 mL of 72% sulfuric acid was layered on top of the crucible. The crucible was agitated intermitantly at room temperature for three hours. After this 3 h dissolution period, the solution was transferred to a 200 ml volumetric flask and distilled water was added to bring the cellulose solution to volume, which is approximately 1N sulfuric acid. Aliquots of the 1N solution were placed in 30 mL Corex tubes and autoclaved for one hour at 121°C. Then, 900μL of autoclaved sample was removed to a test tube and neutralized with 100μL of 8N NaOH. The neutralized hydrolysate was then assayed for glucose, using the glucose oxidase/peroxidase coupled enzyme method described by Dahlqvist (112). Two hundredμL of sample, standard or blank was pipetted into a test tube, to which 3.0 mL of TGO reagent was added, mixed thoroughly in a Vortex mixer and incubated at 37°C for 60 minutes. The tube was chilled to room temperature and the absorbance value was read at 420 nm.

The fresh tris-Glucose Oxidase (TGO) reagent was composed of 100 mL Tris buffer, 200μL glucose oxidase, 0.5 mL peroxidase, 2.2 mL dianisidine, and 1.0 mL triton solution, prepared as follows:

1. Dissolve 61 g tris in 85 mL 5N HCl and dilute to one liter to form 0.5M tris buffer. Check pH and adjust to 7.0.
2. Peroxidase solution of 1.0 mg/mL in H₂O, frozen and stored in aliquots.

3. O-dianisidine dihydrochloride of 10 mg/mL in H₂O, stored at 4°C in a darkened storage area.

4. 20% (w/w) triton X-100 in 100% ethanol.

The flow stages are shown in Figure 7.

* Tris-buffered Glucose Oxidase reagent

**Figure 7.** Acid-Detergent Fiber/Glucose oxidase method for cellulose determination.
2.3.1 Standard Curve Preparation and calculations

Aqueous glucose solutions containing, respectively, 25, 50, 100, 200, 300 and 400μg glucose/mL were prepared and frozen in small aliquots. The standard curve was obtained running the glucose oxidase assay on these solutions. A representative standard curve is shown on Figure 8.

![Figure 8. Glucose standard curve of ADF/glucose oxidase method.](image)

To calculate the cellulose content of the actual samples one must account for dilutions and the glucose to polymer factor (.9). The actual food samples were diluted to 200 mL and 9μL of the diluted solution was neutralized with 1μL of NaOH prior to glucose analysis. Therefore, the cellulose content was calculated by the following formula:

\[
\%\text{ cellulose content (}\%\text{)} = \frac{(a) (200) (10/9) (.9)}{\text{Sample weight}} \times 100\%
\]

where a is μg/mL cellulose content measured against a glucose based standard curve.
3. **In Vitro** Fermentation Method

3.1 Selvendran Method (114)

The basic components of the *in vitro* fermentation system include the sample to be digested, a culture medium, and an appropriate inoculum. The sample preparation procedure was described previously. The culture medium and the inoculum solution (a reducing solution) are prepared separately as described below.

3.1.1 Preparation of Medium

A. Procedure

BSM10 media (115), in the amount of 500 mL, was prepared as follows. One gram of trypticase, 0.25 g of yeast extract, 18.75 mL of mineral solution 1 (1.5% K₂HPO₄·3H₂O), 18.75 mL of mineral solution 2 [0.48% KH₂PO₄, 0.12% NaCl, 1.2% (NH₄)₂SO₄, 0.12% CaCl₂ and 0.25% MgSO₄·7H₂O], 5.0 mL of hemin (.1 mg/mL) and 1.55 mL of volatile fatty acid were placed in a 500 mL round bottom flask and dissolved in 345.45 mL of distilled water. To this mixture was added 50 mL of chicken fecal extract, 25 mL of liver extract, and 0.5 mL of 0.1% resazurin and the solution was brought to a boil.

The following procedure, as described by the Anaerobe Laboratory Manual (101) was then conducted. The flask was fitted with a removable chimney to prevent the media from boiling over and a stir bar was added to obtain an even boil. After boiling the medium, the flask was removed from the heat and the chimney was immediately replaced with a 2-hole stopper and a cannula to deliver a stream of oxygen-free CO₂ into the media. The media were then cooled to room temperature in an ice bath, the pH was adjusted to 6.5 with 8N NaOH or 5N HCl and the oxygen-free CO₂ was switched immediately to oxygen-free N₂. The media was then dispensed into 60 mL serum bottles.
and flushed with oxygen-free N₂. The serum bottles were capped with rubber stoppers and sterilized in an autoclave at 121°C for 15 min. Following sterilization, the media were cooled to room temperature, the stoppers were removed from the serum bottles and the pH was tested for each. The pH was maintained at 6.5 to 6.9 with 8N NaOH or 5N HCl. Then, oxygen-free CO₂ was again passed into each bottle to keep out oxygen, and 3 mL of sterile 8% sodium carbonate solution, 0.6 mL of sterile 2.5% sodium sulfide and 0.5 mL of sterile 3.0% cystein hydrochloride were added. The pH of the resulting solution was then adjusted to 7.1. The flow stages are shown in Figure 9. Other ingredients were prepared as described below.

![Diagram of medium preparation](image)

**Figure 9.** Medium preparation for Selvendran *in vitro* fermentation system.
B. Reagent

1) Volatile fatty acid: 17 mL of acetic acid, 6 mL propionic acid, 4 mL of n-butyric acid, 1 mL of iso-butyric acid, 1 mL of n-valeric acid, 1 mL of iso-valeric acid and 1 mL of 2-methylbutyric acid were mixed.

2) Resazurin: 0.1% (w/v) aqueous solution.

3) Hemin: 10 mg of hemin were dissolved in 1 mL of 1N NaOH and diluted to 100 mL.

4) Chicken fecal extract: Prepared by blending 1:2 quantities of chicken feces and water and autoclaving at 121°C for 30 min. The sludge was removed by centrifugation at 9000x rpm for 10 min. The supernatant was poured off, the pH was adjusted to 7.0 and the mixture was sterilized by autoclaving at 121°C for 15 min.

5) Liver extract: 54 g of dehydrated liver was dissolved in 400 mL of distilled water, heated to 50°C and held at this temperature for one hour. The mixture was then boiled, cooled and centrifuged. The pH value of the supernatant liquid was adjusted to 7.0 and sterilized by autoclaving at 121°C for 15 min.

C. Na₂CO₃ 8% solution preparation

A round bottle flask with 500 mL of distilled water was prepared and fitted with a removable chimney, to which a stir bar was added to obtain an even boil. A preparation procedure similar to that for the media was carried out, except that 40 g of Na₂CO₃ was added and dissolved after the mixture was cooled. The solution (30 mL) was dispensed into 50 mL flasks and each was flushed with oxygen-free N₂. After sterilization, the oxygen-free N₂ was replaced by oxygen-free CO₂ and the samples were stored.
D. Cys-HCl 3% solution preparation

A flask of 200 mL of distilled water was prepared. A procedure similar to that for the Na₂CO₃ preparation was used (C, above), except that 6 g of Cys-HCl was added and dissolved and the N₂ gas was not replaced. The mixture was then stored.

E. Na₂S·9H₂O 2.5% preparation

A procedure similar to that for 3% Cys-HCl solution (D, above) was prepared, except that 6 g of 3% Cys-HCl was replaced by 5 g of Na₂S·9H₂O.

3.1.2 Anaerobic Dilution Solution Preparation

There were two methods used to prepare the anaerobic dilution solution for this experiment. The first has been described by Bryant (116), and is conducted as follows.

To prepare a volume of 300 mL of anaerobic dilution solution, first, 45 mL of mineral solution 1 (0.39% K₂HPO₄·3H₂O), 45 mL of mineral solution 2[0.3% KH₂PO₄, 0.6% NaCl, 0.123% MgSO₄·7H₂O and 0.06% CaCl₂], 0.9 g Na₂CO₃, 0.3 mL of 0.1% resazurin solution and 193.45 mL of distilled water were placed in a 500 mL flask. The flask was fitted with a removable chimney and boiled. Then the flask was removed and the chimney was replaced with a 2-hole stopper and a cannula to deliver a stream of oxygen-free CO₂ into the solution. After chilling the solution in an ice bath, the pH was allowed to drop to 6.5. The gas was then switched immediately to oxygen-free N₂ and the solution was dispensed, 9.46 mL, into culture tubes simultaneously being flushed with oxygen-free N₂. The tubes were sealed with rubber stoppers and the solutions were sterilized at 121°C for 15 min. Following sterilization, the solution was cooled to room temperature and 0.375 mL of 8% sodium carbonate and 0.167 mL of 3% of Cys-HCl was added to each tube. This procedure is shown in Figure 10. The preparations of 8% sodium carbonate and 3% Cys-HCl were identical to the media preparations.
add 45 mL mineral solution 1 and 2
0.9 g Na\(_2\)CO\(_3\), 0.3 mL 0.1% resazurin
and 193.45 mL H\(_2\)O in 500 mL flask

biol
(same as medium preparation)

cool down

adjust pH to 6.5

dispense 9.46 mL solution to culture tube

cap and autoclave for 15 min

adjust pH to 6.9

cool to room temperature

add 0.375 mL 8% Na\(_2\)CO\(_3\)
and 0.167 mL 3% Cys-HCl
to each culture tube

Figure 10. Anaerobic dilution solution preparation for the collection
and dilution of fecal inoculum: Bryant Method
(anaerobic condition maintained by CO\(_2\) syringing).

The second procedure is conducted as follows. A salt solution is first prepared
by dissolving 0.2 g of CaCl\(_2\) and 0.41 g of MgSO\(_4\)-7H\(_2\)O, in 300 mL of distilled water.
An additional 500 mL of distilled water is then added and swirled. To this solution is
added 1.31 g of K\(_2\)HPO\(_4\)-3H\(_2\)O, 1.0 g of KH\(_2\)PO\(_4\), 10.0 g of NaHCO\(_3\), and 2.0 g of
NaCl and the solution was again swirled until all salts were dissolved. Finally, an
additional 200 mL of water is added and with mixing (101). Two hundred mL of anaerobic dilution solution is then prepared by mixing 100 ml salt solution, 0.4 g of gelatin, 100 mL of distilled water, and 0.8 mL resazurin solution (25.0 mg resazurin in 100 mL distilled water). The 200 ml anaerobic dilution solution is placed in a 500 mL flask and degassed as above. The solution was reduced after cooling by the addition of 0.2 g of Cys-HCl. The solution was dispensed into 10 mL culture tubes and sterilized at 121°C for 15 min. The flow stages for preparation of anaerobic dilution solution are shown in Figure 11.

**Figure 11.** Anaerobic dilution solution preparation for the collection and dilution of fecal inoculum: VPI Anaerobe Method (anaerobic condition maintained by CO₂ syringing).
3.1.3 Inoculum and Fermentation

The following procedure has been described by Bayliss et al. (33). Feces are provided by a donor which has not been treated with antibiotics for at least three months. Feces were collected in a plastic bag and kneaded by hand. A sample of approximately 1 g was transferred to a weighed tube containing 10 mL of anaerobic dilution solution; the tube was reweighed, and the contents were mixed. Diluted feces in the amount of 1 mL were added to a serum bottle containing 60 mL of medium and 0.3 g of substrate. The inoculated flask containing sample, medium and inoculum was then incubated at 37°C for specific digestive (fermentation) times with continuous agitation. Following fermentation, the culture contents were transferred to sintered glass crucible filters (size C) that were previously weighed to the nearest 0.1 mg. Following filtration, the filters were dried in an oven to a constant weight, reweighed, and the amount of residue calculated by difference. The dried samples in the crucibles were then assayed for cellulose content as described above. The flow stages are shown in Figure 12.

Figure 12. Inoculation and fermentation, Selvendran in vitro fermentation system.
3.2 Van Soest Method

The Van Soest (117) method of *in vitro* fermentation is described in the following sections.

3.2.1 Fermentation Media Preparation

The fermentation medium and the anaerobic dilution solution for dilution of the fecal inoculum were prepared together. A total of 1200 mL of media is prepared, which accommodates up to 20 samples, and provides sufficient media for inoculum dilution. For this preparation, the reagents must be added in the order listed and should not be made up more than 24 hours before inoculation. The composition of the pertinent mineral mixes and other reagents are given in section 3.2.3. To 600 mL of deoxygenated H$_2$O, previously boiled, cooled under N$_2$ in an Erlenmeyer flask and stirred under N$_2$ for 15 min, 3.0 g of trypsinase and 15μL of micromineral solution were added. After mixing, 300 mL of fermentation buffer, 300 mL of macromineral solution and 1.5 mL of resazurin were added; with continuous mixing. The color of the solution at this time will have gone from blue to purple. The medium is then dispensed at 40 mLs per flask and the sample fiber is added to the medium. The mixture is then stored overnight at 4°C under N$_2$ to ensure complete hydration of the fiber matrix. The fermentation medium prepared in this manner should be used the following day. The fermentation media must be reduced just prior to inoculation. The reduction procedure is given in section 4.2.2. The procedure is shown in Figure 13.
boil 600 mL H₂O 5-10 min
and cool under N₂

add 3 g trypticase, 150 μL micromineral solution

stir under N₂ for 15 min

add 300 mL fermentation buffer
300 mL macromineral solution
and 1.5 mL resazurin

store at 4°C under N₂

**Figure 13.** Medium and anaerobic dilution solution preparation, Van Soest in vitro fermentation system.

3.2.2 Inoculum Collection and Fermentation

Ten mL of reducing solution (see 3.2.3 for preparation) was added to a zip-lock plastic bag containing 200 mL of media under CO₂ and the resulting solution was allowed to go anaerobic at 37°C. Then the feces sample was collected into this pre-weighed zip-lock plastic bag and the fecal weight determined by difference. The contents of the feces bag were transferred into a blender while being gassed with CO₂. Additional reduced media was added to achieve an approximate 5-fold dilution (w/w) of feces. Sufficient feces was collected to ensure a final volume of at least 250 mL. The feces were then blended for 30-60 seconds to achieve a homogeneous slurry. The slurry was squeezed through Nitex nylon with a pore size of 41μ, and the resulting suspension then filtered through glass wool into an Erlenmeyer flask. During these procedures, both
the funnel containing the nylon or glass wool and the receiving flask were gassed with CO₂. The procedure is shown in Figure 14. Ten mL of the resulting filtered fecal suspension was used to inoculate the medium containing the fiber sample. The inoculum was added through an inlet tube in the fermentation flask's rubber stopper using an automatic syringe. All samples were then incubated in duplicate for the desired fermentation period, either 0, 4, 8, 12 or 24 hrs.

---

**Figure 14.** Inoculation and fermentation, Van Soest *in vitro* fermentation system (manipulated under CO2 atmosphere).
3.2.3 Reagent

A. Fermentation buffer solution: ammonium bicarbonate (NH$_4$HCO$_3$), 4 g/L; and sodium bicarbonate (NaHCO$_3$) 35 g/L.

B. Macromineral solution: Na$_2$HPO$_4$, anhydrous, 5.7 g/L; KH$_2$PO$_4$, anhydrous, 6.2 g/L; and MgSO$_4$·7H$_2$O, 0.6 g/L.

C. Micromineral solution: CaCl$_2$·2H$_2$O, 13.2 g/100 mL; McCl$_2$·4H$_2$O, 20.2 g/100 mL; CoCl$_2$·6H$_2$O, 1.0 g/100 mL; and FeCl$_3$·6H$_2$O, 8.0 g/100 mL.

Use a 100 mL volumetric flask; if all salts are not completely solubilized, assure that solution is mixed before removing aliquots.

D. Resazurin solution: 0.1 percent w/v solution.

E. Reducing solution:

1) add 1.25 g cysteine dihydrochloric acid and about 20 pellets of KOH in 100 mL of boiled water; 2) add 1.25 g Na$_2$S·9H$_2$O in 100 mL of boiled water; and 3) after dissolving both solutions separately, mix together, always preparing the mixture fresh.

4. Squash, Beets and Refined Cellulose Fermentation

4.1 AIR preparation for squash and beets (Cummings, 32)

Two different samples, one each of squash and beets were prepared. To remove free proteins, lipids, pigments, simple sugars and acids, diced cortical tissue was extracted with hot ethanol and acetone as described in section 1.3. Approximately 500g of fresh cortical tissue, was blended with approximately 2.3L of 95% ethanol to achieve a final concentration of 80% (v/v) ethanol. The resulting material was filtered using 36μmeter pore size nylon with a Buchner funnel. The residue was then refluxed with
700 mL of 80% ethanol for two hours, the resultant material was filtered, and the process was repeated. The material was then extracted with 700 mL of refluxing acetone for one hour, filtered, then stirred for one-half hour with 700 mL of cold acetone at room temperature and filtered. The last cycle was repeated twice. The resulting material was air dried and sieved. The fraction passing the 355 mm sieve but retained by the 124 mm sieve was used as the AIR in these experiments. The flow stages are shown in Figure 15.

Figure 15. AIR (Alcohol Insoluble Residue) preparation, Cummings method.
4.2 Van Soest Method, *In Vitro* Fermentation

4.2.1 Fiber Substrate Hydration

The fiber samples employed as substrates included a commercially available purified pulp cellulose (Avicel pH101) an AIR sample derived from acorni squash and an AIR sample derived from red beets. Each experimental fermentation flask contained either 500 mg for the AIR sample or 100 mg for the purified cellulose sample (Avicel pH101). The sample sizes were in approximate correspondence with the amount of cellulose contained in the fiber sample. The samples were added to the 125 mL Erlenmeyer fermentation flasks, to which 40 mL of media were added. Each flask was stoppered under N₂ and stored overnight at 40°C to allow hydration of the fiber samples. These samples were then inoculated the following morning to initiate the fermentation (0 time). The procedure is shown in Figure 16.

![Substrate hydration diagram](image)

**Figure 16.** Substrate hydration, Van Soest *in vitro* fermentation system.
4.2.2 Fermentation Flask Equilibration

The fermentation flasks containing the media and fiber substrates were placed in a water bath at 37-39°C. The flasks were purged with CO₂. A low pressure regulator coupled with a gas exit tube which could be submerged to variable depths in water was used to regulate pressures and CO₂ flow rates at the fermentation flasks. While purging with CO₂, 2 mL of reducing solution (section 3.2.3E) was then added to each fermentation flask, and the flask restoppered. The medium containing the substrate and reducing solution was left at 37°C until the medium had gone anaerobic as indicated by a color change from pink to colorless (approximately 30 min). The colorless medium was then ready to receive the fecal inoculum.

4.2.3 Inoculation and Fermentation

The fecal inoculum was prepared during the temperature equilibration and medium reduction period described above (4.2.2). Freshly voided feces were collected as described in 3.2.2. Ten mL of the prepared fecal inoculum was added to each fermentation flask with an automatic syringe while maintaining a CO₂ atmosphere. This addition of inoculum represented time zero for fermentation studies. The CO₂ pressure was then decreased to 3-4 cm water and the flow rate to approximately 60 mL/min. Shaking was avoided for at least the first hour to allow the microbes to attach to the fiber particles. Samples were incubated in duplicate for periods of 4, 8, 12 or 24 hours, given that 24 hrs is an average colonic retention time. The flasks were agitated every four hours for several minutes. At selected times (i.e., 4, 8, 12 and 24 hr intervals), the fermentation was terminated by the addition of 1.5 mL of concentrated H₂SO₄ to the fermentation flasks, resulting in a final H₂SO₄ concentration of approximately 1N. Terminated fermentations were stored by keeping the flask at 4°C for subsequent analysis. The pH of the fermentation was measured with a combination glass electrode.
at time zero and at the completion of the specified fermentation, but prior to the addition of H₂SO₄. Controls for these experiments included a flask containing sample but without inoculum and a zero time sample containing all components but H₂SO₄ was added at zero time.

4.3 ADF/Glucose-Oxidase Method for Determination of Cellulose Content

The cellulose residues were isolated from the AIR with acid detergent, as described by Van Soest (117). The contents of a fermentation flask was allowed to come to room temperature, then transferred to a tall-form 600 mL beaker without spout. One hundred mL of acid detergent solution, section 2.3, plus 2 mL decahydronaphthalene was added and the contents refluxed for one hour. The resulting suspension was then removed, filtered with a "C" sintered glass crucible and washed with acetone. The residue in the crucible was dried overnight in a hood. Crucibles containing fiber residues were placed in beakers, filled with 8.33 mL of 72% sulfuric acid and intermittently stirred at room temperature for three hours. Three hours after the initial acid addition, distilled water was added to each sample with stirring to dilute the sulfuric acid to 1N. Approximately 10 ml of the 1N solution was then transferred to a 30 ml screw cap Corex tube and autoclaved at 121°C for 1 hr. Then 900 μL of the resulting hydrolysate was transferred to a test tube and neutralized with 100 μL of 8N NaOH. The glucose content of the neutralized hydrolysate was then measured using the glucose oxidase/peroxidase coupled enzyme method describe in section 2.3 (112).

Purified cellulose (Avicel) was used as the reference standard for calibration curves to account for potential degradation of glucose during extraction and hydrolysis. The flow stages for this procedure are shown in Figure 17.
Figure 17. Van Soest and Acid-Detergent Fiber/Glucose oxidase method for squash, beet and Avicel cellulose digestibility.
RESULTS AND DISCUSSION

1. Methods of Cellulose Determination

1.1 Updegraff and Modified Updegraff Method

The Updegraff method of cellulose isolation and subsequent quantification was used in the early phases of the study. In most cases the method was not successful due to the formation of precipitates during the final glucose assays. The presence of precipitates made it necessary to further centrifuge the samples prior to taking absorbance measurements. To circumvent this problem a different method of cellulose hydrolysis and glucose quantification was employed, now referred to as the modified Updegraff method. This modified method dissolved the cellulose in 72% H₂SO₄ and added thiourea to the anthrone reagent. The precipitate prevalent in the original method was not observed under these conditions. The cellulose content of cabbage, apple and carrot were determined using the modified Updegraff method (Table 1). The results were obtained by measuring the cellulose content of the AIR and then extrapolating to the other fractions. The cellulose content of the products on a fresh weight basis were all very low, less than 1%, as expected. These values are comparable to those reported by Englyst (9). The cellulose content on a dry weight basis, considering the whole food, was 4.48, 9.83 and 4.01 g per 100 g dry weight for cabbage, apple and carrot, respectively. The cabbage value appears low while the apple value appears high relative to that reported by Englyst (9). The cellulose content of the cabbage, apple and carrot AIR samples, 15.51%, 19.01% and 17.18%, respectively, reflect the extraction of non-fiber components. The cellulose content for AIR of cabbage and apple were similar to results obtained by Bittner et al. (6), who measured the AIR cellulose content of cabbage
at 16.3% and apple at 20.9%. However, the results were approximately 35% less than those of Cummings (32), who obtained AIR cellulose contents for cabbage, apple and carrot of 23.4%, 31.9% and 25.1%, respectively. The latter differences could reflect differences in the methods used to prepare the AIR. The increased cellulose content of the H$_2$O/AIR samples relative to the AIR samples most likely reflects the loss of soluble fiber components during the water extraction step. The cellulose content of the purified cellulose preparation, Avicel, was found to be 99.1%. This value agrees with Sloneker’s (107) results in which the average yield of carbohydrate was in excess of 99%. With respect to sensitivity, an absorbance of 0.189-0.195 and 0.222-0.224 was obtained for 90μg/mL glucose and 100μg/mL Avicel samples, respectively. This method is simple and appears to be of sufficient sensitivity for these assays. However, a number of factors have been reported to influence the final reaction mixture absorbance when using the anthrone method. Assay parameters such as temperature, freshness of the reagents and acid concentrations must be carefully controlled. For the current study, large volumes of concentrated H$_2$SO$_4$ were used, with care taken to avoid hurting the skin.
Table 1. Cellulose content of cabbage, apple, carrot and microcrystalline cellulose fiber preparations determined by the modified Updegraff method.

<table>
<thead>
<tr>
<th></th>
<th>Cabbage</th>
<th>Apple</th>
<th>Carrot</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O (%)</td>
<td>92.40</td>
<td>85.10</td>
<td>88.20</td>
</tr>
<tr>
<td>original food (%)</td>
<td>0.59</td>
<td>0.66</td>
<td>0.34</td>
</tr>
<tr>
<td>original food (g/100g dry weight)</td>
<td>4.48</td>
<td>9.83</td>
<td>4.01</td>
</tr>
<tr>
<td>AIR* (%)</td>
<td>15.51±0.29***</td>
<td>19.01±0.20</td>
<td>17.18±0.10</td>
</tr>
<tr>
<td>H₂O/AIR** (%)</td>
<td>21.51±0.43</td>
<td>19.74±0.97</td>
<td></td>
</tr>
<tr>
<td>microcrystalline cellulose (Avicel)</td>
<td>99.07±7.50%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Alcohol Insoluble Residue  
** treated with H₂O prior to AIR procedure  
*** mean ± standard deviation

1.2 Acid-Detergent Fiber/Glucose-Oxidase Method

Initial tests of the ADF method utilized squash and beet due to the lack of information on the fermentation of fibers from these sources. The cellulose residues were isolated from AIR with acid-detergent, as described by Van Soest (117). The cellulose content for fresh squash and beets was respectively, 0.74% and 0.51%, while the cellulose content of the AIR from these samples was 26.71% and 23.22%, respectively. These results are presented in Table 2. The cellulose content of the fresh squash and beets on a dry weight basis was found to be 10.07 and 6.51 g per 100g dry weight. Englyst measured the cellulose content of beets to be 6.1% on a dry weight
basis. To my knowledge, the cellulose content of the edible portion of squash has not been reported. For sensitivity, an absorbance of 0.318-0.324 and 0.333-0.337 was obtained for 100μg/mL of glucose and Avicel, respectively. The precision of this method is also affected by the freshness of the reagents, temperature and acid concentrations. The method is simple to perform, is accurate, and uses relatively less hazardous chemicals than the modified Updegraff method.

Table 2. Cellulose content of squash and beet fiber preparations determined by the ADF*/glucose oxidase method.

<table>
<thead>
<tr>
<th></th>
<th>Squash</th>
<th>Beet</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O (%)</td>
<td>86.30</td>
<td>87.30</td>
</tr>
<tr>
<td>original food (%)</td>
<td>0.74</td>
<td>0.51</td>
</tr>
<tr>
<td>original food (g/100g dry weight)</td>
<td>10.07</td>
<td>6.51</td>
</tr>
<tr>
<td>AIR** (%)</td>
<td>26.71±0.95***</td>
<td>23.22±0.89</td>
</tr>
</tbody>
</table>

* Acid-Detergent Fiber  
** Alcohol Insoluble Residue  
*** mean ± standard deviation

1.3 Comparison of Updegraff and ADF/Glucose-Oxidase Method

It is important to consider the two analytical methods with respect to their appropriateness for measuring of the cellulose content of fibers used in fermentation studies. The modified Updegraff method appeared to allow complete recovery of
cellulose based on the results of assays of purified microcrystalline cellulose. However, the method includes repeated centrifugations and washings to remove unwanted materials and then a rather ineffective drying step. During the extraction and washing steps I found the sample was often difficult to pellet. Consequently, when the supernatant was decanted some sample/cellulose was lost. Unfortunately, this centrifugation/decantation step is repeated several times, so the results are likely to underestimate the cellulose content. Another problem is that during the drying procedure a gel-like material was often formed which did not completely dry. These limitations make the modified Updegraff method less convenient and less precise than what is needed.

The ADF/glucose-oxidase method did not appear to have any major disadvantages. The entire sample, including a fermentation culture, could be transferred to the refluxing chamber for extraction of non-cellulosic materials. This extraction step is easy to control and does not have to be repeated. The resultant suspension is filtered and the cellulose content easily measured. The method appears more convenient and more accurate than the Updegraff methods. It is my conclusion that the ADF/glucose-oxidase method should be used for the determination of cellulose content in fiber-fermentation studies. Table 3 provides a comparative summary of the two analytical methods discussed here.
Table 3. Comparison of methods of cellulose analysis.

<table>
<thead>
<tr>
<th>Method</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>modified Updegraff</td>
<td>- sensitive</td>
<td>- less precise,</td>
</tr>
<tr>
<td></td>
<td>- accurate for Avicel</td>
<td>- less convenient,</td>
</tr>
<tr>
<td></td>
<td>- affected by temperature,</td>
<td></td>
</tr>
<tr>
<td></td>
<td>freshness of reagents</td>
<td></td>
</tr>
<tr>
<td></td>
<td>and acid concentration</td>
<td></td>
</tr>
<tr>
<td>ADF* /glucose</td>
<td>- sensitive</td>
<td>- affected by temperature,</td>
</tr>
<tr>
<td>oxidase</td>
<td>- convenient</td>
<td>freshness of reagents</td>
</tr>
<tr>
<td></td>
<td>- accurate</td>
<td>and acid concentration</td>
</tr>
</tbody>
</table>

* Acid-Detergent Fiber

2. *In Vitro* Fermentation Substrates

The AIR preparation was repeatedly extracted with a series of hot solvents, a procedure designed to remove free sugars, lipids, proteins and pigments. The resultant materials were colorless. The results in Tables 1 and 2 clearly show that non-cellulosic food components were preferentially extracted by the ethanol and acetone treatments. The higher content of cellulose in the H2O/AIR preparation suggests the removal of some of the soluble fiber fraction during the initial water extraction. The AIR preparations appear the most appropriate for fermentation studies since much of the non-fiber fraction of the food has been removed and yet these preparations would likely contain the complete fiber profile.
3. *In Vitro* Fermentation Methods

3.1 Selvendran Method

According to the Selvendran method described in section 3.1.1 of Materials and Methods, the medium was prepared by adding all the ingredients to a container and boiling it for a period of time to keep the mixture oxygen free. At the same time oxygen-free CO₂ was delivered to the mixture, the pH was adjusted to 6.5, and the medium was dispensed into serum bottles and autoclaved under anaerobic conditions. The optimum pH of the human colon is 6.5-7.1. For this method, after the medium was autoclaved, the pH increased to 7.5. The pH was maintained from 6.5 to 7.1 by the addition of 8N of NaOH or 5N of HCl. It was necessary to prepare the reducing dilution solution, 8% of Na₂CO₃, 3% of Cys-HCl and 2.5% of Na₂S-9H₂O solutions, separately, then add it to the medium. For preparation of the anaerobic dilution solution all ingredients were prepared and added as described by Bryant et al. (116) or the Anaerobe Laboratory Manual (101), based on the Selvendran (117)*in vitro* fermentation method. After autoclaving the media had a significantly higher pH, was colorless (reduced), and contained a white precipitate. These results were observed for both methods of preparation. The white precipitate was presumed to be CaCO₃ that could be formed at the high sterilization temperatures. Personal communication with other labs indicates this precipitate is largely ignored.

For preparation of the Selvendran inoculum, the procedure described by Bayliss et al. (33) was used. The feces were collected in a plastic bag and kneaded by hand. Then ~1 g of the feces were transferred to a weighed tube containing 10 mL of anaerobic dilution solution, reweighed and mixed. One mL of the diluted feces was added to the culture medium containing the substrate to start the fermentation. Fermentation studies using this system indicated a considerable lag period prior to cellulose fermentation.
This may be due to the relatively small fecal inoculum used. When the inoculum was added to the medium containing the substrate, it may be speculated that the microflora required a period of time in which to grow to a critical population size. This growth period may serve to delay fermentation in these in vitro experiments. The Selvendran method also uses a closed system which may also affect fermentation rates due to VFA's and other volatile components not being removed.

For the analysis of the Selvendran in vitro fermentation system I used carrot AIR, carrot H₂O/AIR and purified cellulose (Avicel) as fiber samples. Table 4 indicates that the Avicel preparation was resistant to fermentation over the time studied. The 48 fermentation studies of carrot indicate nearly complete fermentation of the cellulose component (Table 4) in carrots. To get an idea of the rate of cellulose fermentation, the carrot samples were tested over a 24 fermentation period. The results of the extent of fermentation of the carrot AIR and H₂O/AIR were only 14.98% and 28.17% respectively. These relatively low values, relative to the 48 hr values, possibly reflect the lag period discussed above. Perhaps this may be attributable to an inadequate methodology.

Table 4. Relative digestibility of carrot and Avicel as measured by the Selvendran in vitro fermentation system.

<table>
<thead>
<tr>
<th>Incubation Time (hr)</th>
<th>AIR* (%) Digested</th>
<th>H₂O/AIR** (%) Digested</th>
<th>Avicel (%) Digested</th>
</tr>
</thead>
<tbody>
<tr>
<td>48</td>
<td>95.09±1.67***</td>
<td>96.90±0.51</td>
<td>0</td>
</tr>
<tr>
<td>24</td>
<td>14.98±2.25</td>
<td>28.17±0.67</td>
<td>0</td>
</tr>
</tbody>
</table>

* Alcohol Insoluble Residue
** treated with water prior to AIR procedure
*** mean ± standard deviation
Simultaneously to this work the Van Soest method of in vitro fermentation was being investigated. The fibers other than carrot were analyzed using the latter system due to its simplicity.

3.2 Van Soest Method

In accordance with the Van Soest in vitro fermentation method described in section 3.2.1 of Materials and Methods, the medium and reducing dilution solution were prepared together. As the fermentation media were prepared; the reducing agents were added directly to the media. Following media preparation, the media did not require autoclaving and the pH levels were stable. Even when the inoculum was added and during the incubation time the pH value remained at the optimum pH of the human colon. This was because a large amount of fermentation buffer was added. Table 5 provides the pH values for squash AIR using the Van Soest in vitro fermentation system.

<table>
<thead>
<tr>
<th>Incubation time (hr)</th>
<th>Inoculum</th>
<th>pH Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>N</td>
<td>7.50</td>
</tr>
<tr>
<td>0</td>
<td>Y</td>
<td>7.65±0.08*</td>
</tr>
<tr>
<td>6</td>
<td>Y</td>
<td>6.82±0.05</td>
</tr>
<tr>
<td>12</td>
<td>Y</td>
<td>6.84±0.06</td>
</tr>
<tr>
<td>24</td>
<td>Y</td>
<td>6.85±0.04</td>
</tr>
</tbody>
</table>

* mean ± standard deviation
The Van Soest inoculum preparation was considerably different from the Selvendran method. Feces were collected in a pre-weighed zip-lock plastic bag containing 200 mL of reduced and warmed media and then reweighed. The feces were transferred to a blender, to which the remaining media was added to achieve about a 5-fold feces dilution. The feces were then blended to achieve a homogeneous slurry and squeezed through nylon and glass wool under CO₂ pressure. The resultant solution provides 10 mL of inoculum for the medium containing the substrate. The inoculum contained a greater microflora population than the Selvendran inoculum and since the fermentation system was open, the VFAs could be immediately removed. Thus, this method provided the bacteria with a good growth environment.

From the pH values (Table 5), it may be observed that the microflora grew very well. Tables 6 and 7 show that the extent of fermentation of the substrate increased with increasing incubation time. The average cellulose digestibility of AIR for squash was 6.04%, 10.58%, 17.11% and 96.18% for incubation 4, 8, 12 and 24 hrs, respectively. The average cellulose digestibility of AIR for beets was, respectively, 17.52%, 23.52%, 30.53% and 96.06% for the same range of incubation times. However, for the Avicel substrate, digestibility was negligible, indicating the general inability of microflora to degrade this type of purified preparation (Table 8).
Table 6. Digestibility of AIR cellulose from squash.

<table>
<thead>
<tr>
<th>Incubation Time (hr)</th>
<th>Inoculum</th>
<th>(%) Range of Digestibilities**</th>
<th>(%) Average Digestibility</th>
<th>Recovery***</th>
<th>average recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Y</td>
<td>0</td>
<td>0</td>
<td>94.75 - 95.65</td>
<td>95.21±0.56</td>
</tr>
<tr>
<td>0</td>
<td>N</td>
<td>0</td>
<td>0</td>
<td>97.09 - 100.31</td>
<td>99.84±0.09</td>
</tr>
<tr>
<td>4</td>
<td>Y</td>
<td>5.50 - 6.82</td>
<td>6.04±0.69***</td>
<td>89.08 - 90.16</td>
<td>89.46±0.61</td>
</tr>
<tr>
<td>4</td>
<td>N</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>8</td>
<td>Y</td>
<td>8.15 - 11.83</td>
<td>10.58±2.11</td>
<td>83.36 - 87.65</td>
<td>85.14±2.24</td>
</tr>
<tr>
<td>8</td>
<td>N</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>12</td>
<td>N</td>
<td>0</td>
<td>0</td>
<td>97.85 - 99.69</td>
<td>98.77±0.06</td>
</tr>
<tr>
<td>24</td>
<td>Y</td>
<td>94.73 - 97.42</td>
<td>96.18±1.36</td>
<td>2.47 - 5.03</td>
<td>3.64±1.29</td>
</tr>
<tr>
<td>24</td>
<td>N</td>
<td>0</td>
<td>0</td>
<td>97.70 - 99.54</td>
<td>98.77±0.05</td>
</tr>
</tbody>
</table>

* using Van Soest fermentation system
** based on a control run terminated at zero time
*** based on the calculated amount of cellulose added to each fermentation flask
**** mean ± standard deviation
Table 7. Digestibility of AIR cellulose from beet roots*.

<table>
<thead>
<tr>
<th>Incubation Time (hr)</th>
<th>Inoculum</th>
<th>(% Range of Digestibilities**)</th>
<th>(% Average Digestibility)</th>
<th>Recovery*** (%)</th>
<th>average recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Y</td>
<td>0</td>
<td>0</td>
<td>99.19 - 107.03</td>
<td>104.24±5.24</td>
</tr>
<tr>
<td>0</td>
<td>N</td>
<td>0</td>
<td>0</td>
<td>96.25 - 99.87</td>
<td>98.25±0.08</td>
</tr>
<tr>
<td>4</td>
<td>Y</td>
<td>15.07 - 19.33</td>
<td>17.52±1.83****</td>
<td>82.80 - 88.14</td>
<td>85.92±2.80</td>
</tr>
<tr>
<td>4</td>
<td>N</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>8</td>
<td>Y</td>
<td>22.11 - 24.99</td>
<td>23.52±1.44</td>
<td>75.15 - 83.46</td>
<td>79.72±4.22</td>
</tr>
<tr>
<td>8</td>
<td>N</td>
<td>---</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>12</td>
<td>Y</td>
<td>27.62 - 33.44</td>
<td>30.53±4.12</td>
<td>71.08 - 71.32</td>
<td>71.20±0.17</td>
</tr>
<tr>
<td>12</td>
<td>N</td>
<td>0</td>
<td>0</td>
<td>98.25 - 102.50</td>
<td>99.50±0.08</td>
</tr>
<tr>
<td>24</td>
<td>Y</td>
<td>95.74 - 96.50</td>
<td>96.06±0.39</td>
<td>3.76 - 4.57</td>
<td>4.11±0.42</td>
</tr>
<tr>
<td>24</td>
<td>N</td>
<td>0</td>
<td>0</td>
<td>98.25 - 100.50</td>
<td>99.00±0.04</td>
</tr>
</tbody>
</table>

* using Van Soest fermentation system  
** based on a control run terminated at zero time  
*** based on the calculated amount of cellulose added to each fermentation flask  
**** mean ± standard deviation
Table 8. Digestibility of microcrystalline cellulose (Avicel).

<table>
<thead>
<tr>
<th>Sample Entry</th>
<th>Incubation Time(hr)</th>
<th>Digestibility (%)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
<td>0</td>
<td>100.27</td>
</tr>
<tr>
<td>2</td>
<td>4</td>
<td>0</td>
<td>103.93</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>0</td>
<td>106.57</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
<td>0</td>
<td>105.29</td>
</tr>
<tr>
<td>5</td>
<td>24</td>
<td>0</td>
<td>97.15</td>
</tr>
</tbody>
</table>

3.3 Comparisons of In Vitro Fermentation System

There are major differences between the Selvendran and Van Soest in vitro fermentation systems. First, Selvendran's medium and anaerobic dilution solution were prepared separately, while Van Soest's medium and anaerobic dilution solutions were prepared together. The latter method is obviously more convenient. Second, the pH values for the former system required several adjustments which were not required in the Van Soest method. The pH of the Van Soest media was very constant, which is likely attributed to the large amount of bicarbonate buffer used. Third, after the medium and anaerobic dilution solutions were prepared, the Selvendran procedure required autoclaving, while the Van Soest procedure did not. Fourth, the Van Soest method provided a substantial and even fecal inoculum able to maintain a large bacterial population for fermentation. It did not appear to have the pronounced lag period typical of the Selvendran method. Finally, with respect to incubation conditions, the Selvendran fermentation system was closed so that VFAs produced as a result of metabolism, which could possibly influence the growth rate of bacteria, were not removed. In the Van Soest method these VFAs are removed immediately.
Since the Van Soest *in vitro* fermentation method provides many advantages over the Selvendran method, it is the one that I would recommend for general use. Table 9 summarizes the differences in the two methods.

**Table 9** Comparison of Selvendran and Van Soest *in vitro* fermentation methods.

<table>
<thead>
<tr>
<th></th>
<th>Selvendran method</th>
<th>Van Soest method</th>
</tr>
</thead>
<tbody>
<tr>
<td>medium and inoculation solution</td>
<td>prepare separately</td>
<td>prepare together</td>
</tr>
<tr>
<td>sterile technique</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>requires pH adjustments</td>
<td>yes</td>
<td>no</td>
</tr>
<tr>
<td>inoculum</td>
<td>small</td>
<td>large and homogeneous</td>
</tr>
<tr>
<td>fermentation system</td>
<td>closed</td>
<td>open</td>
</tr>
</tbody>
</table>

4. Fermentation of Squash, Beets and Avicel Cellulose

The experimental results from this study confirm that cellulose can be fermented by fecal bacteria. The average cellulose digestibility of the AIR for squash with an incubation time of 4, 8, 12 and 24 hrs was 6.04±.69%, 10.58±2.11%, 17.11±6.37% and 96.18±1.36%, respectively (Table 6). In contrast, no cellulose fermentation was detected in the control (i.e. no inoculum), the cellulose recovery being nearly 100%. For the beet substrate, the digestibility was 17.52±1.83%, 23.52±1.44%, 30.53±4.12%
and 96.06±0.39% for incubation times of 4, 8, 12 and 24 hrs, respectively, (Table 7).
The control results for beets were similar to those for squash.

The changes in digestibility and the recovery of cellulose over time are indicated graphically in Figure 18 for squash and Figure 19 for beets. The changes in recovery of cellulose over time for Avicel are shown graphically in Figure 20. Combined results are shown in Figure 21. The disappearance of the digestible fraction of cellulose behaved as a substrate limiting reaction between 0 and 12 hr of digestion. That is, the rate of digestion of cellulose in the fermentation flasks was proportional to the amount of digestible cellulose remaining at any time. After 12 hrs only 17.11±6.57% of the squash cellulose and 30.53±4.12% of the beet cellulose were degraded, indicating a lag phase. After 24 hrs 96.18%±1.36% of the squash and 96.06±0.93% of the beet cellulose were degraded. The apparent lag phase observed in these experiments is approximately one-half the size of the one observed for the Selvendran method. The Avicel was not fermented over any time period.

The experimental results indicate that high levels of cellulytic activity occur in the large intestine. The time course of fermentation differed markedly with the fiber source. However, the cellulose component of both beets and squash was almost completely fermented by 24 hrs. There may be a small fraction of the cellulose which is extremely resistant to microbial degradation and hence would take a very long time to ferment. This may be due to a highly crystalline fraction of cellulose or due to stearic hinderance by other molecules, such as highly branched polysaccharides, macromolecules cross-linked by phenolics, and lignin (119). The results demonstrate that human gut microflora can metabolize dietary cellulose. The fermentation of cellulose by these bacteria is likely to produce metabolic end products, such as VFAs, which are ultimately metabolized by the human host.
Figure 18. Time course of digestion and recovery of the cellulose component of squash.

Figure 19. Time course of digestion and recovery of the cellulose component of beet roots.
Figure 20. Time course of digestion and recovery of the cellulose component of microcrystalline cellulose.

Figure 21. Time course of digestion and recovery of the cellulose component of squash, beet roots and microcrystalline cellulose.
RECOMMENDATIONS FOR FUTURE RESEARCH

In this study a single individual provided all fecal inocula so that relative fermentation rates of different fibers could be obtained. It would now be appropriate to determine the relative fermentative activity inherent in different individuals. This type of comparative research should also include measurement of VFA production under the same conditions. It is also apparent that a survey of different foods is required before one could reliably estimate the fermentability of celluloses derived from different food groups.
BIBLIOGRAPHY


72. Sceley, R. C. 1969. "Feed carbohydrate", the contributions of the end products of their digestion to energy supply in the ruminant. In: Energy Metabolism of Farm Animals. 93.


113. Roe, J. H.. 1955. The determination of sugar in blood and spinal fluid with 

114. Selvendran, R. R.. 1975. Analysis of cell wall material from plant tissues: 
extraction and purification. Phytochemistry. 14:1011.


116. Bryant, M. P. and L. A. Burkey. 1953. Culture methods and some characteristics 
of some of the more numerous groups of bacteria in the bovine rumen. J. Dairy 
Sci.. 36:205.

reagents, procedures, and some applications). Agriculture Handbook No. 379. 
Agricultural Research Service, United States Department of Agriculture.

118. Wrong, D. M., A. J. Vince, and J. C. Waterlow. 1985. The contribution of 
endogenous urea to fecal ammonia in man, determined by $^{15}$N labelling of plasma 


Research Service, United States Department of Agriculture. Agriculture Handbook 
No. 8.