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The Influence of Dietary Fiber and Short-Chain Fatty Acids on Metabolism in Isolated Rat Colonocytes

by

Kathleen Elizabeth Marsman

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfilment of the requirements for the degree of Doctor of Philosophy

in

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The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled The Influence of Dietary Fiber and Short-Chain Fatty Acids on Metabolism in Isolated Rat Colonocytes by Kathleen Elizabeth Marsman in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Nutrition and Metabolism.

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ABSTRACT

Dietary fiber has trophic effects on the large intestine of monogastric species, showing increased mass and epithelial cell proliferation. The short-chain fatty acids (SCFA) acetate, propionate and butyrate are major products of colonic fiber fermentation. SCFA are absorbed and either oxidized by epithelial cells or transported to hepatic or peripheral circulation. These experiments investigate the effects of dietary fiber and SCFA on dynamic measurements of metabolism in isolated colonocytes. It was hypothesized that 2 weeks of fiber supplementation would increase colonocyte oxidation of SCFA and that concurrent increases in oxygen utilization, protein synthesis and cell proliferation would be observed in isolated colonocytes. Sprague-Dawley rats consumed elemental diets supplemented with 0%, 15% or 30% fiber. Colonocytes were isolated and oxidation rates of glucose, glutamine, and SCFA were assessed by measuring $^{14}CO_2$ evolving from ^{14}C-labeled substrates. TCA cycle anaplerotic flux, theoretical ATP yields, oxygen consumption, cell proliferation and protein synthesis were measured. Increased glutamine oxidation and decreased glutamate formation were observed with fiber supplementation, but overall ATP production was unaffected. Although cell proliferation was greater for fiber-fed animals, protein synthesis was unaffected. Additionally, colonocytes from the fiber group consumed no more oxygen than those from the fiber-free group. Thus, the effect of in vitro SCFA on colonocyte metabolism was investigated. It was hypothesized that SCFA would increase proliferation, protein synthesis and oxygen consumption. Providing 15 or 30 mmol/L of mixed SCFA (5 or 10 mmol/L each acetate, propionate, butyrate) decreased proliferation, independent of incubation media osmolarity. SCFA decreased protein synthesis, but this effect was mimicked using equimolar NaCl. This is

the first report of osmotic regulation of colonocyte protein synthesis. No change in oxygen consumption was observed. It was concluded that the changes in colonocyte metabolism induced through fiber supplementation were subtle and consistent with an increase in proliferative state of the cells. Inhibitory effects of SCFA on proliferation and protein synthesis illustrate the potential of SCFA to act as metabolic signals to the intestine, but do not explain the trophic effects of fiber on colonic mucosa in vivo.

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ATP - Adenosine 5'-triphosphate

DIDS - 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid

DPM - Disintegrations per minute

EDTA- Ethylenediaminetetraacetic acid

KB - Ketone Bodies

KRH - Krebs-Ringer HEPES

SCFA - Short-chain fatty acids

SEM - Standard error of the mean

TCA - Tricarboxylic acid

TCAA - Trichloroacetic acid

CHAPTER 1

INTRODUCTION

1 Dietary Fiber

1.1 Definition

Dietary fiber is defined as plant non-starch polysaccharides and lignin which are resistant to digestive hydrolysis by the enzymes of the upper intestinal tract in man (Trowell et al., 1976). Dietary fibers can be classified analytically into either water-soluble or water-insoluble categories (Anderson, 1986). Fiber sources such as lignin (from grains, seeds, and the woody parts of vegetables), cellulose and hemicellulose (found in vegetables, fruits, wheat bran and whole grains) are water-insoluble. Pectins and gums, which can be found in citrus fruits, vegetables, oat bran, and legumes, are water soluble fibers. The solubility of a fiber source does not necessarily predict the physiological effects of consuming such a fiber source. However, the solubility of a fiber source tends to parallel its fermentability (Nyman and Asp, 1982).

When consumed, dietary fibers move through the intestinal tract undigested and are passed on to the large intestine where they are substrates for microbial fermentation. Other undigested food components or luminal secretions from the small intestine can be fermented in the large intestine or provide bulk. These components include waxes, cutins and tannins from plant cell walls, malabsorbed starch, and intestinal mucins (Morand et al., 1992; Southgate, 1993; Stephen et al., 1981).

1.2 Fiber Fermentation

When dietary fiber arrives at the large intestine, intestinal bacteria degrade the fiber either completely or in part, depending on various factors such as the physical, chemical, and fermentative properties of the fiber source, the colonic transit time and the composition of

the intestinal bacteria (Stephen and Cummings, 1980b). The products of colonic fermentation include the following compounds: carbon dioxide, methane, H_2, water, and the short-chain fatty acids formate, acetate, propionate and butyrate, corresponding to 1, 2, 3, and 4 carbons in chain length, respectively (Miller and Wolin, 1979). The majority of the short-chain fatty acids produced are either acetate, propionate, and butyrate (Hoverstad, 1986). The ratios in which these organic acids are produced varies between species (Bugaut, 1987), between individuals (Ehle et al., 1982), and depending on fiber source (McBurney and Thompson, 1989). The short-chain fatty acids produced by the intestinal bacteria can reduce the pH of the intestinal contents by 0.2 - 0.6 pH units, depending on such factors as the fermentative properties of the fiber source and the location being assessed within the lumen of the large intestine (Lupton et al., 1985; Lupton et al., 1988; McBurney, 1991).

It has been estimated that microbial mass contributes half of the fecal mass in humans (Stephen and Cummings, 1980a), an amount which can be altered by the type and quantity of fiber in the diet. Less fermentable fibers, such as wheat bran, increase fecal bulk due to the presence of both unfermented fiber and increased fecal mass in the lumen (Cummings, 1982). Fermentable fibers, such as pectin and oat bran, have only a small impact on fecal mass (Nyman and Asp, 1982), but they significantly increase short-chain fatty acid production (McBurney, 1991) and bacterial cell mass is increased (Stephen and Cummings, 1980b).

The approximate molar percent ratio of short-chain fatty acids from fiber fermentation is 60% acetate, 24% propionate, 16% butyrate (Cummings, 1981). Based on the fermentation equations derived from Miller and Wolin (1979) and adjusted for the molar percent ratio, complete fermentation of 100 g of dietary fiber would yield 0.62 moles of acetate, 0.25 moles of propionate, and 0.16 moles of butyrate. The gross energy provided from acetate, propionate and butyrate is 0.87 (209), 1.50 (365), and 2.18 (522) MJ/mol (kcal/mol), respectively (CRC, 1984) and thus, the complete fermentation of 100 g of fiber

would yield 1.27 MJ (305 kcal).

1.3 Effects of Fiber on Colonic Mass and Proliferation

Colonic mass increases with short-term fiber supplementation (Dowling et al., 1967; Jacobs and Lupton, 1984; Palacio et al., 1989). Fiber sources which are most readily fermented (such as guar and pectin) have the greatest hyperplastic effect in the proximal colon (Tasman-Jones, 1993), whereas less fermentable fiber sources such as wheat bran, increase distal colonic mass (Jacobs and Schneeman, 1981). This effect may be due to the bulking effect of fiber (for less fermentable fibers) in combination with the trophic effect of the fermentation products on mucosal cell proliferation. Upregulation of colonic mass has been observed with both mixed fibers and inert kaolin in germ-free rats, indicating that absorption of fermentation products is not the sole mechanism responsible (Goodlad et al., 1989).

Studies assessing the effect of fiber on cell proliferation have most often measured in vivo bromodeoxyuridine or ^{3}H-thymidine incorporation into colonic tissue. These studies have found that both highly fermentable and less fermentable dietary fibers affect proliferation rate (Boffa et al., 1992; Jacobs, 1983; Jacobs and Lupton, 1984; Jacobs and Schneeman 1981; Jacobs and White, 1983; Lee et al., 1993; Lupton and Jacobs, 1987; Lupton and Kurtz, 1993; Lupton et al., 1988; Palacio et al., 1989; Zhang and Lupton, 1994). Most consistently, however, increased proliferation and/or crypt proliferation zones in rats have been observed when fermentable fiber sources are supplemented. Edwards et al. (1992) showed that long-term (18 months) fiber supplementation increased cell proliferation in the proximal colon and that the compartment containing proliferating cells was expanded to higher regions of the crypts. Expanded proliferative zones were also found by Lupton and Jacobs (1987) in rats after 4 weeks of feeding.

2 *Short-Chain Fatty Acids*

2.1 Absorption

Short-chain fatty acids are readily absorbed by intestinal epithelial cells but the mechanism for absorption has not been fully elucidated. It is established that the disappearance of short-chain fatty acids from the colonic lumen is coupled with the appearance of bicarbonate ions (Ruppin et al., 1980; Umesaki et al., 1979) which implies the existence of a bicarbonate-dependent transporter system. It is most likely that short-chain fatty acids are absorbed as the protonated form, facilitating diffusion through the lipid membrane of the mucosal cell (Bugaut, 1987) but both non-ionic diffusion and carrier mediated mechanisms of uptake seem to be present (Rowe and Bayless, 1992). Jenkins and Milla (1988) found that the anion exchange mechanism in the human colon is poorly developed in infancy but is fully functional by one year of age.

Work using apical membrane vesicles from the rat colon found that butyrate uptake proceeded though a carrier-mediated anion-exchange process that was stimulated by either a bicarbonate or a pH gradient (Mascolo et al., 1991; Stein et al., 1995). The kinetics were substrate-dependent with a K_m of 27 mmol/L of butyrate. Additionally, both propionate and acetate inhibited butyrate uptake, implying a competitive uptake process for the three short-chain fatty acids (Mascolo et al., 1991). The anion-exchange inhibitor 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) did not alter butyrate uptake in either rat or rabbit colon (Mascolo et al., 1991; Sellin et al., 1993) which indicates that the carrier-mediated ion-exchange mechanism of short-chain fatty acid uptake differs from other DIDS-sensitive ion-exchange mechanisms. Reynolds et al. (1993) found that basolateral membrane transport of short-chain fatty acids proceeded through both non-ionic diffusion and a carrier-mediated anion-exchange mechanism that was DIDS-sensitive, showing that carrier-mediated apical and basolateral mechanisms differ.

Once the non-ionic form of a short-chain fatty acid is inside the epithelial cell, it is

dissociated and this causes transient intracellular acidification (Butzner et al. 1994; DeSoignie and Sellin, 1994). In response, the cell uses the apical Na^+/H^+ exchange to remove protons, which stimulates luminal sodium absorption (Butzner et al., 1994; Rowe et al., 1992; Sellin and DeSoignie, 1990), and the basolateral Na^+/K^+-ATPase to deal with sodium influx (von Engelhardt et al., 1993). At high levels of extracellular propionic acid, Feldman et al. (1989) found that intracellular K^+ accumulated and caused cell swelling, most likely due to influx of K^+ from the basolateral Na^+/K^+-ATPase.

2.2 Metabolism

Roediger (1982) published the first report of nutrient oxidation and product formation by rat colonocytes. It was shown that butyrate could be oxidized to CO_2 or could form ketone bodies; that ketone bodies could be oxidized by colonocytes; that lactate was formed from glucose; that glutamine formed either CO_2 or glutamate; and that a number of interactions between in vitro substrates may alter various product formation rates. It was suggested that butyrate may spare glutamine from oxidation in vitro. Ardawi and Newsholme (1985) subsequently examined rat colonocyte fuel utilization and found that most of the glucose utilized by colonocytes could be accounted for as lactate ($\geq$ 80%) and confirmed that ketone bodies could be oxidized by these cells. It was also found that fasting the animal reduced the activity of some enzymes required in both glucose and glutamine utilization. This initial work established the main fuels and end-products in isolated colonocytes and inspired subsequent studies of colonocyte metabolism during malnutrition or fasting (Butler et al., 1990; Butler et al., 1992; Firmansyah et al., 1989), interactions between intestinal fuels (Fleming et al., 1991), the kinetics of substrate utilization (Clausen and Mortensen, 1994; Kight and Fleming, 1993), alterations in fuel metabolism as a result of disease (Chapman et al., 1994) and alterations in oxidative metabolism as a result of dietary fiber consumption (Clausen and Mortensen, 1994; Darcy-Vrillon et al., 1993).

2.2.1 Oxidation Routes of Major Colonic Fuels

The main route of glucose oxidation in colonocytes is through glycolysis to lactate, which can be either transported out of the epithelial cell or metabolized further through acetyl-CoA to CO_2 in TCA cycle metabolism (Roediger, 1982). Glutamine oxidation proceeds through phosphate-dependent glutaminase, forming ammonia and glutamate, which can enter TCA cycle metabolism as α-ketoglutarate and be oxidized to CO_2 or leave TCA cycle metabolism as an intermediate product, such as alanine (Ardawi and Newsholme, 1985). The short-chain fatty acids, once absorbed, are co-acylated in the mitochondria (Bugaut, 1987). The short-chain acyl-CoA synthase remains to be characterized. Butyrate metabolism proceeds through ketone body formation (Ardawi and Newsholme, 1985) or the molecule can enter TCA cycle metabolism as 2 acetyl-CoA molecules and be converted to CO_2 (Roediger, 1982). Acetate is oxidized in TCA cycle metabolism through acetyl-CoA. Propionate, once activated, proceeds through methyl-malonyl-CoA to enter TCA metabolism as succinyl-CoA. Thus, both propionate and glutamine can replenish TCA cycle intermediates or can lead to synthesis of amino acids such as alanine and aspartate. However, glucose, acetate and butyrate do not generate TCA cycle intermediates but generally enter oxidative metabolism as acetyl-CoA (the presence of the malate shunt does allow formation of malate from pyruvate, although this is more energetically expensive than replenishing TCA cycle intermediates through other molecules). An overview of the routes of entry of these substrates into oxidative metabolism is shown in Figure 1-1.

The kinetics of substrate utilization by colonocytes have been assessed in simple incubation systems in which the substrate of interest is the only oxidizable compound in the buffer/salt solution (Clausen and Mortensen, 1994; Kight and Fleming, 1993). It was shown that the K_m values for saturation kinetics of short-chain fatty acids and glucose were less than 1 mmol/L (Clausen and Mortensen, 1994; Kight and Fleming, 1993), but that glutamine oxidation kinetics did not saturate, even at 20 mmol/L (Kight and Fleming, 1993). Roediger (1982) found that the addition of 10 mmol/L butyrate to incubation

media decreased colonocyte oxidation of glutamine by 40%. Fleming et al. (1991) found that including either glutamine, acetate or butyrate in incubation media reduced glucose oxidation by 25 - 50% and that glutamine oxidation was reduced by glucose, acetate and butyrate by 25 - 30%. These results suggest that both glutamine and glucose oxidation can be reduced by the presence of other oxidative fuels. In neither instance was this accompanied by a decrease in oxygen consumption.

More recently, substrate competition experiments showed that butyrate inhibits propionate oxidation (and vice versa) potentially through a competitive route of oxidation. However, glucose did not inhibit oxidation of short-chain fatty acids, nor was glucose oxidation inhibited by the presence of short-chain fatty acids (Clausen and Mortensen, 1994).

2.2.2 Alterations in Colonic Fuel Utilization

Malnutrition or fasting can alter colonocyte metabolism (Butler et al., 1990; Butler et al., 1992; Clausen and Mortensen, 1994; Firmansyah et al., 1989). Clausen and Mortensen (1994) found increased net ketone body production from butyrate in colonocytes isolated from rats that had been fasted for 72 hours. Butyrate and glucose were oxidized to CO_2 at approximately the same rate in colonocytes from fed animals. Butler et al. (1992) found that butyrate gave rise to 5 - 7 times more CO_2 than glucose in colonocytes from fasted rats. Firmansyah et al. (1989) showed that both fasting and malnutrition decreased lactate formation, and glucose, glutamine, ketone body and butyrate oxidation, but increased colonic mucosal concentrations of ketone bodies. Roediger (1980) and Chapman et al., (1994) found that colonocytes from humans with ulcerative colitis have an impaired ability to metabolize butyrate, although glucose and glutamine oxidation rates were not altered.

Recently, Darcy-Vrillon et al., (1993) assessed the effect of dietary fiber on colonocyte metabolism in pigs supplemented with 12% fiber and found no increases in substrate oxidation, although a trend toward increased butyrate oxidation (25% increase) was observed for the high fiber group. Pyruvate kinase activity was reduced slightly with the

high fiber diet. Clausen and Mortensen (1994) found that supplementing a rat chow diet with either 10% pectin or 20% tributyrin increased the K_m of butyrate oxidation in isolated colonocytes when compared to cells from chow-fed rats, but no other alterations in kinetic parameters were found. However, these studies have all used salt/buffer incubation solutions containing one or two oxidizable substrates at most. The effect of diet on colonocyte metabolism still remains to be fully elucidated in the presence of multiple substrates as would be seen in vivo.

2.3. Physiological Effects on the Colon

2.3.1 Proliferation

Short-chain fatty acids have been shown to enhance in vivo cell proliferation rates (Boffa et al., 1992; Zhang and Lupton, 1994). Luminal butyrate concentrations closely correlated with proliferation rates (Boffa et al., 1992). Cell proliferation did not increase in germ-free rats fed a fiber supplemented diet, although an increase was seen in conventional rats fed the same diet (Goodlad et al., 1989).

In isolated epithelial cells, short-chain fatty acids have the effect of decreasing cell proliferation. Sakata (1987) observed a decrease in colonocyte proliferation in the presence of short-chain fatty acids. This has also been observed in human colon cancer cell lines (Dexter et al., 1984; Gamet et al., 1992; Niles et al., 1988) and in isolated rumen epithelial cells (Sakata et al., 1980). Work assessing the in vitro effect of short-chain fatty acids on cell proliferation has found butyrate to be largely responsible for the reduced rate (Gamet et al., 1992). Frankel et al. (1994a) found a trend toward decreased proliferation in vitro when colonic mucosal biopsies obtained from humans with either ulcerative colitis, crohn's disease, or diverticulitis were exposed to 10 mmol/L butyrate. This effect was independent of culture medium osmolarity. Comparisons between normal epithelial cells and abnormal cells such as colon cancer cell lines or diseased biopsies must be done with caution. Butyrate stimulates cell differentiation into mature epithelial cells which may be

advantageous to abnormal cells but of less benefit to normal cells (Gamet et al., 1992). Comparisons between normal epithelia and colon cancer cell lines have not been reported in the literature, in part because normal colonocytes do not culture well. Osmotic regulation of cell cycle kinetics of human cells in culture has been reported and cell proliferation decreases during hypertonic stress (Pellicciari et al., 1995; Petronini et al., 1992).

Intraluminal infusion of short-chain fatty acids had a trophic effect on cell proliferation in colonic regions removed from the infusion site (Sakata and von Engelhardt, 1983). However, when rats were either vagotomized or sympathectomized, both the direct and indirect trophic effects of short-chain fatty acids vanished. Frankel et al. (1994b) also found that cecal infusion of short-chain fatty acids increased jejunal DNA, surface area and crypt depth in normal but not in denervated rats. Thus, there is evidence that short-chain fatty acids may moderate cell proliferation both directly and indirectly in vivo.

Some of the indirect trophic effects of short-chain fatty acids on the colon may be due to enhanced blood flow to the colon, which would increase the delivery of oxygen and nutrients to the region. Short-chain fatty acids dilated isolated human colonic resistance arteries (Mortensen et al., 1990) and increased blood flow to the colon in dogs (Kvietys and Granger, 1981). Short-chain fatty acids cause vasodilation of rat caudal (tail) artery in vitro (Nutting et al., 1991) and this can be blocked by DIDS (an anion exchange inhibitor). Injection of butyrate in guinea pigs mediated prostaglandin synthesis which increased contractile activity in smooth muscle and increased blood pressure (Kristev et al., 1991).

The contrast between in vivo and in vitro effects of short-chain fatty acids on colonocyte proliferation has not been explained in the literature. Exposing the colon to short-chain fatty acids in vivo is accompanied by various physiological effects which are absent in an isolated cell model. It may be that the mechanism whereby short-chain fatty acids affect proliferation has more to do with alterations in blood flow (thus nutrient delivery) or

changes in local expression and secretion of growth factors or hormones which alter proliferation rates. Work by Tappenden et al. (1995) found that jugular infusion of short-chain fatty acids in parenteral nutrition solution increased mRNA for both proglucagon and ornithine decarboxylase. Peptide products of intestinal proglucagon are thought to be enterotrophic and ornithine decarboxylase is a rate-limiting enzyme in the synthesis of polyamines, molecules which are required for cell proliferation and differentiation (Taylor et al., 1992). Denervation, vagotomy and sympathectomy reduce trophic effects of short-chain fatty acids on the intestine which suggests that short-chain fatty acids elicit signals involving the nervous system (Frankel et al., 1994b; Sakata and von Engelhardt, 1983). The in vitro model does not involve these mechanisms which may explain the contrast between proliferation results measured in vivo vs. in vitro.

2.3.2 Protein Synthesis

Häussinger et al. (1994, review) has established that protein synthesis and cell function in hepatocytes is altered by changes in extracellular osmolarity. Stoll et al. (1992) established that the rate of protein synthesis in isolated hepatocytes was osmotically regulated regardless of whether raffinose or NaCl was used to adjust incubation media osmolarity. Hyperosmolar media reduced protein synthesis in cultured SV-3T3 cells (Petronini et al., 1992).

In vitro colonocyte protein synthesis, measured by ^{3}H-leucine incorporation, did not respond to 10 mmol/L butyrate, independent of osmolarity, when measured in mucosal tissues taken from humans with normal, Crohn's and diverticulitis mucosa although it was elevated in mucosa from humans with active ulcerative colitis (Frankel et al., 1994a). Munks and Turner (1994) found that 20 mmol/L butyrate decreased heat-shock protein synthesis in drosophila, but it was not assessed whether this effect was attributable to osmotic regulation. Indeed it may be that regulation of this protein does not parallel total protein synthesis. Higashiguchi et al. (1993) found that glutamine enhanced protein synthesis in rat enterocytes, independent of osmotic regulation. It has been suggested that

the changes in intracellular pH induced by short-chain fatty acid absorption may be linked to the osmotically regulated alterations in cell function (DeSoignie and Sellin, 1994) but this hypothesis has yet to be investigated.

Rates of fractional protein synthesis in all segments of the small intestine and colon were assessed in vivo by Stein et al. (1994). It was found that various formulations of parenteral nutrition reduce fractional synthesis rates in jejunum, ileum, and both the proximal and distal colon. This suggests that increased luminal nutrient supply increases the rate of protein synthesis. Because a high-fiber diet would increase colonic luminal short-chain fatty acids, it may be that protein synthesis would be increased by a high-fiber diet. Including butyric acid in the parenteral nutrition formulation did not increase colonic protein synthesis (Stein et al., 1994). Ahnen et al. (1988) assessed in vitro ^{3}H-leucine incorporation into isolated rat colonocytes as an indicator of protein synthesis and found that less mature colonocytes exibited twice as much protein synthesis when compared to a mature population of colonocytes. The effect of diet was not assessed.

2.3.3 Oxygen Consumption

The uptake of oxygen by isolated cells is one of the best indicators of total metabolic oxidative activity, since $^{14}CO_2$ evolution yields information pertaining only to the complete combustion of the initial ^{14}C-labeled substrate. Roediger (1982) found that adding 5 mmol/L glutamine, 10 mmol/L glucose or 10 mmol/L butyrate to colonocytes in a substrate-free salt buffer increased oxygen uptake. Oxygen uptake was further increased by the addition of butyrate to either glucose- or glutamine-containing solutions. Ardawi and Newsholme (1985) also reported that oxygen consumption increased with the addition of glucose, glutamine, acetoacetate or butyrate to colonocyte incubation media, and that the addition of butyrate to glucose further increased oxygen uptake. Additionally, Fleming et al. (1991) found that butyrate enhanced oxygen uptake when added to a medium containing glucose but this effect was not seen when acetate was added, supporting their findings that butyrate was the preferred substrate for colonocytes. The kinetics

established by Clausen and Mortensen (1994) showed only minor non-competitive inhibitory effects of glucose on butyrate oxidation and of butyrate on glucose oxidation in a two substrate incubation system. The effects of fiber and short-chain fatty acids on oxygen uptake using colonocytes in complete media remains to be determined.

2.3.4 Anaplerotic Flux

Another measurement of metabolism which can be used in isolated cell systems is the evaluation of anaplerosis. The propionate (or acetate) CO_2 ratio, the glucose (or pyruvate) CO_2 ratio, and the A+T probability can be assessed using ^{14}C-labeled substrates and calculated as outlined by Kelleher (1985) (See Appendix 2 for calculations). The CO_2 ratios represent the ratio of carbons from different positions in the molecule which are converted to CO_2. A+T is the probability that a compound will complete one turn of the TCA cycle by either remaining in the cycle or leaving as a 4-carbon intermediate and re-entering via acetyl-CoA. A reduced A+T probability suggests a reduced likelihood that a molecule, once it enters oxidative metabolism, will stay in for another turn of the TCA cycle. The higher the propionate CO_2 ratio is above 1, the less completely compounds are oxidized in TCA cycle metabolism. A glucose CO_2 ratio that approaches the propionate CO_2 ratio indicates that decarboxylation of pyruvate is the only mechanism whereby pyruvate enters TCA cycle metabolism, since the labeled glucose carbons would then correspond in position to those labeled on the propionate molecule (Kelleher and Bryan, 1985). Otherwise, it can be concluded that carboxylation of pyruvate is also a significant form of entry into TCA cycle metabolism. As yet, no assessment of the effect of fiber or short-chain fatty acids on anaplerotic flux in colonocytes has been conducted.

3 Experimental Strategy

3.1 Diet Treatments

Fiber-free elemental diets result in decreased intestinal mass (Janne et al., 1977; Morin et al., 1980), an effect which may be reversed by including fiber in the diet (Jones et al.,

1989; Maxton et al., 1987). Janne et al. (1977) found that rats consuming an elemental diet had a 41% reduction in colonic mass after 1 week and a 44% reduction after 4 weeks, illustrating that long term feeding is not required to induce colonic mass reduction. No significant mass reduction was observed in any region of the small intestine. Morin et al. (1980) found that 8 days of elemental diet consumption reduced colonic DNA and protein content by 26% and 31%, respectively. Maxton et al. (1987) compared an elemental diet to a complete (intact protein and starch) defined formula diet and found that both the animals consuming elemental and complete defined formula diets had reduced ileal and jejunal mass (by approximately 30%) when compared to chow-fed animals. Colonic mass was not assessed. Barber et al. (1990) found that adding psyllium fiber to a complete defined formula diet maintained bowel mass in rats, whereas the unsupplemented diet reduced upper intestinal mass by one third as compared to chow-fed animals. Koruda et al. (1986) also found that supplementing pectin in an elemental diet for eight days following massive small bowel resection increased colonic mass by 22%. These studies all indicate that colonic mass can be modulated over short-term feeding periods by supplementing an elemental diet with fiber.

For the dietary fiber supplementation experiments described in subsequent chapters, an elemental diet is supplemented with dietary fiber at levels of either 0%, 15% or 30% by weight. A mixed fiber source (containing pea, oat and sugar-beet fiber) of intermediate fermentability (McBurney, 1991) was used. A two week diet consumption period was chosen to ensure that any adaptive changes in intestinal mass and metabolism would occur.

3.2 In vitro Evaluation of Colonocyte Metabolism

Many difficulties are associated with maintaining non-cancerous colonocyte cell lines (Baten et al., 1992), so experiments to establish metabolic activity in colonocytes have been conducted using freshly isolated colonocytes (Ardawi and Newsholme, 1985; Butler et al., 1992; Clausen and Mortensen, 1994; Darcy-Vrillon et al., 1993; Firmansyah et al.,

1989; Fleming et al., 1991; Roediger, 1982). In these studies, colonocytes were suspended in salt/buffer solutions containing one or two metabolic substrates at most. Oxidation studies have assessed the amount of $^{14}CO_2$ evolving from a ^{14}C-labeled substrate and in some cases the formation of various metabolic end-products. Although this simple system allows for isolated examination of substrate utilization, it may be that a combination of extracellular compounds and metabolic substrates are required for optimal cell function. For example, cell proliferation and protein synthesis may be limited by size of the intracellular amino acid pool in a model where no extracellular amino acids are provided. Also, if the substrate of interest is oxidized in TCA cycle metabolism through acetyl-CoA, its metabolism may be limited by a lack of appropriate compounds to replenish TCA cycle intermediates through anaplerotic flux. Others have used complete media systems in the assessment of protein synthesis in liver cells (Stoll et al., 1992), or in vitro cell proliferation determination in human colonic epithelial cells (Del Castillo et al., 1991; Frankel et al., 1994a; Scheppach et al., 1992) and transformed colon cell lines (Dexter et al. 1984; Gamet et al., 1992; Hague et al., 1995; Niles et al., 1988; Ryder et al., 1994; Siddiqui and Kim, 1984; Tanaka et al., 1989). Thus, complete culture media is more appropriate for the study of dynamic measurements of metabolism, and was employed in the experiments described herein.

4 Rationale

Physiological states such as fasting and malnutrition can alter oxidative metabolism in colonocytes (Butler et al., 1992; Firmansyah et al., 1989). This may be due to lack of lumnal nutrients. The main source of luminal nutrients in the colon are the short-chain fatty acids derived from fiber fermentation. If a fiber-free diet is fed, there will be a concurrent reduction in luminal fuel availability which could cause cells to down-regulate oxidative metabolism as they appear to do after fasting. Conversely, if a high amount of fiber is included in the diet, the increase in short-chain fatty acids from fermentation may up-regulate fuel oxidation. Should up-regulation of oxidative metabolism occur, concurrent increases in oxygen uptake and cellular biosynthesis may also take place. The

effect of diet and luminal fuel on these parameters is unknown.

Previous studies assessing the interaction of different oxidative substrates in isolated colonocytes have done so in salt/buffer incubation media containing only the two substrates in question (Fleming et al., 1991; Roediger, 1982). It was found by these researchers that the addition of butyrate to incubation buffer reduced both glutamine and glucose oxidation. Oxygen uptake was increased when butyric acid was added to glutamine- or glucose-containing incubation media (Roediger, 1982). Whether these changes reflect the use of single substrate systems which do not include other oxidizable fuels or substrate competition leading to fuel displacement has not been determined. Metabolism studies conducted in complete culture media could best answer this question. Previous in vivo measurements of protein synthesis suggest that luminal nutrients would stimulate colonic protein synthesis (Stein et al., 1994). In vivo infusion of short-chain fatty acids enhances cell proliferation (Boffa et al., 1992). The in vitro effects of short-chain fatty acids on protein synthesis and cell proliferation in isolated colonocytes remain to be elucidated.

If high-fiber diets increase the oxidative metabolism of colonocytes and cell proliferation rates, leading to a greater colonic mass with a higher metabolic rate, this would disproportionately increase intestinal energy expenditure vs. whole body energy expenditure of the organism. However, if luminal fuels such as short-chain fatty acids could displace serosally-derived fuels such as glucose and glutamine in colonocytes, an organism could benefit by sparing nitrogen-containing fuel for other organs during times of limited glutamine supply.

In summary, high-fiber diets have been shown to increase colonic mucosal mass which increases intestinal energy expenditure Malnutrition and fasting decrease colonocyte metabolism. It is unknown whether high-fiber diets and increased short-chain fatty acid availability increase colonocyte oxidative metabolism. If so, this would further increase

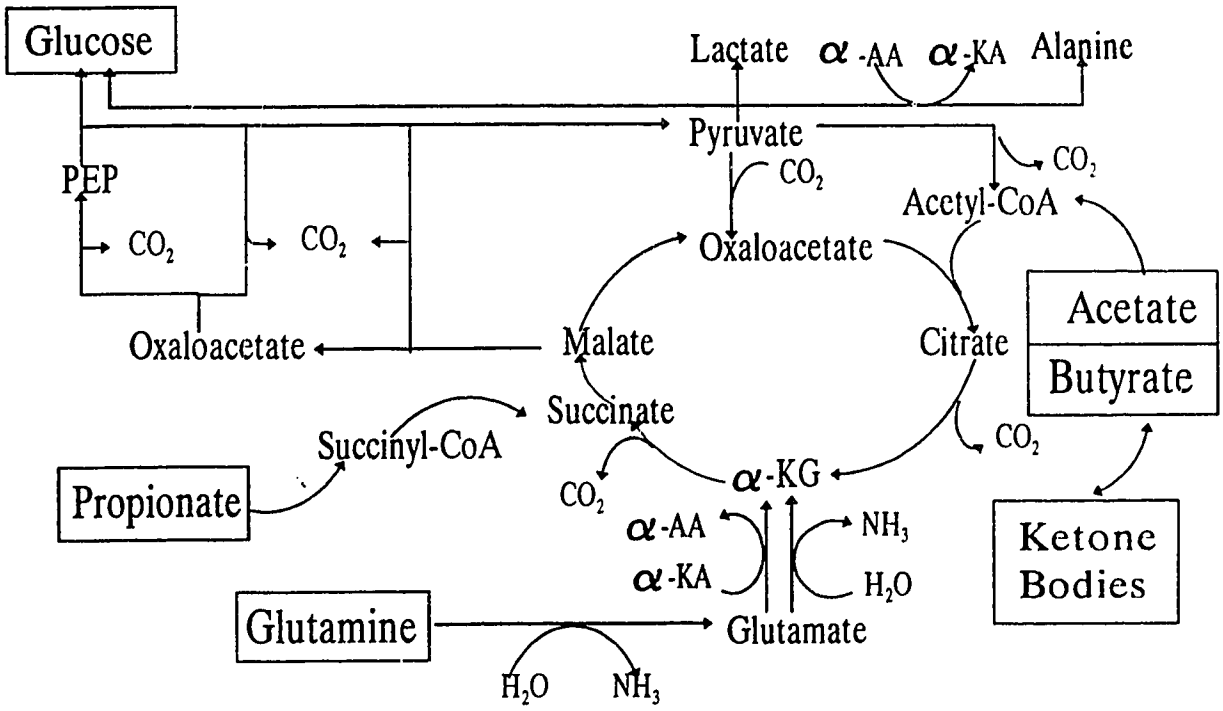
colonic energy expenditure as a proportion of whole-body energy expenditure. It is also unknown whether high-fiber diets and increased short-chain fatty acid availability induce the preferential use of short-chain fatty acids. If colonocytes do use short-chain fatty acids to displace oxidation of glucose or glutamine, this could reduce interorgan catabolism and transfer of serosally-derived nutrients to the colon.

5 Hypotheses

1. Colonocytes isolated from rats fed high-fiber vs. fiber-free diets will have:
   - increased oxidative utilization of short-chain fatty acids, glucose and glutamine
   - increased rates of oxygen uptake, cell proliferation and protein synthesis

2. In isolated colonocytes, the addition of short-chain fatty acids to incubation media will:
   - inhibit glucose and glutamine oxidation
   - increase oxygen uptake, cell proliferation and protein synthesis

Figure 1-1 Routes of entry into TCA cycle metabolism for fuels commonly used by colonocytes.



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CHAPTER 2

DIETARY FIBER INCREASES OXIDATIVE METABOLISM IN COLONOCYTES BUT NOT IN DISTAL SMALL INTESTINAL ENTEROCYTES ISOLATED FROM RATS[1,2]

INTRODUCTION

Dietary fiber, resistant starch and intestinal mucins are the main substrates for bacterial fermentation in the monogastric cecum and colon (Demigné and Rémésy, 1982; Englyst and Cummings, 1985; Stephen et al., 1981). The major products of this fermentation, short-chain fatty acids: acetate, propionate and butyrate, are almost completely absorbed by the cells of the cecum and colon, and are either utilized by intestinal cells or transported to hepatic or peripheral tissues to be metabolized (Bugaut, 1987). Concentrations and total amounts of short-chain fatty acids in cecal and colonic contents are higher when fermentable substrate levels are increased through dietary fiber supplementation (reviewed by Fleming, 1993).

Colonocyte metabolism can be altered by physiological and dietary changes. Both colonic mass and cell proliferation rate increase with short-term fiber supplementation (Goodlad et al., 1987; Jacobs and Lupton, 1984; Palacio et al., 1989). This may be due to short-chain fatty acid production from fiber fermentation since luminal short-chain fatty acid infusion also increases colonic mass and proliferation (Kripke et al., 1989; Sakata and von Engelhardt, 1983). Demigné and Rémésy (1985) found that adaptation to a high fiber diet stimulated absorption of short-chain fatty acids and minerals in rat colon. A recent study

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[1]A version of this chapter has been published. Kathleen E. Marsman and Michael I. McBurney. *J. Nutr.* 1995; **125**: 273 - 282.

[2]A version of this chapter was presented at Experimental Biology '94, April 1994, Anaheim, California and published in abstract form [Beaulieu (Marsman), K.E. and McBurney, M.I. Supplemental dietary fiber increases substrate oxidation in isolated rat colonocytes. *FASEB J.* 1994; **8**: A811 abs. #4704; McBurney, M.I. and Beaulieu (Marsman), K.E. Replacing digestible carbohydrate with dietary fiber decreases substrate oxidation in isolated rat enterocytes. *FASEB J.* 1994; **8**: A722 abs. #4188].

examining the effect of high vs. low fiber diets on oxidative metabolism by isolated pig colonocytes found a decrease in the maximal activity of pyruvate kinase due to the high fiber diet but did not find changes in substrate oxidation (Darcy-Vrillon et al., 1993). Food deprivation alters maximal activities of certain key metabolic enzymes in isolated enterocytes (Budohoski et al., 1982) and colonocytes (Ardawi and Newsholme, 1985). Colonocytes from either malnourished or food-deprived rats exhibited reduced rates of glucose, glutamine and butyrate oxidation as compared to colonocytes from animals with free access to food (Firmansyah et al., 1989). Food deprivation followed by re-feeding increased oxidation of glucose in isolated rat colonocytes (Butler et al., 1992). Reduced metabolism by isolated intestinal cells may be due to a down-regulation of oxidative metabolism when luminal nutrient availability is reduced.

The oxidative metabolism of colonocytes has been characterized in chow-fed rats using buffered salt solutions as incubation media for isolated cells (Ardawi and Newsholme, 1985; Fleming et al., 1991; Roediger, 1982). These reports have established that SCFA (butyrate > acetate > propionate) are oxidized at higher rates than glucose or glutamine in isolated colonocytes and that the addition of short-chain fatty acids (butyrate in particular) to incubation solutions decreases colonocyte glutamine oxidation.

Through dietary supplementation of fermentable fiber, exposure of colonocytes to short-chain fatty acids increases. This may cause adaptations in short-chain fatty acid utilization. We hypothesized that short-term consumption of a high-fiber diet would increase short-chain fatty acid oxidation by colonocytes and would increase the capacity of short-chain fatty acids to replace glucose and glutamine as oxidative fuels. The metabolism of and interactions among short-chain fatty acids, glucose and glutamine in both distal small intestinal enterocytes and colonocytes were examined in this study.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats of the Buffalo strain weighing 220 - 270 g were obtained from the University of Alberta Animal Science colony and housed in a temperature and humidity controlled room with a 12 hour light/dark cycle. For a brief period (5 d) prior to and during the 14 d experimental period, animals were housed individually in wire mesh-bottomed cages. The protocol was approved by the Faculty Animal Welfare Policy Committee at the University of Alberta.

Diets

Prior to the experimental period, animals consumed a nonpurified diet (Rodent Laboratory Diet PMI #5001, PMI Feeds, Inc. St. Louis, MO). During the experimental period, animals consumed either a fiber-free elemental diet (ICN Chemically Defined Diet #960346, ICN Biomedicals, Mississauga, Canada) or the same elemental diet with 300 g fiber supplement per kg in place of an equal amount of glucose and sucrose. The fiber source used was a mixture of Fibrad (Ross Laboratories, Columbus, OH) and Alphacel (ICN, Mississauga, Canada) in a ratio of 5:1. Complete composition of the experimental diets is given in Table 2-1. Fibrad is a moderately fermentable fiber source, producing 4.50 mmol SCFA/g organic matter under in vitro fermentation conditions (McBurney, 1991). The mixed fiber source was found to have a digestible energy value of 5 kJ/g as determined in vitro (Marsman and McBurney, unpublished data, Appendix 1). Animals consumed the experimental diets (21 animals consumed elemental and 14 consumed fiber) for 14 d and had free access to diet during that time. A cohort of 14 animals continued to consume the nonpurified diet under the same housing conditions as the experimental animals to determine whether metabolism of intestinal cells differed between animals fed the experimental diets vs. the nonpurified diet.

Chemicals

[U-^{14}C]-D-glucose and [1-^{14}C]-sodium propionate were obtained from ICN Biomedicals

Inc. (Irvine, CA). [1-^{14}C]-sodium butyrate, [1-^{14}C]-sodium acetate, and L-[U-^{14}C]-glutamine (>96% pure) were obtained from NEN Research Products (DuPont, Wilmington, DE). L-[U-^{14}C]-glutamic acid and L-[U-^{14}C]-aspartic acid were obtained from Amersham (Arlington Heights, IL). Glacial acetic acid, sodium propionate, *n*-butyric acid, L-glutamine, D-glucose, lithium acetoacetate, sodium-ß-hydroxybutyrate, methyl benzethonium hydroxide, sodium citrate, calcium-free bovine albumin (fraction V), DL-dithiothreitol, HEPES buffer, EDTA, Percoll, sodium lactate, and DNA (sodium salt from calf thymus) were obtained from Sigma Chemica' (St. Louis, MO). Trypan blue dye was obtained from Eastman Kodak (Rochester, NY). RPMI 1640 cell culture media (without glutamine and HEPES) and broad-spectrum antibiotic-antimycotic (10.0×10^6 U/L penicillin C sodium, 10 g/L streptomycin sulfate, 25 mg/L amphotericin B and 8.5 g/L NaCl) were obtained from GIBCO Life Technologies (Grand Island, NY).

Enterocyte and Colonocyte Isolation

Enterocytes (from the distal 30 cm of the small intestine) and colonocytes were isolated using EDTA and mechanical stirring according to the method of Roediger and Truelove (1979) as modified by Ardawi and Newsholme (1985) with modifications. Calcium-free Krebs-Ringer HEPES (KRH) buffer (Whitesell and Abumrad, 1985) was prepared in advance, suction filtered for sterility using 0.22 micron AcetatePlus filter discs (MSI, Westboro, MA) and was adjusted to pH 7.4. Antibiotic-antimycotic was added to the buffer (10 mL/L). The following solutions were prepared fresh daily, adjusted to pH 7.4 and gassed continually with O_2:CO_2 (19:1) for 15 min prior to use. Solution A was prepared by adding sodium bicarbonate (24 mmol/L) to the calcium-free KRH buffer. Solution B was prepared by adding 3 g/L calcium-free albumin and 5 mmol/L EDTA to solution A. Solution C was prepared by adding 10 g/L calcium-free albumin and 5 mmol/L DL-dithiothreitol to solution A. RPMI 1640 culture media (containing calcium) was supplemented with 25 mmol/L HEPES, 10 g/L calcium-free albumin, 1 mmol/L L-glutamine and 10 mL/L antibiotic-antimycotic on the day of use for the final cell suspension.

Animals were killed by CO_2 inhalation followed by cervical dislocation. Intestinal segments were removed, rinsed of luminal contents with cold solution A and weighed. Segments were ligated distally, inverted, distended with solution A, and ligated proximally. Distended segments were placed in plastic containers with 30 mL of solution B and gassed continually with O_2:CO_2 (19:1) in a shaking water bath (50 oscillations/min) at 37 °C for 20 min. Segments were then transferred to plastic containers with 25 mL of cold solution C and agitated constantly with a disposable plastic pipette for 10 min to free cells. Cells were kept on ice from this step onward. The distended intestinal segment was then removed and the cell suspension centrifuged for 5 min at 600 g. Cells were washed twice with oxygenated solution C and resuspended in supplemented culture media. At this stage, colonocytes from animals consuming the same diet were pooled (2 colons from the fiber diet, 3 colons from the elemental diet because fewer cells were obtained from the elemental colons). Enterocytes were not pooled for either diet group. Cells were further purified with a discontinuous percoll density gradient (15% and 40% percoll by volume in 0.15 mol/L NaCl) and centrifuged at 700 g for 15 min according to a modification of Lundqvist et al. (1992). Isolated cells were washed once and re-suspended in supplemented culture media at concentrations of 10 - 20 x 10^9 cells/L. Cells were counted using a hemocytometer and viability was estimated using trypan blue exclusion. Complete cell removal from the underlying musculature was confirmed by histological examination of segments after cell removal.

Cell Incubations

Incubations were carried out in siliconized glass tubes (Vacutainer, Beckton Dickson, Rutherford, NJ) containing 0.5 mL polypropylene microcentrifuge tubes (Fisher Scientific, Ottawa, ON, Canada) as center wells for CO_2 collection. Aliquots of the cell suspensions (100 µL containing 1 - 2 x 10^6 cells) were added to 1.0 mL of the eight incubation solutions outlined in Table 2-2. Isotopes were present in incubation solutions at levels of 6.3 - 10.0 MBq/L. L-[U-^{14}C]-glutamine was purified by elution through hydrated analytical grade anion exchange resin (AG 1-X8 200-400 mesh acetate form, Bio-Rad

Laboratories, Richmond, CA) prior to its addition to incubation solutions, as described by Wu et al. (1991). Incubation tubes were gassed with O_2:CO_2 (19:1) for 5 s, sealed with a rubber stopper and incubated for 1 h at 37 °C in a shaking water bath. Oxidation rates of glutamine, glucose and SCFA were linear with time up to 90 min for enterocytes and colonocytes from rats fed nonpurified diet. Incubations were stopped by injecting 100 µL citric acid (1.5 mol/L, pH 5.0) into the sealed tubes. Citric acid was used instead of 10 % perchloric acid because the latter led to high blank values due to the volatility of SCFA. Citric acid was as effective as perchloric acid at stopping cell metabolism and volatilizing $^{14}CO_2$ (Marsman & McBurney, unpublished data). Blanks were prepared by adding 100 µL cell suspension and citric acid simultaneously after the 1 h incubation period.

Assay of Metabolites

To collect $^{14}CO_2$, 200 µL of benzethonim hydroxide (a CO_2 trapping agent) were injected through the rubber stoppers into the center wells of incubation tubes and blanks after the incubations were stopped. Tubes were incubated at 37 °C in a shaking water bath for 1 h. Center wells were then removed, placed in scintillation vials with 4.0 mL of Hionic-Fluor scintillation fluid (Packard, Meriden, CT) and ^{14}C was counted using a Beckman LS 5801 counter (Beckman Instruments, Irvine, CA). The DNA contents of the initial cell suspensions were determined fluorometrically using the method of Prasad et al. (1972) with calf thymus DNA as a standard. Stopped incubation tubes were stored at -40 °C prior to lactate, glutamate, and aspartate analysis. Incubation tubes were thawed and centrifuged at 700 g for 15 min, and supernatants were removed for either lactate or glutamate and aspartate analysis. Lactate was determined enzymatically in neutralized incubation media using Lactate Kit #735 (Sigma Chemical, St. Louis, MO). Lactate present in blank tubes was subtracted from lactate in sample tubes. Aspartate and glutamate were separated from neutralized incubation media by ion exchange chromatography based on a method modified from Lee and Davis (1979). A Harvard 22 syringe pump (Harvard Apparatus, South Natick, MA) was used to pump solutions of increasing acetic acid concentration (0.1 - 0.5 mol/L) through analytical grade anion

exchange resin (AG 1-X8 200-400 mesh acetate form, Bio-Rad Laboratories, Richmond, CA) columns at a rate of 1.5 mL/min. Retention times for glutamate and aspartate were determined by counting 1 mL fractions of column eluent when standard amounts of [U-^{14}C]-glutamate and [U-^{14}C]-aspartate were eluted from the column. Column elution fractions (1 mL) were counted with 3.0 mL EcoLite scintillation fluid (ICN, Costa Mesa, CA). Amounts of ^{14}C labelled aspartate and glutamate present in blanks were subtracted from incubation tube values.

Calculations

$^{14}CO_2$ production was used to calculate either the total CO_2 produced or the proportion of substrate for which at least carbon-1 was converted to $^{14}CO_2$. For [U-^{14}C]-labeled substrates, the total quantity of CO_2 produced from oxidation of that substrate is reported. For [1-^{14}C]-labeled substrates, the quantity of substrate oxidized is reported, but whether each carbon in the molecule was completely oxidized cannot be determined using [1-^{14}C]-labeled compounds since oxidation to CO_2 varies with the position of the carbon and may also be influenced by the incubation system (Kight and Fleming, 1993). For [1-^{14}C]-labeled compounds, the amount of substrate in which at least carbon-1 was oxidized (*nmol substrate/(10^6 cells · h)*) can be calculated as:

$$\frac{(A-B) \times D \times E}{C \times F \times T}$$

For [U-^{14}C]-labeled compounds, the amount of product (CO_2, glutamate, or aspartate) produced from the metabolism of a substrate *(nmol CO_2/(10^6 cells · h))* can be calculated as:

$$\frac{(A-B) \times D \times E \times N}{C \times F \times T \times P}$$

where:

A = activity of product formed per incubation tube (*DPM*)

B = activity of product formed per blank tube (*DPM*)

C = activity present in incubation tube (*DPM*)

D = concentration of substrate in final incubation volume (*nmol/L*)

E = final incubation volume (*L*)

F = number of cells in incubation tube ($x10^6$ *cells*)

N = number of carbons per molecule of substrate

P = number of carbons per molecule of product

T = incubation time (*h*)

Statistical Analysis

One observation was determined as the mean of triplicate measurements of either oxidation or product formation. Seven observations (one from each test day) comprise the means shown. Differences between means were determined using a two-way ANOVA model in the general linear model procedure in SAS (Version 6.04, SAS Institute, Cary, NC). Because of day-to-day variability between observations, test day (or experimental block) was analyzed as a main effect in the ANOVA model for a randomized complete block design. For comparisons between the experimental diets within a segment and for a particular solution, the main effects analyzed in the ANOVA model were diet and test day. For comparisons between the enterocytes and colonocytes within a diet and for a particular solution, the main effects analyzed in the ANOVA model were segment and test day. For a particular segment and within a diet group, to determine whether differences were due to the presence of either SCFA or ketone bodies in the incubation media, the main effects analyzed in the ANOVA model were solution and test day. Statistical significance was defined as $P \leq 0.05$. Values given are means ± SEM, n=7.

RESULTS

Consumption, Growth and Intestinal Segment Characteristics

Animals consumed 17.8 ± 2.8 g/d and 16.1 ± 1.4 g/d of the elemental diet and the fiber diet, respectively. Animals gained 2.4 ± 0.6 g/d and 1.9 ± 0.9 g/d on the elemental diet and the fiber diet, respectively. Neither diet consumption rate nor weight gain differed between groups. The distal small intestinal segment (30 cm) from which enterocytes were isolated weighed 2.1 ± 0.2 g and 2.1 ± 0.2 g for the elemental and the fiber diet groups, respectively. Although the small intestinal segment mass did not differ with diet, colonic mass was less for animals consuming the elemental diet (1.4 ± 0.1 g) than for those consuming the fiber diet (1.8 ± 0.1 g). At the end of the 1 h incubation period, the viabilities of isolated cells from animals consuming the elemental diet (enterocytes, 93.4%; colonocytes, 92.9%) and animals consuming the fiber diet (enterocytes, 93.9%; colonocytes, 93.1%) did not differ. The DNA content of the isolated cells averaged 7.59 ± 0.52 mg/10^6 cells for enterocytes and 6.91 ± 0.52 mg/10^6 cells for colonocytes and did not differ between diet groups.

Metabolic Changes Due to Diet

Although there was a trend toward lower substrate oxidation for all SCFA in enterocytes from fiber-fed animals vs. elemental-fed animals, only the acetate oxidation rate was significantly affected (30% lower) by fiber consumption (Figure 2-1). Fiber supplementation led to higher oxidation rates by isolated colonocytes for all SCFA tested (acetate, 51%; propionate, 39%; butyrate, 22%) (Figure 2-1). Rates of colonocyte CO_2 production from glutamine and glucose were also higher (46% and 45%, respectively) (Figure 2-2).

In enterocytes, the proportions of glutamine from the 1 mmol/L incubation solution that could be accounted for as either CO_2, aspartate or glutamate, were 3.11 ± 0.78 % and 3.64 ± 0.36 % for the fiber group and the elemental group, respectively. These means did not differ between diets. The rates of formation of CO_2 (Figure 2-2), glutamate and

aspartate (Figure 2-3) from glutamine were not altered by diet, regardless of whether SCFA were included in the incubation solution. Lactate production by enterocytes was lower in the fiber group, only when SCFA and ketone bodies were absent from the incubation media (Figure 2-4).

In colonocytes, the conversion rates of glutamine to either CO_2 (Figure 2-2) or glutamate (Figure 2-3) but not aspartate (Figure 2-3) were affected by diet. Although the rate of CO_2 formation from glutamine in colonocytes was greater for the fiber-fed group, glutamate formation from glutamine was lower. Production of CO_2, glutamate and aspartate from glutamine by colonocytes used, in total, 0.40 ± 0.07 % and 0.43 ± 0.09 % of the glutamine in solution for the fiber group and the elemental group, respectively, and these means were not different. Glutamate was the major fate of glutamine carbons in the incubation solutions for both colonocytes and enterocytes regardless of diet, although this metabolite was formed in far greater quantities in enterocytes than colonocytes. No change due to diet was found in colonocyte lactate production (Figure 2-4).

Substrate Interactions

In enterocytes, the fiber group had less lactate production than the elemental group. However, this difference was only significant when ketone bodies and short-chain fatty acids were absent from the incubation media (solution 2, Table 2-2). This trend was present for enterocytes in incubation solutions containing short-chain fatty acids and ketone bodies (solutions 4 and 8, Table 2-2), but was not significant ($0.1 \geq P \geq 0.05$). Including short-chain fatty acids in the incubation media led to a 24% higher glucose oxidation rate by enterocytes for the elemental group only (Figure 2-2). Glutamine conversion to CO_2 by enterocytes was not altered by including short-chain fatty acids in incubation media (Figure 2-2), nor was glutamine conversion to glutamate or aspartate affected (Figure 2-3).

The presence of ketone bodies (KB) or short-chain fatty acids in the incubation media did

not alter enterocyte lactate production (Figure 2-4). However, including ketone bodies in the incubation media lessened diet differences in enterocyte acetate oxidation (nmol acetate/(10^6 cells · h)) (elemental -KB, 1.96 ± 0.39; fiber -KB, 1.38 ± 0.19; elemental +KB, 1.84 ± 0.39; fiber +KB, 1.42 ± 0.24) and diet differences were no longer statistically significant.

Neither colonocyte glucose nor glutamine oxidation rates were affected by the presence of short-chain fatty acids in the incubation media (Figure 2-2). When ketone bodies were included in the incubation media, diet differences in colonocyte acetate oxidation were no longer statistically significant (nmol acetate/(10^6 cells · h)) (elemental -KB, 2.90 ± 0.55; fiber -KB, 4.38 ± 0.71; elemental +KB, 2.81 ± 0.53; fiber +KB, 3.39 ± 0.51). Including ketone bodies in the incubation media resulted in a 23% lower colonocyte acetate oxidation rate for the fiber group, but did not significantly alter acetate oxidation in the elemental group.

Segment Differences

Rates of acetate and butyrate oxidation were greater (acetate 48%; butyrate 894%) for colonocytes than enterocytes in the elemental-fed animals but propionate oxidation rates did not differ between segments (Figure 2-1), nor did the rates of glucose oxidation (Figure 2-2). Regardless of whether short-chain fatty acids (SCFA) were present in incubation media, the rates of glutamine oxidation were lower in colonocytes than enterocytes (+SCFA, 34%; -SCFA, 38%) for the elemental-fed animals (Figure 2-2). Differences in substrate metabolism between enterocytes and colonocytes were more pronounced for the fiber-fed animals than for the elemental-fed animals. For the fiber-fed animals, rates of short-chain fatty acid oxidation were greater (acetate, 218%; propionate, 161%; butyrate 2175%) in colonocytes than enterocytes (Figure 2-1). The rate of glucose oxidation was 109% greater in colonocytes than in enterocytes from the fiber-fed animals (Figure 2-2). Glutamine oxidation did not differ between segments for the fiber-fed animals (Figure 2-2).

Glutamate production from glutamine by colonocytes was only 9% of the production by enterocytes for elemental-fed animals, regardless of the presence or absence of short-chain fatty acids in incubation media (Figure 2-3). Aspartate production was lower (+SCFA, 60%; -SCFA, 43%) in colonocytes than in enterocytes for the elemental-fed rats (Figure 2-3). Lactate production rates did not differ between enterocytes and colonocytes for animals consuming the elemental diet (Figure 2-4).

For fiber-fed animals, glutamate production from glutamine by colonocytes was only 7% of the enterocyte value both in the presence and absence of short-chain fatty acids in incubation media, but aspartate production was not different between segments (Figure 2-3). In contrast to the elemental-fed animals, lactate production rates for the fiber-fed group were much greater in colonocytes than enterocytes (Figure 2-4).

Nonpurified Diet Animals

Enterocytes and colonocytes isolated from rats fed nonpurified diet had substrate oxidation and product formation rates similar to those of cells isolated from animals fed either of the purified experimental diets. Oxidation rates by cells from animals consuming the nonpurified diet were not different from the oxidation rates by cells from fiber-fed animals for any of the substrates tested in either enterocytes or colonocytes.

Enterocyte short-chain fatty acid oxidation rates for animals consuming the nonpurified diet were (nmol substrate/(10^6 cells · h)): acetate, 1.81 ± 0.36; propionate, 0.89 ± 0.16; butyrate, 0.32 ± 0.07. Rates of enterocyte glutamine and glucose oxidation were (nmol CO_2/(10^6 cells · h)) 8.40 ± 1.40 and 8.10 ± 1.56, respectively, in the presence of short-chain fatty acids. Colonocyte short-chain fatty acid oxidation rates for animals fed the nonpurified diet were (nmol substrate/(10^6 cells · h)): acetate, 4.10 ± 0.49; propionate, 1.32 ± 0.14; butyrate, 6.54 ± 1.08. Colonocyte glutamine and glucose oxidation rates were (nmol CO_2/(10^6 cells · h)) 5.90 ± 0.60 and 12.3 ± 1.80, respectively, in the presence of short-chain fatty acids.

DISCUSSION

Increased rates of colonocyte short-chain fatty acids, glutamine and glucose oxidation, and increased disparity between oxidation rates of distal small intestinal enterocytes and colonocytes were found with fiber consumption.

Chemically defined elemental diets cause intestinal atrophy in both the distal small intestine and colon (Morin et al., 1980). We supplemented a chemically defined diet with fiber to evaluate whether fiber could attenuate this atrophy. Our diets contained 0% and 30% fiber, replacing glucose and sucrose with fiber to formulate the 30% fiber diet. The extreme level of fiber used in this study was chosen to ensure that diet differences, should they exist, would be detected. The absence of complex carbohydrates in the elemental diet eliminated the possibility of resistant or malabsorbed starch fermentation in the large intestine on the fiber-free diet. Because the fiber was supplemented in place of digestible carbohydrate, no dilution of the protein content of the diet occurred, thus reducing the likelihood of coprophagy.

We assessed the effect of the elemental vs. the fiber diet on enterocytes from the distal small intestine to determine whether or not the effect of fiber was specific to the colon or if other regions of the intestine which are not directly exposed to luminal short-chain fatty acids from fiber fermentation are also influenced. The trend toward decreased enterocyte acetate oxidation and lactate formation for animals fed the fiber diet may be explained by the decreased digestible carbohydrate consumption by the fiber-fed group. Enterocytes from rats denied access to food for 48 h had reduced glycolytic and citric-acid cycle enzyme activities than enterocytes from rats in the fed state (Budohoski et al., 1982). The down-regulation of oxidative metabolism in enterocytes from fiber-fed rats is most likely a response to reduced small intestinal nutrient availability rather than an effect of fiber consumption per se. Thus, although fiber consumption increased oxidation rates in colonocytes, this effect was specific for the colon.

Darcy-Vrillon et al. (1993) reported that fiber supplementation did not change substrate oxidation rates in pig colonocytes. Although they found butyrate and glutamine oxidation rates were approximately 25% and 10% greater (respectively) on the high-fiber diet, these increases were not statistically significant. Their experimental diets contained 4% and 16% fiber, exchanging cornstarch for fiber. Small but significant amounts of cornstarch can reach the large bowel through starch malabsorption or because of the presence of resistant starch (Morand et al., 1992). Their low-fiber diet contained more cornstarch (550 g/kg diet) than the high fiber diet (430 g/kg), which may have made the fermentable load more equivalent between diets.

Rates of oxidation for acetate, propionate, butyrate and glucose but not glutamine were higher in colonocytes than in enterocytes. This is consistent with the work of others, which compared oxidative metabolism of cells isolated from colon vs. jejunum in rats consuming a nonpurified diet (Fleming et al., 1991). Interestingly, in our study, propionate and glucose oxidation rates did not differ between segments for animals consuming the elemental diet, illustrating that the fiber diet amplified regional disparity along the gastrointestinal tract. Fiber fermentation and short-chain fatty acid production may be involved in maintaining this increasing gradient of substrate oxidation along the intestinal tract.

A decreasing gradient of glutamate formation from distal small intestine to colon was found in this study. The observation that glutamate formation is greater in enterocytes than colonocytes is consistent with reports of increased phosphate-dependent glutaminase enzyme activity in the small intestine as compared to the colon (Ardawi and Newsholme, 1985; Budohoski et al., 1982). An increasing gradient in lactate production from distal small intestine to colon was found in the fiber-fed animals only. This was consistent with the glucose oxidation gradient, found only in the fiber-fed animals.

The concentrations of acetate, butyrate and glucose but not glutamine used in this study

have been shown to saturate oxidation kinetics in both jejunal enterocytes and colonocytes in a buffered salt solution system (Kight and Fleming, 1993). Consistent with the work of Kight and Fleming (1993), we have found that no glutamine concentration below 20 mmol/L saturated oxidation kinetics in our incubation system for colonocytes from animals fed a nonpurified diet. The concentration of glutamine used in our incubation media (1 mmol/L) was chosen to approximate physiological concentrations, rather than to provide a saturating concentration.

We have examined enterocyte and colonocyte substrate oxidation rates when cells from animals fed nonpurified diets were incubated either in supplemented RPMI 1640 (as used in this study) or in KRH salt solution containing albumin, bicarbonate, short-chain fatty acids, glucose and glutamine at the same levels as the supplemented RPMI 1640. We found that butyrate, glutamine and glucose oxidation rates did not significantly differ between RPMI 1640 vs. KRH in colonocytes, and glucose and butyrate oxidation did not differ between RPMI 1640 vs. KRH in enterocytes. Enterocyte glutamine oxidation was 27 % lower in RPMI 1640 vs. KRH ($P \leq 0.05$), which may be because RPMI 1640 contains other amino acids whereas glutamine was the only amino acid in the supplemented KRH.

In the presence of no other oxidizable substrates, glutamine carbons would be used both for acetyl-CoA and to replenish 4-carbon citric acid cycle intermediates, in addition to other cellular functions. Should other sources of acetyl-CoA become available, glutamine would not be the sole source of oxidizable carbon. Therefore, we used complete cell culture media containing glucose, short-chain fatty acids, glutamine and other amino acids, to prevent short-chain fatty acid displacement of glutamine or glucose from occurring as an artifact of an inadequate supply of alternative substrates.

When incubating colonocytes in buffered salt solutions with only one or two metabolizable substrates, adding butyrate decreased the amount of glutamine oxidized (Fleming et al.,

1991; Roediger, 1982). This led to the hypothesis that short-chain fatty acids may displace glutamine as an oxidative substrate and thus exert a glutamine-sparing effect. However, because these previous studies did not use complete media for incubations, cell metabolism may have been limited by the absence of other nutrients (eg.- the absence of amino acids would limit protein synthesis). In the present study, we found that supplementing short-chain fatty acids at 5 mmol/L did not reduce glutamine oxidation This is likely due to the presence of many different oxidizable substrates in the media at levels meeting or exceeding physiological concentrations.

Glucose oxidation was not decreased by adding short-chain fatty acids to the incubation solution. Interestingly, in enterocytes isolated from elemental diet group, the presence of short-chain fatty acids in the incubation medium led to a 15% higher rate of glucose oxidation. This contradicts previous reports (Ardawi and Newsholme, 1985; Fleming et al., 1991) which examined oxidation of glucose alone (in a buffered salt solution) and in the presence of butyrate and found that adding butyrate decreased glucose oxidation. In our study, the higher enterocyte glucose oxidation rate when short-chain fatty acids were present in the media may have been due to propionic acid, which could help replenish 4-carbon citric acid cycle intermediates, through succinyl-CoA formation, allowing more oxidation via the citric acid cycle.

In colonocytes from animals fed the elemental diet, formation of glutamate from glutamine was lower by 10% when short-chain fatty acids were present in the media. Aspartate formation from glutamine by colonocytes from the fiber-fed animals was lower by 35% when short-chain fatty acids were included in the media. Although there was an effect of short-chain fatty acids on glutamate and aspartate formation (or perhaps removal or subsequent metabolism) from glutamine, short-chain fatty acids did not spare glutamine carbons from oxidation.

The lower formation rate of glutamate from glutamine by colonocytes from the fiber-fed

vs. the elemental-fed animals indicates that either the rate of glutamine conversion to glutamate was lower or that subsequent rate of metabolism of glutamate was higher in the fiber-fed animals. The 46% higher glutamine oxidation rate for fiber-fed animals suggests that the latter explanation is responsible in part for the reduced glutamate production.

A lower rate of colonocyte acetate oxidation was observed when ketone bodies were included in the incubation media for the fiber-fed animals only. This may have been due to displacement of acetate by ketone bodies because of the similar route of entry of these compounds into citric acid cycle metabolism. Alternatively, ketone bodies may act as signals to decrease metabolism (Robinson and Williamson, 1980). This finding is consistent with other work demonstrating that ketone bodies can inhibit glucose utilization (Henry et al., 1990). Because isolated colonocytes can produce ketone bodies in the presence of butyrate (Ardawi & Newsholme, 1985; Roediger, 1982) an inhibition of butyrate oxidation would also be expected in the presence of ketone bodies. Since the acetate sparing effect of ketone bodies was only significant for colonocytes from fiber-fed animals, it may be related to the higher luminal exposure to short-chain fatty acids, and the higher rate of acetate oxidation in colonocytes from animals fed the fiber diet vs. the elemental diet.

The increase in colonic mass with fiber supplementation is associated with an increased crypt cell production rate (Jacobs and Lupton, 1984). Fermentable fiber consumption results in greater increases in crypt cell production rates than does the consumption of non-fermentable sources of inert bulk, such as kaolin (Goodlad et al., 1987). Characterization of isolated colonocytes taken from crypt (immature cells) or villous (mature cells) has established that immature colonocytes have increased rates of protein synthesis, and proliferation ([^{3}H]-thymidine incorporation) vs. mature colonocytes, indicative of increased metabolic activity (Ahnen et al., 1988). Thus, an increase in proliferative cells in the crypt may have contributed to the observed increase in oxidation rates for the fiber-fed group in this experiment.

It remains to be determined whether the changes observed in this study are due to the adaptation of colonocyte oxidation capacity when luminal short-chain fatty acid supply increases, or are due to cellular changes associated with increased colonocyte proliferation. The mechanism by which these changes occur is an area worthy of further investigation.

TABLE 2-1

Composition of the experimental diets

Ingredient	Elemental	Fiber
	g/kg diet	
Amino Acid Mix[1]	178.5	178.5
Corn Oil	100.0	100.0
Glucose	443.0	243.0
Sucrose	221.5	121.5
Alphacel	--	50.0
Fibrad[2]	--	250.0
Mineral Mix[3]	50.0	50.0
Vitamin Mix[4]	7.0	7.0
Digestible Energy[5] *(kJ/g)*	17.5	14.2

[1]Supplied (g/kg diet): L-arginine HCl, 13.5; L-histidine HCl·H_2O, 4.5; L-isoleucine, 8.2; L-leucine, 11.1; L-lysine HCl, 18.0; L-methionine, 8.2; L-phenylalanine, 11.6; L-threonine, 8.2; L-tryptophan, 1.7; L-valine, 8.2; L-alanine, 3.5; L-aspartic acid, 3.5; L-glutamic acid, 35.0; glycine, 23.3; L-proline, 3.5; L-cystine, 3.5; L-serine, 3.5; L-tyrosine, 3.5; L-asparagine, 6.0.

Continued...

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[2]Ross Laboratories, Columbus, OH. Contained a mixture of pea fiber, oat fiber, sugar-beet fiber, xanthan gum and soy lecithin.

[3]Supplied (per kg diet): $CaCO_3$, 14.64 g; $CaHPO_4 \cdot 2H_2O$, 215 mg; KH_2PO_4, 17.15 g; NaCl, 12.53 g; $MgSO_4 \cdot 7H_2O$, 4.99 g; Fe(III)citrate $\cdot 5H_2O$, 311 mg; $MnSO_4 \cdot H_2O$, 60 mg; $ZnCl_2$, 10 mg; $CuSO_4$, 78 mg; KI, 250 µg; $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 1.2 mg; $Na_2SeO_3 \cdot 5H_2O$, 750 µg.

[4]Supplied (per kg diet): retinyl acetate, 1.376 mg; ergocalciferol, 10 µg; DL-α-tocopherol acetate, 100 mg; menadione, 500 µg; thiamin HCl, 5.0 mg; riboflavin, 5.0 mg; nicotinamide, 25 mg; calcium pantothenate, 20 mg; pyridoxine HCl, 5.0 g; cyanocobalamin, 30 µg; folic acid, 500 µg; choline chloride, 2.0 g; biotin, 200 µg; ascorbic acid, 50 mg.

[5]Digestible energy was calculated from gross energy measurements of dietary components and in vivo digestible energy measurements for the fiber sources.

TABLE 2-2

Substrate concentrations that enterocytes and colonocytes were exposed to in incubation solutions[1]

Component	Solution number							
	1	2	3	4	5	6	7	8
	mmol/L							
Glutamine	**1.0***	1.0	**1.0***	1.0	1.0	1.0	1.0	1.0
Glucose	11.0	**11.0***	11.0	**11.0***	11.0	11.0	11.0	11.0
Acetate	--	--	5.0	5.0	**5.0***	5.0	5.0	**5.0***
Propionate	--	--	5.0	5.0	5.0	**5.0***	5.0	5.0
Butyrate	--	--	5.0	5.0	5.0	5.0	**5.0***	5.0
Ketone Bodies[2]	--	--	--	--	--	--	--	1.0

[1]All solutions were made with RPMI 1640 culture media (pH 7.4) and contained 10 g/L bovine serum albumin and 25 mmol/L HEPES buffer.

[2]Ketone bodies were provided as acetoacetate (0.3 mmol/L) and ß-hydroxybutyrate (0.7 mmol/L).

*Indicates ^{14}C labeled substrate in the solution.

Figure 2-1 Short-chain fatty acid oxidation by enterocytes and colonocytes from rats fed elemental vs. fiber diets. Oxidation (nmol substrate oxidized) was quantified by measuring $^{14}CO_2$ production from [1-^{14}C]-acetate (5 mmol/L), [1-^{14}C]-propionate (5 mmol/L) and [1-^{14}C]-butyrate (5 mmol/L) during a 1 h incubation at 37 °C. Complete composition of incubation solutions can be found in Table 2-2, solutions 5 - 7, respectively. Values are means ± SEM, n=7. Within a substrate, means with different letters are different ($P \leq 0.05$). *Indicates mean is different from enterocyte value for that substrate and diet ($P \leq 0.05$).

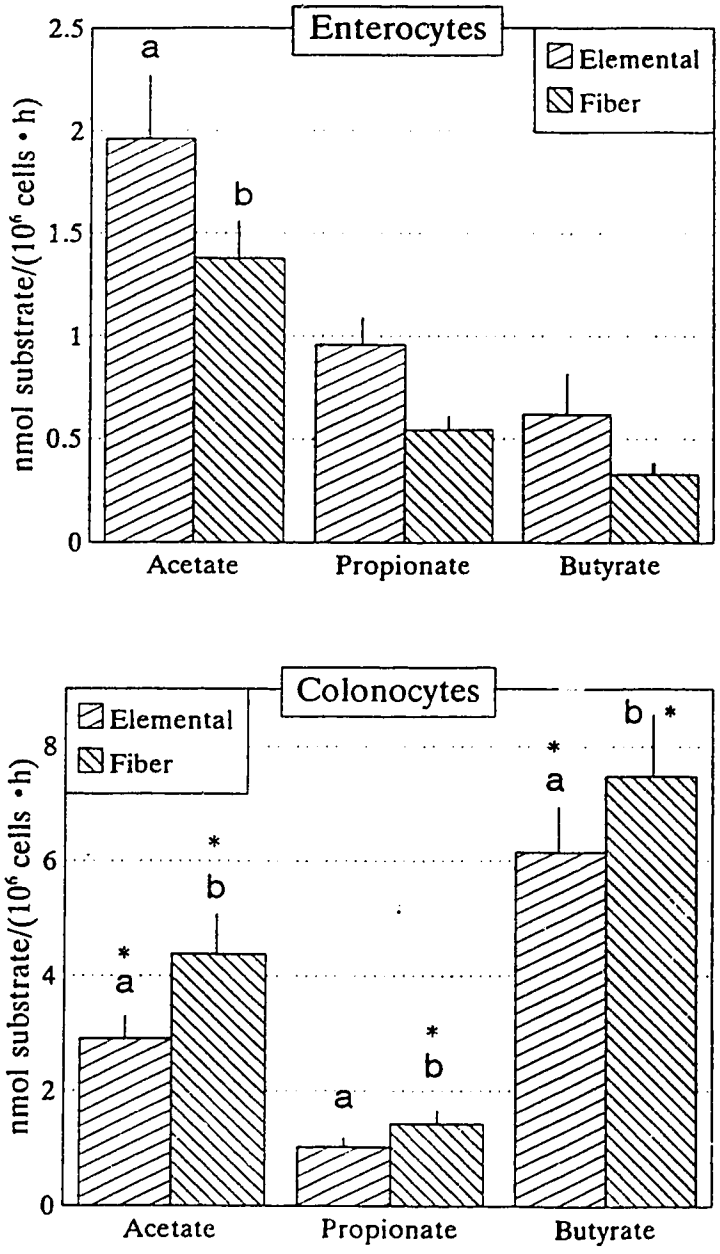


Figure 2-2 The effect of short-chain fatty acids (SCFA) on CO_2 production from glutamine and glucose oxidation by enterocytes and colonocytes from rats fed elemental vs. fiber diets. CO_2 production was quantified by measuring $^{14}CO_2$ from [U-^{14}C]-glutamine (1 mmol/L) and [U-^{14}C]-glucose (11 mmol/L) oxidation during a 1 h incubation at 37 °C. Acetate, propionate and butyrate were each present at 5 mmol/L in +SCFA solutions. Table 2-2 provides complete composition of incubation solutions 1 (glutamine -SCFA), 3 (glutamine +SCFA), 2 (glucose -SCFA) and 4 (glucose +SCFA). Values are means ± SEM, n=7. Within a solution, means with different letters are different ($P \leq 0.05$). *Indicates mean is different from enterocyte value for that solution and diet ($P \leq 0.05$). **Indicates mean is different from -SCFA for that diet ($P \leq 0.05$).

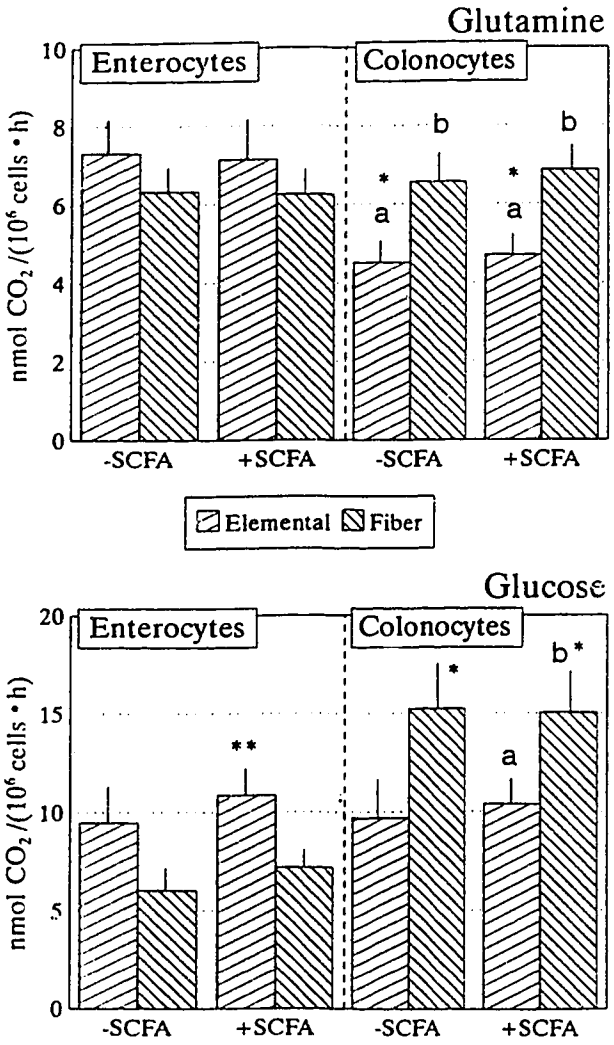


Figure 2-3 The effect of short-chain fatty acids (SCFA) on glutamate and aspartate formation from glutamine by enterocytes and colonocytes from rats fed elemental vs. fiber diets. Product formation was measured as [^{14}C]-glutamate and [^{14}C]-aspartate produced from [U-^{14}C]-glutamine (1 mmol/L) during a 1 h incubation at 37 °C. Acetate, propionate and butyrate were each present at 5 mmol/L in +SCFA solutions. Table 2-2 provides complete composition of incubation solutions 1 (glutamine -SCFA) and 3 (glutamine +SCFA). Values are means ± SEM, n=7. Within a solution, means with different letters are different ($P \leq 0.05$). *Indicates mean is different from enterocyte value for that solution and diet ($P \leq 0.05$). **Indicates mean is different from -SCFA solution for that diet ($P \leq 0.05$).

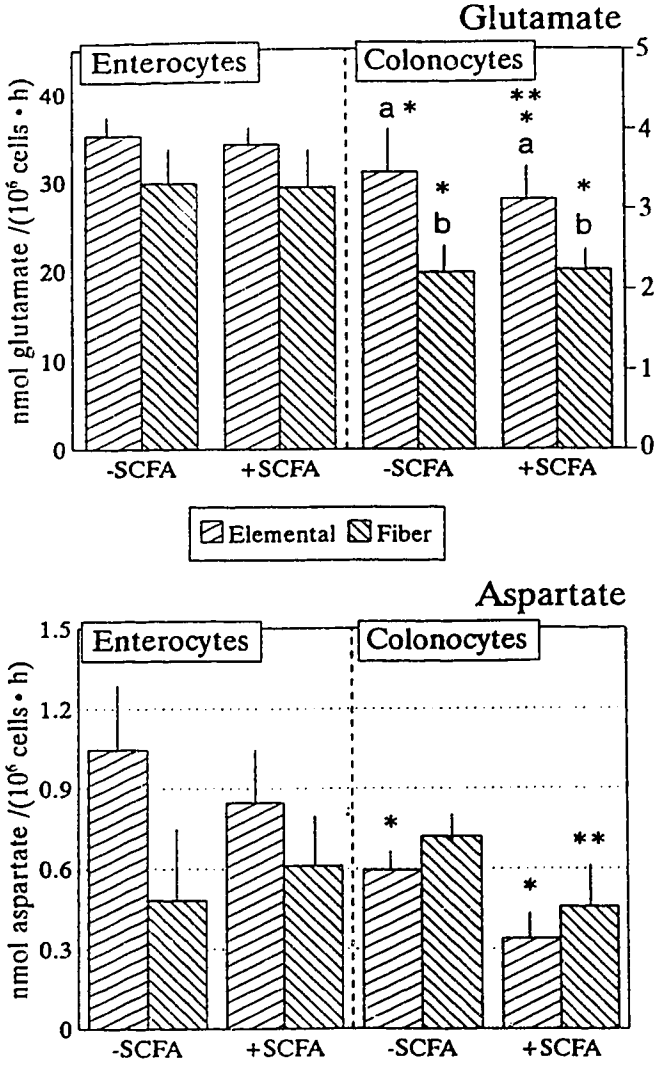
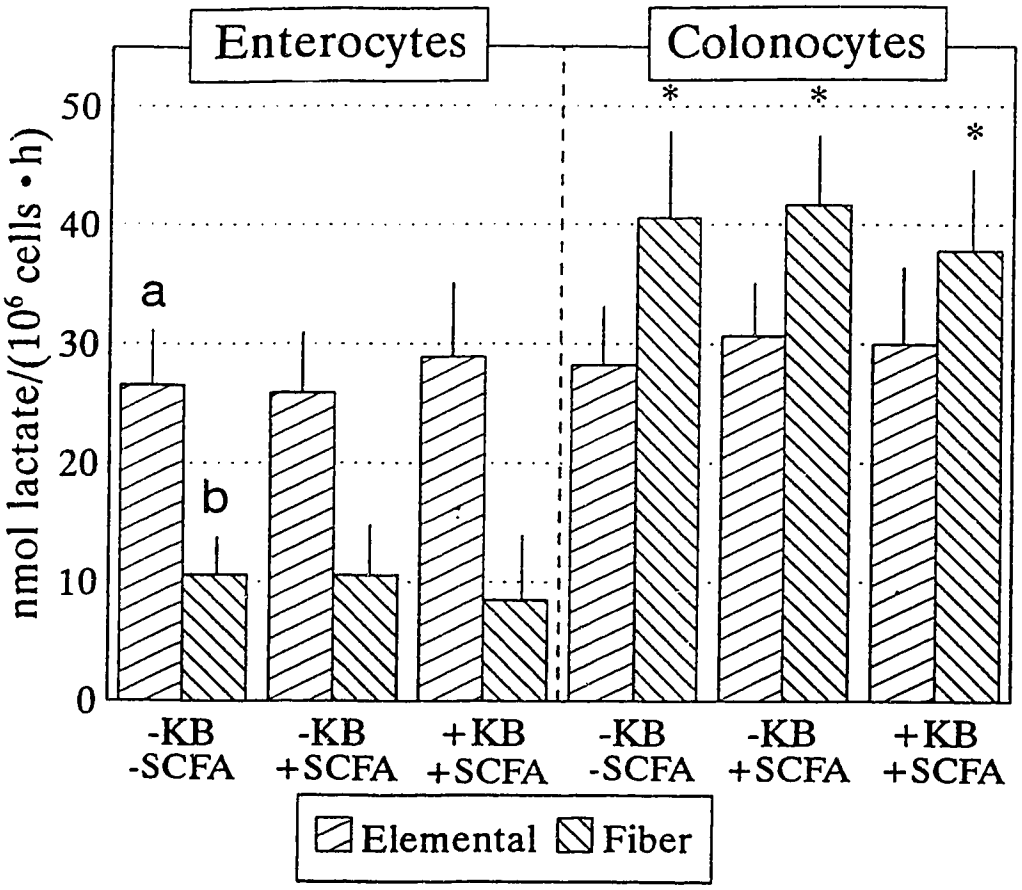


Figure 2-4 The effect of ketone bodies and short-chain fatty acids on lactate formation by enterocytes and colonocytes from rats fed elemental vs. fiber diets. Formation was measured as lactate present in incubation media after 1 h at 37 °C. Acetate, propionate and butyrate were each present at 5 mmol/L in +SCFA solutions; ketone bodies were present in +KB solutions at 1.0 mmol/L. Table 2-2 provides complete composition of incubation solutions 2 (-KB -SCFA), 4 (-KB +SCFA), and 8 (+KB +SCFA). Values are means ± SEM, n=7. Within a solution, means with different letters are different ($P \leq 0.05$). *Indicates mean is different from enterocyte value for that solution and diet ($P \leq 0.05$).



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CHAPTER 3

INFLUENCE OF DIETARY FIBER CONSUMPTION ON OXIDATIVE METABOLISM AND ANAPLEROTIC FLUX IN ISOLATED RAT COLONOCYTES[1,2]

INTRODUCTION

Short-chain fatty acids are the main products of dietary fiber fermentation by microbes in the monogastric colon (Fleming, 1993). The two, three and four carbon fatty acids, acetate, propionate and butyrate are readily absorbed by colonocytes and either oxidized or passed on via the portal vein to hepatic or peripheral metabolism (Bugaut, 1987).

Supplemental dietary fiber results in trophic effects on the colon. Colonic mass, villous height and crypt labeling index (a marker of cellular proliferation) can be increased through supplementation with either fermentable fibers or inert bulk (Goodlad et al., 1987; Jacobs and Lupton, 1984; Palacio et al., 1989). A similar effect is shown with intraluminal colonic short-chain fatty acid infusion (Sakata and von Engelhardt, 1983). Such changes in the colonic epithelium may be associated with changes in colonocyte metabolism.

Similarly, certain physiological states such as fasting can alter colonocyte oxidative metabolism. Others have reported that oxidation rates were reduced in colonocytes isolated from food-deprived rats *vs* fed rats (Firmansyah et al., 1989). Glucose metabolism was reduced in colonocytes obtained from rats which were deprived of food, whereas re-feeding increased glucose metabolism (Butler et al., 1992). Differences also

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[1]A version of this chapter has been submitted for publication. Kathleen E. Marsman and Michael I. McBurney. Submitted to *Comp. Biochem. Physiol.* August 1995.

[2]A version of this chapter was presented at Experimental Biology '95, April 1995, Atlanta, Georg and published in abstract form [Marsman, K.E. and McBurney, M.I. Dietary fiber increases substrate oxidation but not anaplerotic flux through TCA cycle in rat colonocytes. *FASEB J.* 1995; **9**: A728 abs. #4221]. A version of this chapter was presented at Canadian Federation of Biological Societies 38th annual meeting, June 1995, Saskatoon, Saskatchewan and published in abstract form [Marsman, K.E. and McBurney, M.I. The effects of dietary fiber on oxidative metabolism, theoretical ATP yield and anaplerotic flux in isolated rat colonocytes. *CFBS Proc.* 1995; **38**: 13 abs. #019].

exist along the length of the large intestine; epithelial cells from the rat colon oxidized more glucose than those from the cecum (Fleming et al., 1991).

We have previously reported that a diet containing 300 g/kg of a mixed fiber source resulted in an increase in oxidative metabolism in isolated rat colonocytes (Marsman and McBurney, 1995). Others have shown that fiber consumption can reduce pyruvate kinase activity and glycolytic capacity (Darcy-Vrillon et al., 1993) and reduce net production of ketone bodies from short-chain fatty acids (Clausen and Mortensen, 1994). Thus, we investigated whether consuming diets containing 0, 150 or 300 g/kg fiber would result in differences in oxidation and metabolism of glucose, glutamine or short-chain fatty acids by isolated colonocytes. We also investigated whether any observed changes were due to alterations in anaplerotic flux, as measured by the CO_2 ratios method. Theoretical ATP yield was calculated and approximate contributions of individual substrates to oxidative metabolism were assessed. We hypothesized that fiber consumption would reduce anaplerotic flux. Thus since fewer intermediate compounds would enter and exit TCA cycle metabolism, the oxidation rates of glucose, glutamine and short-chain fatty acids would increase and that this alteration would lead to an increase in the theoretical ATP yield from each of the individual substrates.

MATERIALS AND METHODS

Animals and Diets

Thirty-nine male Sprague-Dawley rats weighing 300 - 350 g were obtained from the University of Alberta Biological Sciences colony and housed in a temperature and humidity controlled room with a 12 h light/dark cycle. For a period not less than 5 d prior to and during the 14 d experimental period, animals were housed individually in wire mesh-bottomed cages and were given free access to both water and diet. The protocol was approved by the Faculty Animal Welfare Policy Committee at the University of Alberta.

Prior to the experimental period, animals consumed a nonpurified diet (Rodent Laboratory Diet PMI #5001, PMI Feeds, Inc. St. Louis, MO). During the 14 d experimental period, animals consumed an elemental diet supplemented with either 0, 150 or 300 g/kg fiber in place of an amount of glucose and sucrose such that all diets had the same protein to energy ratio. The fiber source was a mixture of Fibrad (Ross Laboratories, Columbus, OH) and Alphacel (ICN, Mississauga, Canada) in a ratio of 5:1. Complete composition of the experimental diets is given in Table 3-1. The in vivo digestible energy value of the fiber mixture was determined experimentally to be 5 kJ/g (Marsman and McBurney, unpublished data, Appendix 1). Under in vitro fermentation conditions Fibrad produced 4.5 mmol SCFA/g organic matter (McBurney, 1991).

Chemicals

[2-^{14}C]-D-Glucose and [U-^{14}C]-L-glutamine were obtained from American Radiolabeled Chemicals Inc. (St. Louis, MO). [6-^{14}C]-D-Glucose was obtained from Amersham (Buckinghamshire, England). [U-^{14}C]-D-Glucose, [2-^{14}C]-sodium propionate and [1-^{14}C]-sodium *n*-butyrate were obtained from ICN Biomedicals Inc. (Irvine, CA). [1-^{14}C]-Sodium propionate was obtained from DuPont NEN Research Products (Wilmington, DE). L-[U-^{14}C]-Glutamic acid and L-[U-^{14}C]-aspartic acid were obtained from Amersham (Arlington Heights, IL). Sodium acetate, sodium propionate, sodium *n*-butyrate, L-glutamine, D-glucose, lithium acetoacetate, sodium-ß-hydroxybutyrate, benzethonium hydroxide, sodium citrate, calcium-free bovine albumin (fraction V), DL-dithiothreitol, HEPES, EDTA, Percoll, sodium lactate, and DNA (sodium salt from calf thymus) were obtained from Sigma Chemical (St. Louis, MO). Trypan blue dye was obtained from Eastman Kodak (Rochester, NY). RPMI 1640 cell culture media (without glutamine and HEPES) and broad-spectrum antibiotic-antimycotic (10.0 x 10^6 U/L penicillin C sodium, 10 g/L streptomycin sulfate, 25 mg/L amphotericin B and 8.5 g/L NaCl) were obtained from GIBCO Life Technologies (Grand Island, NY).

Colonocyte Isolation

Colonocytes were isolated using EDTA and mechanical stirring according to the method of Roediger and Truelove (1979) as modified by Ardawi and Newsholme (1985) with modifications (Marsman and McBurney, 1995). Calcium-free Krebs-Ringer HEPES buffer (Whitesell and Abumrad, 1985) was prepared in advance, suction filtered for sterility using 0.22 micron AcetatePlus filter discs (MSI, Westboro, MA) and was adjusted to pH 7.4. Antibiotic-antimycotic was added to the buffer (5 mL/L). The following solutions were prepared fresh daily, adjusted to pH 7.4 and gassed continually with O_2:CO_2 (19:1) for 10 min prior to use. Solution A was prepared by adding sodium bicarbonate (24 mmol/L) to the calcium-free Krebs-Ringer HEPES buffer. Solution B was prepared by adding 3 g/L calcium-free albumin and 5 mmol/L EDTA to solution A. Solution C was prepared by adding 10 g/L calcium-free albumin and 5 mmol/L DL-dithiothreitol to solution A. RPMI 1640 culture media (calcium-containing) was supplemented with 25 mmol/L HEPES, 10 g/L calcium-free albumin, 1 mmol/L L-glutamine and 10 mL/L antibiotic-antimycotic.

Animals were killed by CO_2 inhalation followed by cervical dislocation. Colons were removed, rinsed of luminal contents with solution A and weighed. Segments were ligated distally, inverted, distended with solution A and ligated proximally. Distended segments were placed in plastic containers with 40 mL of solution B and gassed continually with O_2:CO_2 (19:1) in a shaking water bath (50 oscillations/min) at 37 °C for 20 min. Segments were then transferred to plastic containers with 40 mL of cold solution C and agitated constantly with a disposable plastic pipette for 15 min to free cells. Cells were kept on ice from this step onward. The distended intestinal segment was then removed and the cell suspension was centrifuged for 5 min at 600 x g. Cells were washed once with supplemented culture media and pressed through a 100 mesh screen. Cells were further purified with a discontinuous percoll density gradient (40% and 15% percoll by volume in 0.15 mol/L NaCl) and centrifuged at 700 x g for 15 min according to a modification of Lundqvist et al. (1992). Isolated cells were washed once and re-suspended in

supplemented culture media at concentrations of 10 - 20 x 10^9 cells/L. Cells were visualised with trypan blue dye and counted using a hemocytometer. Viability was estimated by measuring supernatant lactate dehydrogenase activity (Del Castillo et al., 1991) using kit #228 (Sigma Chemical, St. Louis, MO). Cell removal from underlying musculature was previously confirmed by histological examination of segments after cell removal (Marsman and McBurney, 1995).

Colonocyte Incubation Procedure

The incubation media was prepared by supplementation of RPMI 1640 media with 10 g/L calcium-free bovine serum albumin, 25 mmol/L HEPES buffer, 5 mL/L antibiotic-antimycotic and the following substrates (unless otherwise stated): 1 mmol/L glutamine, 11 mmol/L glucose, 5 mmol/L acetate, 5 mmol/L propionate, and 5 mmol/L butyrate. Where ketone bodies were added to the incubation solution, they were present as 1 mmol/L lithium acetoacetate and 1 mmol/L sodium-ß-hydroxybutyrate. The concentrations given are the concentrations during the cell incubations. Isotopes were present in incubation solutions at levels of 52 - 200 MBq/L and specific activities ranged from 170 to 296 MBq/mole. L-[U-^{14}C]-Glutamine was purified by elution through hydrated analytical grade anion exchange resin (AG 1-X8 200-400 mesh acetate form, Bio-Rad Laboratories, Richmond, CA) prior to its addition to incubation solutions (Wu et al., 1991). Incubation solutions were adjusted to pH 7.4 and oxygenated with O_2:CO_2 (19:1) prior to use.

Incubations were carried out in siliconized glass tubes (Vacutainer, Beckton Dickson, Rutherford, NJ) containing polypropylene microcentrifuge tubes (Fisher Scientific, Ottawa, ON, Canada) as center wells for CO_2 collection. Aliquots of the cell suspensions (100 µL) were added to 1.0 mL of incubation solution. Incubation tubes were gassed with O_2:CO_2 (19:1) for 5 s, sealed with a rubber stopper and incubated for 45 min at 37 °C in a shaking water bath. Oxidation rates of glutamine, glucose, propionate, and butyrate were linear with time up to 90 min for colonocytes from rats fed nonpurified diet

(Marsman and McBurney, 1995). Incubations were stopped by injecting 100 µL citric acid (1.5 mol/L, pH 5.0) into the sealed tubes. Citric acid was used instead of 10 % perchloric acid because the latter led to high blank values due to the volatility of short-chain fatty acids. Citric acid was as effective as perchloric acid at stopping cell metabolism and volatilizing $^{14}CO_2$ (Marsman & McBurney, unpublished data). Blanks were prepared by adding 100 µL cell suspension and citric acid simultaneously after the incubation period.

Analytical Procedures

To collect $^{14}CO_2$, 300 µL of benzethonium hydroxide (a CO_2 trapping agent) were injected through the rubber stoppers into the center wells of incubation tubes and blanks after the incubations were stopped. Tubes were subsequently incubated at 37 °C in a shaking water bath for 1 h. Center wells were then removed, placed in scintillation vials with 4.0 mL of Hionic-Fluor scintillation fluid (Packard, Meriden, CT) and ^{14}C was counted using a Beckman LS 5801 counter (Beckman Instruments, Irvine, CA).

Acidified incubation tubes were stored at -40 °C prior to lactate, aspartate and glutamate analysis. Incubation tubes were thawed and centrifuged at 700 g for 15 min, supernatants were removed and lactate was determined enzymatically in neutralized incubation media using lactate kit #735 (Sigma Chemical, St. Louis, MO). Lactate present in blank tubes was subtracted from lactate in sample tubes.

Aspartate and glutamate were separated from neutralized incubation media by ion exchange chromatography (Lee and Davis, 1979). Solutions of increasing acetic acid concentration (0.1 - 0.5 mol/L) were used to elute incubation media through analytical grade anion exchange resin (AG 1-X8 200-400 mesh acetate form, Bio-Rad Laboratories, Richmond, CA) columns using gravity filtration. Retention times for glutamate and aspartate were determined by counting fractions of column eluent when standard amounts of [U-^{14}C]-glutamate and [U-^{14}C]-aspartate were eluted from the column. Column elution fractions (1 mL) were counted with 3.0 mL EcoLite scintillation fluid (ICN, Costa Mesa,

CA). Amounts of [^{14}C]-labeled aspartate and glutamate present in blanks were subtracted from incubation tube values.

Alkaline phosphatase activity of the cell suspension was determined spectrophotometrically using kit #245 (Sigma Chemical, St. Louis, MO). DNA content of the cell suspensions was determined fluorometrically (Prasad et al., 1972) with calf thymus DNA as a standard.

Calculations

For each solution, the amount of ^{14}C trapped in center wells of blank tubes was subtracted from the ^{14}C trapped in sample tubes prior to calculation of $^{14}CO_2$ production from each substrate. $^{14}CO_2$ production was used to calculate either the total CO_2 produced or the proportion of substrate for which the labeled carbon was converted to $^{14}CO_2$. For [U-^{14}C]-labeled substrates, the total quantity of CO_2 produced from oxidation of that substrate is reported. For substrates with the ^{14}C label present on specific carbons, the molar quantity of substrate from which the labeled carbon appeared as CO_2 is reported. For aspartate formation, the amount of ^{14}C-labeled product formed was then multiplied by the ratio of the number of carbons in the substrate to the number of carbons in the product to derive the total amount of product formed.

Theoretical quantities of ATP produced from substrate oxidation were calculated for glucose, glutamine, butyrate, and propionate based on the following values. Complete oxidation of 1 mole of glucose to CO_2 yields 38 ATP and formation of 2 lactate molecules from 1 mole of glucose yields 2 ATP. Complete oxidation of all five glutamine carbons to CO_2 yields 24 moles of ATP (Newsholme and Newsholme, 1989). Total CO_2 produced from glutamine oxidation was calculated as total moles of CO_2 produced from [U-^{14}C]-glutamine minus one mole of CO_2 for every mole of aspartate formed from [U-^{14}C]-glutamine, due to the stoichiometric production of 1 mole of CO_2 for every mole of aspartate formed from glutamine. Assuming that glutamate metabolism proceeds mainly

through aminotransferase reactions rather than glutamate dehydrogenase (Okine et al., 1995) an additional 9 moles of ATP are generated for every mole of glutamine converted to aspartate.

The propionate (acetate) CO_2 ratio, glucose (pyruvate) CO_2 ratio and the A+T probability were calculated as outlined by Kelleher (Kelleher, 1985). The ATP yield from propionic acid was calculated for propionate entering TCA cycle metabolism as succinyl-CoA and forming three moles of CO_2 from complete oxidation, producing 18 moles of ATP (Martin et al., 1983). Based on the number of moles of propionic acid for which at least C-1 was converted to CO_2, the acetate CO_2 ratio was used to calculate the frequency with which the other carbons were converted to CO_2. The ATP yield from butyrate when metabolized through 2 acetyl-CoA molecules in the TCA cycle is 27 moles of ATP per mole of butyrate (Martin et al., 1983). Based on the number of moles of butyrate for which at least C-1 was converted to CO_2, the acetate CO_2 ratio was used to calculate the frequency with which the other carbons were converted to CO_2.

Statistics

One observation was determined as the mean of replicate measurements of either oxidation, product formation, or enzyme activity. Differences among means were determined using a two-way ANOVA model followed by a Duncan's Multiple Range test in the general linear model procedure in SAS (Version 6.04, SAS Institute, Cary, NC). Because of day-to-day variability between observations, test day (or experimental block) was analyzed as a main effect in the ANOVA model for a randomized complete block design. To determine whether differences were due to the presence of either ketone bodies or the concentration of glutamine in the incubation media a paired comparisons test was used within a diet group. Statistical significance was defined as $P \leq 0.05$. Values given are means ± SEM, for n = 13, unless otherwise stated.

RESULTS

Consumption, Growth and Cell Characteristics

Animals consumed slightly greater levels of energy with the fiber diets, but this was not reflected in a significantly greater weight gain (Table 3-2). Colonic mass increased linearly with fiber level in the diet. For each 15% increment in fiber content of the diet, the colon increased in mass by 20%, as compared to the fiber-free diet (Table 3- 2). Viability and DNA content of the colonocytes in suspension were unaffected by diet (Table 3-3). Cell number correlated highly to DNA content regardless of diet ($R = 0.715$, $P \leq 0.0001$). The activities of lactate dehydrogenase and alkaline phosphatase were not different among diet treatments (Table 3-3). Lactate dehydrogenase was activity positively correlated with lactate formation ($R = 0.603$, $P \leq 0.01$).

Substrate Oxidation and Product Formation

Total CO_2 produced from glutamine oxidation was higher for the 15% fiber group than 0% group, regardless of whether glutamine was present at 1 mmol/L or 5 mmol/L in the incubation solutions (Figure 3-1). Also, for the 1 mmol/L glutamine level, the 30% diet showed the same increase.

Net glutamate formation from glutamine decreased as fiber in the diet increased (Figure 3-2). Both glutamate and aspartate formation rates were higher in the presence of 5 mmol/L glutamine vs. 1 mmol/L glutamine, regardless of diet (Figure 3-2).

Consumption of either the 15% or 30% fiber diet tended to increase oxidation rates for glucose, as compared to the 0% fiber diet, but this was only significant for carbon 2 for the 15% fiber diet (Table 3-4). For the 15% diet, increased oxidation of [2-^{14}C]-glucose (Table 3-4) by 37% was found as compared to the 0% fiber diet. Lactate formation was lower in the colonocytes from the 30% fiber diet than the 0% diet (Table 3-4).

Neither propionate nor butyrate oxidation was affected by diet (Table 3-5). However,

when ketone bodies were added to the incubation solutions at a total of 2 mmol/L, butyrate oxidation was reduced by 13% for the 0% diet, but not for either of the fiber-fed groups (Table 3-5).

Anaplerotic Flux and CO_2 Ratios

The acetate and pyruvate CO_2 ratios, as well at the A+T probability calculated from propionate oxidation in this system are presented in Table 3-6 . The CO_2 ratios represent the ratio of carbons from different positions in the molecule which are converted to CO_2. A+T is the probability that a compound will complete one turn of the TCA cycle by either remaining in the cycle or leaving as a 4-carbon intermediate and re-entering via acetyl-CoA (Kelleher, 1985). "A" is the probability that TCA cycle carbon is incorporated into citrate via acetyl-CoA. "T" is the probability that TCA cycle carbon is incorporated into citrate via oxaloacetate. A reduced A+T probability (also a measure of anaplerotic flux) translates into a reduced likelihood that a molecule, once it enters TCA cycle metabolism, will stay in for another turn of the Kreb's cycle. No significant differences among diets were found, but there was a trend toward a higher acetate CO_2 ratio and a reduced A+T probability as fiber level increases in the diet.

Theoretical ATP Yield

The theoretical ATP yields from oxidation of substrates are shown in Table 3-7. Although ATP production from glutamine increased when fiber was included in the diet at either the 15% or 30% level, this did not translate into an over-all increase in ATP production for the high-fiber diets. Dietary fiber did not influence ATP production from any other substrates. For all colonocyte suspensions, regardless of diet, glucose and butyrate were primary contributors to theoretical ATP yield, both substrates combined yielded 85 - 87% of total ATP produced by the 4 substrates assessed. Glutamine and propionate each produced between 6 - 8 % of total ATP. Therefore, glutamine and propionate are minor contributors to total theoretical ATP production in this system.

DISCUSSION

Isolated Cell Characteristics

Cell viability and DNA content of the isolated colonocytes were unaffected by fiber level in the diet. Neither alkaline phosphatase nor lactate dehydrogenase activities of colonocyte suspensions were significantly different among diet treatments. In this study alkaline phosphatase was measured in whole-cell homogenate, and thus includes both intercellular and membrane-associated alkaline phosphatase. A ten-fold enrichment of this enzyme was found in brush-border membrane compared to mucosal homogenate (Stein et al., 1995). Ahnen et al. (1988) found that mature cells had a 60% increase in alkaline phosphatase activity compared to immature colonocytes from cell homogenates obtained by sequential isolation of colonocyte populations along the crypt-villous axis. Gamet et al. (1992) found that both propionate and butyrate increased alkaline phosphatase activity in HT29 colon cancer cells in vitro using whole cell homogenate, indicating increased cell differentiation In our study, the trend toward higher alkaline phosphatase activity as fiber level increased in the diet may indicate an increase in the maturity or differentiation of the isolated cells. However, the oxidative capacity of colonocytes distributed along the crypt-villous axis was beyond the scope of this study and remains to be examined. For no substrate did oxidation rate correlate to alkaline phosphatase activity. We have examined colons histologically and confirmed the completeness of removal of colonocytes from the underlying mucosa using this technique (Marsman & McBurney, unpublished observation).

DNA as a Denominator

Product formation, oxidation rates and theoretical ATP yields are expressed per unit DNA because of the inaccuracies in other denominators such as cell counts, measuring protein, or determining dry weight. Using this isolation method, most cells from villous and crypt regions are completely separated as individual cells, but some cells remain in clumps. Extending incubation time during the isolation procedure only marginally decreases the number of these clumps and can have the effect of reducing cell viability (Marsman &

McBurney, unpublished observation). Quantitation of DNA in a cell suspension produces highly repeatable values, whereas counting cells is more variable due to the presence of cell clumps and the number of fields that can practically be counted. Expressing the data in terms of cell protein content or cell dry weight is difficult when cells are suspended in solutions containing 10 g/L albumin, since both the protein and the dry weight contribution of the albumin alone is greater than that of the cells in suspension. Dry weight determination also requires a far greater number of cells to get accurate values and would require a greater number of animals to be used.

Glutamine Metabolism

The present results confirm our previous finding that oxidation of glutamine to CO_2 increases as fiber level in the diet increases (Marsman and McBurney, 1995). However, the increase in CO_2 formation from 1 mmol/L glutamine for both the 15% and 30% fiber diets vs. the 0% fiber diet found in this study represents a small contribution to overall cell energy (as emphasized by the theoretical ATP yield calculation). Because this increase in glutamine oxidation was not accompanied by a decrease in anaplerotic flux, the increase can not be attributed to more complete oxidation of each glutamine molecule. The concurrent decrease in net glutamate production suggests that more glutamine was oxidized to CO_2 without remaining in the glutamate pool.

Glucose and Short-Chain Fatty Acid Metabolism

Glucose oxidation was not significantly affected by fiber levels in the diet. Our previous work found that including 300 g/kg fiber in an elemental diet resulted in a 45% higher rate of total CO_2 formation from glucose vs. a fiber-free diet (Marsman and McBurney, 1995). The present study does not confirm that previous exposure to dietary fiber results in a significantly higher rate of glucose oxidation. We had also shown higher rates of short-chain fatty acid oxidation with a 30% fiber diet, but the present study showed no difference. The previous work was quantitated as oxidation per 10^6 cells, the diets fed varied in composition, and the animals used were approximately 100 g lighter. The diets

fed in our previous study were also elemental diets supplemented with the same fiber source but were different from those in the current study in that we replaced dietary sugars with a equal weight of fiber and thus the diets were neither isocaloric nor isonitrogenous. In this experiment, we predetermined the digestible energy value of the fiber source (Marsman and McBurney, unpublished data) and the diets were formulated to be identical in nitrogen to energy ratio.

Due to the differences in diet formulation, the previous experiment resulted in higher energy and nitrogen:energy intakes with the fiber-free diet. In the present study, animals consuming the 15% and 30% fiber diets consumed more energy than the fiber-free group. However, these differences in caloric consumption do not explain discrepancies between the two studies, especially since caloric restriction (fasting) resulted in a decrease in substrate oxidation by colonocytes (Firmansyah et al., 1989). However, in our previous work the high-fiber diet had more nutrients per unit energy than the fiber-free diet and this may have contributed to the increases observed previously with the high fiber diet. In that no significant diet effect was found in glucose or short-chain fatty acid metabolism, the current study concurs with the lack of diet effect observed by others (Clausen and Mortensen, 1994; Darcy-Vrillon et al., 1993).

CO_2 Ratios and A+T Probability

The CO_2 ratios method can be used to determine the probability of a molecule staying in for further oxidation once it has entered TCA cycle metabolism, or the likelihood of a molecule leaving as another intermediate product (anaplerotic flux) (Kelleher, 1985). Also, differences in the relative rates of re-entry of pyruvate carbon into TCA cycle metabolism via carboxylation vs. decarboxylation can be determined (Kelleher and Bryan, 1985). In this study, we used the C-1:C-2 propionate CO_2 ratio and the C-2:C-6 glucose CO_2 ratio which are analogous to the acetate CO_2 ratio and the pyruvate CO_2 ratio, respectively (Kelleher, 1985; MacDonald, 1993).

The acetate CO_2 ratio can be used to estimate anaplerotic flux, an indicator of the completeness of oxidation. For example, if the acetate CO_2 ratio is 2.54:1 (or 2.54), it means that for every C-2 of acetate (or propionate) oxidized to CO_2, there are 2.54 C-1 carbons oxidized to CO_2. After C-1 is removed to CO_2 in a turn through the Kreb's cycle, each subsequent turn removes one half of the remaining C-2 to CO_2. Thus we can make inferences about the completeness with which a particular molecule is oxidized during a specific incubation time period. More anaplerotic flux (a larger acetate CO_2 ratio) would mean that more molecules are entering and leaving TCA cycle metabolism as products other than CO_2 and that, once entering oxidative metabolism, molecules are less completely converted to CO_2.

Although the acetate CO_2 ratio did not differ significantly among diets, there was a trend toward less complete oxidation (increased acetate CO_2 ratio) as fiber level in the diet increased, suggesting greater anaplerotic flux with higher intakes of fiber. The A+T probability supports this observation; the probability that a molecule, once entering TCA cycle metabolism, will stay in for subsequent oxidation, was decreased as fiber level increased but this trend was not significant.

Since C-2 and C-6 from glucose are metabolized through glycolysis to C-2 and C-3 of pyruvate, respectively, they can be used to determine the pyruvate CO_2 ratio. A pyruvate CO_2 ratio that approaches the acetate CO_2 ratio indicates that C-2 and C-3 of pyruvate are metabolically indistinguishable from C-1 and C-1 of acetate in TCA cycle metabolism and that the major route of entry of pyruvate is through pyruvate dehydrogenase. The more the carboxylation of pyruvate contributes to the entry of this molecule into TCA cycle metabolism, the more the pyruvate ratio will differ from the acetate CO_2 ratio, since the labeled carbons are incorporated into different positions of the citrate molecule than are C-1 and C-2 of acetate (Kelleher and Bryan, 1985). Because we found that the pyruvate CO_2 ratio was less than the acetate CO_2 ratio, we can conclude that decarboxylation is not the only route of entry for pyruvate carbon into TCA cycle metabolism.

Short-chain fatty acid oxidation studies in isolated colonocytes have previously been done with C-1 labeled molecules (Clausen and Mortensen, 1994; Darcy-Vrillon et al., 1993; Fleming et al., 1991; Roediger, 1982) due in large part to the availability of the compounds so labeled. However, because the acetate CO_2 ratio is not 1:1, the oxidation of C-1 over-represents the other carbons in the short-chain fatty acid molecule (except, theoretically, C-3 of butyric acid) and if a measurement of total oxidation is required, the acetate CO_2 ratio should be established. This is especially important because changes in incubations media composition can also alter the acetate CO_2 ratio. Mallet et al. (1986) found that the succinate CO_2 ratio (analogous to the acetate CO_2 ratio) was decreased closer to 1:1 when more metabolic substrates were provided to enterocytes.

ATP Yield

Because acetate CO_2 ratios were taken into account to calculate theoretical ATP yield, differences between diets determined for both propionate and butyrate by ATP production were less pronounced than the differences determined using oxidation rates. Dietary preconditioning did not affect ATP yield from glucose, propionate or butyrate. Although ATP yield from glutamine increased with fiber level in the diet, when the four ATP parameters calculated (glucose, propionate, butyrate, and glutamine) were added together, the ATP generated in this system was clearly unaffected by diet.

Both ATP and CO_2 production from glutamine were greater when glutamine levels were increased from 1 to 5 mmol/L, which is supported by a previous (unpublished) observation in our lab indicating that glutamine oxidation kinetics are not saturated in this system, even up to 20 mmol/L glutamine. Kight and Fleming (1993) also found the same lack of saturation for colonocyte glutamine oxidation kinetics using a salt/buffer incubation media. However, because both the 1 and 5 mmol/L levels of glutamine used in this study surpass the normal serum levels of 0.4 - 0.6 mmol/L (Windmueller and Spaeth, 1978), the ATP from glutamine calculated in the present study probably over-estimates in vivo ATP production from glutamine. We conclude that glutamine is a minor contributor to

colonocyte energetics in this system when compared to glucose and butyrate. Roediger (1982) and later, Fleming et al. (1991) reported glutamine to be a major fuel source for colonocytes, but they used 5 mmol/L glutamine for their studies and assessed the oxidation of glutamine either in the absence of other oxidizable substrates or in the presence of one other substrate at most. We used a concentration closer to physiological levels in complete culture media. Thus, the currently accepted view that glutamine is an important fuel source for colonocytes must be re-visited. Certainly, however, the potential importance of glutamine for purposes other than for oxidative energy production in colonocytes should not be overlooked, such as the roles of glutamine in polyamine synthesis and ureagenesis. In regard to theoretical ATP yield in this complete culture media system, the relative importance of the metabolic substrates assessed is:

GLUCOSE & BUTYRATE >> GLUTAMINE & PROPIONATE

Although acetate was not examined in the current work, based on our previous work, the contribution of acetate to overall ATP yield in this system would approach that of propionate (Marsman and McBurney, 1995).

Final Conclusion

The supplementation of fiber in an elemental diet for two weeks resulted in alterations of glutamine metabolism and oxidation, but not glucose, propionate or butyrate metabolism in isolated colonocytes. However, the change in glutamine oxidation must be viewed in relation to the energy contribution of other oxidizable substrates and in this context, the increase is not energetically significant. Previous consumption of fermentable fiber sources does not seem to induce physiologically significant alterations in anaplerotic flux, ATP yield or metabolism by colonic epithelial cells. Regardless of diet, colonocytes use fuels in the preferential order of glucose & butyrate >> propionate & glutamine.

TABLE 3-1

Composition of the experimental diets

Ingredient	0% Fiber	15% Fiber	30% Fiber
	g/kg diet		
Amino Acid Mix[1]	200.0	179.3	158.6
Fat[2]	100.0	89.7	79.3
Glucose	427.2	352.0	276.7
Sucrose	213.6	176.0	138.4
Alphacel	--	25.0	50
Fibrad[3]	--	125.0	250
Mineral Mix[4]	44.1	39.5	35
Vitamin Mix[5]	12.6	11.3	10
Choline Chloride	2.5	2.2	2
Digestible Energy[6] (*MJ/kg*)	17.9	16.0	14.2
Amino Acid Content (*g/MJ*)	11.2	11.2	11.2
Nitrogen Content (*g/MJ*)	1.72	1.72	1.72

[1]Supplied (g/kg mixture): L-arginine · HCl, 67.5; L-histidine, 22.5; L-isoleucine, 41; L-leucine, 67.5; L-lysine HCl, 90; L-methionine, 41; L-phenylalanine, 58; L-threonine, 41; L-tryptophan, 13; L-valine, 47.5; L-alanine, 17.5; L-aspartic acid, 17.5; L-glutamic acid, 239.5; glycine, 116.5; L-proline, 27.5; L-cystine, 22.5; L-serine, 17.5, L-tyrosine, 22.5; L-asparagine, 30.

Continued...

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[2]Contained (g/kg mixture): hydrogenated beef tallow, 392; linseed oil 20; and safflower oil, 588. Polyunsaturated:saturated fatty acid ratio was 1.0, as determined chromatographically.

[3]Ross Laboratories, Columbus, OH. Contained a mixture of pea fiber, oat fiber, sugar-beet fiber, xanthan gum and soy lecithin.

[4]Supplied (g/kg mixture): calcium phosphate dibasic, 500; potassium citrate monohydrate, 220; sodium chloride, 74; potassium sulfate, 52; magnesium oxide, 24; ferric citrate pentahydrate 6.0; manganous carbonate, 3.5; zinc carbonate, 1.6; potassium iodate 0.01; cupric carbonate, 0.3; sodium selenite, 0.01; chromium potassium sulfate, 0.55; sucrose, 118.

[5]Supplied (per kg mixture): thiamine hydrochloride, 600 mg; riboflavin, 600 mg; pyridoxine hydrochloride, 700 mg; nicotinic acid, 3.0 g; D-calcium pantothenate, 1.6 g; folic acid, 200 mg; D-biotin, 20 mg; cyanocobalamin, 1.0 mg; retinyl palmitate (250,000 IU/g), 1.6 g; DL-α-tocopherol acetate (250 IU/g), 20 g; cholecalciferol (400,000 IU/g), 250 mg; menaquinone, 5.0 mg; sucrose, 972.9 g.

[6]Digestible energy was calculated from gross energy measurements of dietary components and in vivo digestible energy measurements for the mixed fiber source.

TABLE 3-2

Growth and Diet Consumption Parameters[1]

		0% Fiber	15% Fiber	30% Fiber
Weight Gain	*g/d*	3.3 ± 0.3	3.7 ± 0.4	3.7 ± 0.3
Diet Consumption	*g/d*	22.2 ± 0.5[a]	26.3 ± 0.5[b]	30.5 ± 0.5[c]
Digestible Energy	*kJ/d*	398 ± 9[a]	421 ± 8[b]	433 ± 6[b]
Colonic Mass	*g*	1.74 ± 0.06[a]	2.09 ± 0.06[b]	2.43 ± 0.07[c]

[1] Values are means ± SEM, n=13. Within a row, means with different letters differ significantly ($P \leq 0.05$).

TABLE 3-3

Properties of isolated colonocytes[1]

	0% Fiber	15% Fiber	30% Fiber
Cell Viability *(%)*	92.1±0.7	90.7±0.7	90.7±0.7
DNA Content *$\mu g/10^6$ cells*	6.03±0.32	6.15±0.36	5.68±0.27
Alkaline phosphatase *$\mu U/(\mu g\ DNA)$*	17.91±2.78	22.16±3.09	23.31±3.1
Lactate dehydrogenase *mU/(µg DNA)*	8.07±0.42	7.86±0.45	7.76±0.46

[1] Values are means ± SEM, n=13. Within a row, no means were significantly different ($P \leq 0.05$).

TABLE 3-4

Product formation from 11 mmol/L glucose by isolated colonocytes[1]

	0% Fiber	15% Fiber	30% Fiber
		nmol/(μg DNA · h)	
Lactate	12.4 ± 0.6[a]	12.7 ± 1.0[ab]	11.0 ± 0.6[b]
Total CO_2	2.83 ± 0.16	3.65 ± 0.44	3.29 ± 0.25
CO_2 from C-2	0.393 ± 0.021[a]	0.538 ± 0.061[b]	0.453 ± 0.033[ab]
CO_2 from C-6	0.378 ± 0.023	0.488 ± 0.057	0.420 ± 0.028

[1] Values are means ± SEM, n=13. Within a row, means with different letters differ significantly ($P \leq 0.05$).

TABLE 3-5

Oxidation rates of 5 mmol/L propionate and butyrate by isolated colonocytes[1]

	0% Fiber	15% Fiber	30% Fiber
	nmol substrate oxidized/(mg DNA · h)		
[1-^{14}C]-propionate	405.1 ± 36.9	448.4 ± 59.0	444.7 ± 72.1
[2-^{14}C]-propionate	261.5 ± 35.7*	281.2 ± 49.7*	213.0 ± 31.3*
[1-^{14}C]-butyrate	1655 ± 106	1605 ± 113	1620 ± 141
[1-^{14}C]-butyrate + ketone bodies	1471 ± 107**	1594 ± 106	1585 ± 141

[1] Values are means ± SEM. For [2-^{14}C]-propionate, n=9; for all other parameters, n=13. Within a row, no means were significantly different ($P \leq 0.05$). *Indicates values which are significantly different from [1-^{14}C]-propionate within a diet. **Indicates values which are significantly different from [1-^{14}C]-butyrate oxidation due to the addition of 2 mmol/L ketone bodies.

TABLE 3-6

The effect of diet on anaplerotic flux in isolated colonocytes[1]

	0% Fiber	15% Fiber	30% Fiber
[1-^{14}C]:[2-^{14}C]-Propionate (Acetate CO_2 Ratio)	1.87±0.28	2.20±0.34	2.54±0.44
[2-^{14}C]:[6-^{14}C]-Glucose (Pyruvate CO_2 Ratio)	1.05±0.03	1.11±0.03	1.08±0.03
A+T Probability	0.75±0.05	0.69±0.07	0.62±0.06

[1] Values are means ± SEM, n=13. Within rows, no means were significantly different ($P \leq 0.05$).

TABLE 3-7

Theoretical ATP yield from substrate oxidation by isolated colonocytes[1]

	0% Fiber	15% Fiber	30% Fiber
	nmol ATP/(μg DNA · h)		
11 mmol/L glucose	30.38±1.08	35.78±3.58	31.82±1.96
1 mmol/L glutamine	4.67±0.24[a]	5.36±0.33[b]	6.04±0.34[b]
5 mmol/L glutamine	10.71±0.48[a]	12.95±0.56[b]	12.17±1.08[ab]
5 mmol/L propionate	5.42±0.57	5.68±0.82	4.96±0.77
5 mmol/L butyrate	35.73±2.32	33.23±2.66	31.38±2.46
5 mmol/L butyrate +ketone bodies[2]	31.67±2.31	33.21±2.68	30.74±2.46
Total[3]	76.2±4.1	80.1±7.4	74.2±5.5

[1] Values are means ± SEM. For 1 mmol/L glutamine, n=13 and for 5 mmol/L glutamine, n=10. Within a row, means with different letters differ significantly ($P \leq 0.05$).

[2] This value estimates ATP produced from 5 mmol/L butyrate when 1 mmol/L of each acetoacetate and ß-hydroxybutyrate are added to the incubation media.

[3] This value is the sum of ATP produced from 11 mmol/L glucose, 1 mmol/L glutamine, 5 mmol/L propionate and 5 mmol/L butyrate when assessed together in the same system.

Figure 3-1 The effect of diet on CO_2 production rate from glutamine by isolated colonocytes. Values are means ± SEM. For 1 mmol/L glutamine, n=13 and for 5 mmol/L glutamine, n=10. CO_2 production rates were calculated from the rate of $^{14}CO_2$ formation from [U-^{14}C]-glutamine with glutamine concentrations of either 1 mmol/L or 5 mmol/L. Complete incubation media composition is outlined in the methods section. Within a concentration level, bars with different letters are significantly different ($P \leq 0.05$). *Indicates value is significantly different from 1 mmol/L glutamine.

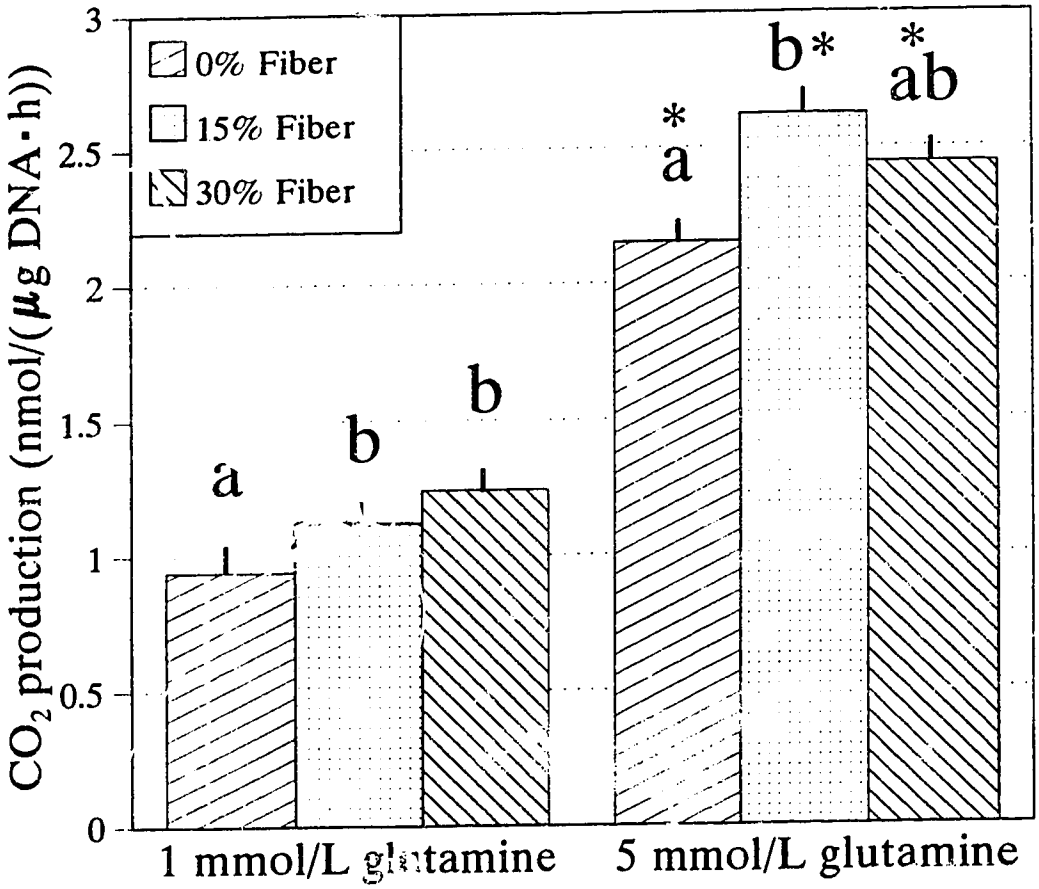
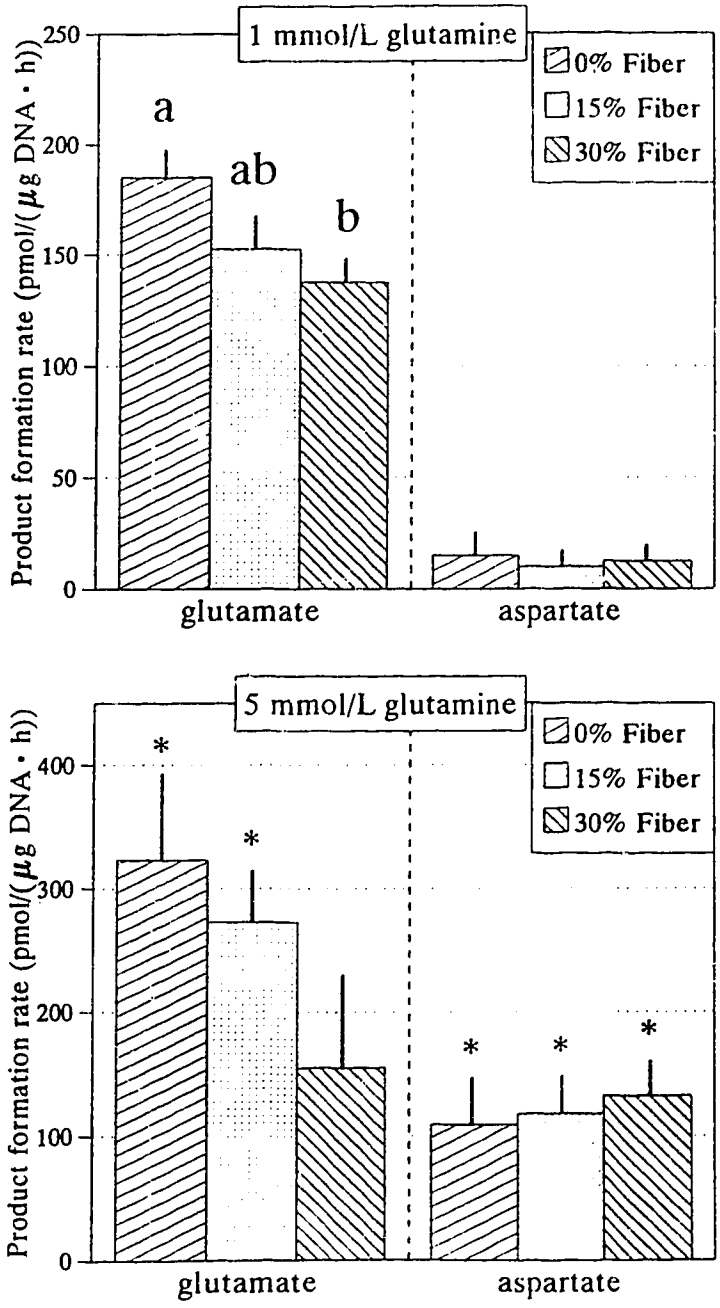


Figure 3-2 The effect of diet on glutamate and aspartate production from glutamine by isolated colonocytes. Values are means ± SEM. For 1 mmol/L glutamine, n=13 and for 5 mmol/L glutamine, n=10. Glutamine concentrations of either 1 mmol/L or 5 mmol/L were used. Complete incubation media composition is outlined in the methods section. Within a substrate, bars with different letters are significantly different ($P \leq 0.05$). *Indicates value is significantly different from 1 mmol/L glutamine.



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CHAPTER 4

DIETARY FIBER AND SHORT-CHAIN FATTY ACIDS AFFECT CELL PROLIFERATION AND PROTEIN SYNTHESIS IN ISOLATED RAT COLONOCYTES[1]

INTRODUCTION

Dietary fiber and short-chain fatty acids, the products of colonic microbial fiber fermentation, have been shown to alter colonic mass and in vivo cell proliferation rate (Edwards et al., 1992; Goodlad et al., 1987; Jacobs and Lupton, 1984). The presence of short-chain fatty acids in the intestinal lumen correlates with proliferation rate in the regional mucosa (Boffa et al., 1992; Lupton and Kurtz, 1993; Zhang and Lupton, 1994). It has also been shown that intraluminal infusion of short-chain fatty acids leads similar trophic effect in colonic regions removed from the infusion site (Sakata and von Engelhardt, 1983).

In vitro assessment of the effect of short-chain fatty acids on cell proliferation has shown the opposite effect compared with in vivo results. Short-chain fatty acids reduce proliferation in isolated rumen epithelia (Sakata et al., 1980), rat cecal tissue pieces (Sakata, 1987), and in human colon cancer cell lines (Dexter et al., 1984; Gamet et al., 1992; Niles et al., 1988). Butyrate increased in vitro protein synthesis in mucosa from humans with ulcerative colitis but not in mucosa from normals (Frankel et al., 1994a). In vitro protein synthesis in isolated rat enterocytes was increased when glutamine but not mixed amino acids were added to the incubation media (Higashiguchi et al., 1993). The effect of fiber consumption, the interaction between in vivo exposure to high-fiber diets and the relative importance of short-chain fatty acids vs. glutamine on in vitro colonocyte protein synthesis rates remain to be investigated.

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[1]A version of this chapter has been submitted for publication. Kathleen E. Marsman and Michael I. McBurney. Submitted to *J. Nutr.* August 1995.

The objective of this study was to assess the effect of 2 weeks of dietary fiber supplementation on protein synthesis, cell proliferation, and oxygen uptake in isolated rat colonocytes. Additionally, the in vitro effects of short-chain fatty acids on colonocyte proliferation and the influence of both short-chain fatty acids and glutamine on protein synthesis were assessed.

METHODS AND MATERIALS

Animals and Diets

Male Sprague-Dawley rats weighing 300 - 375 g were obtained from the University of Alberta Biological Sciences colony and housed in a temperature and humidity controlled room with a 12 h light/dark cycle. For 2 d prior to and during the experimental period, animals were housed individually in wire mesh-bottomed cages. The protocol was approved by the Faculty Animal Welfare Policy Committee at the University of Alberta. For the oxygen uptake and proliferation measurements, 22 animals were used. For protein synthesis determination, 16 animals were used.

Prior to the experimental period, animals consumed a nonpurified diet (Rodent Laboratory Diet PMI #5001, PMI Feeds, Inc. St. Louis, MO). Animals consumed the experimental diets for 14 d and had free access to diet during that time. Composition of the experimental diets is given in Table 4-1. During the experimental period, animals consumed either a fiber-free elemental diet (0% fiber) or an elemental diet supplemented with 150 g fiber/kg diet (15% fiber) in place of an isocaloric amount of glucose and sucrose to ensure that both diets had the same protein:energy ratio. The in vivo digestible energy value of the fiber mixture was determined experimentally to be 5 kJ/g (Marsman and McBurney, unpublished data, Appendix 1). Fibrad produced 4.5 mmol of short-chain fatty acids per g of organic matter under *in vitro* fermentation conditions (McBurney, 1991).

Chemicals

L-[2,6-^{3}H]-Phenylalanine and methyl-[^{3}H]-thymidine were obtained from Amersham (Arlington Heights, IL). Sodium acetate, sodium propionate, sodium *n*-butyrate, L-glutamine, lithium acetoacetate, sodium-ß-hydroxybutyrate, calcium-free bovine albumin (fraction V), DL-dithiothreitol, HEPES, EDTA, Percoll, insulin, and DNA (sodium salt from calf thymus) were obtained from Sigma Chemical (St. Louis, MO). RPMI 1640 cell culture media (without glutamine and HEPES) and broad-spectrum antibiotic-antimycotic (10.0×10^6 U/L penicillin C sodium, 10 g/L streptomycin sulfate, 25 mg/L amphotericin B and 8.5 g/L NaCl) were obtained from GIBCO Life Technologies (Grand Island, NY). Soluene and Hionic Fluor were obtained from Packard (Meriden, CT) and EcoLite was obtained from ICN (Costa Mesa, CA)

Colonocyte Isolation

Colonocytes were isolated using EDTA and mechanical stirring according to the method of Roediger and Truelove (1979) with modifications (Marsman and McBurney, 1995). Calcium-free Krebs-Ringer HEPES buffer (Whitesell and Abumrad, 1985) was prepared in advance, suction filtered for sterility using 0.22 micron AcetatePlus filter discs (MSI, Westboro, MA) and was adjusted to pH 7.4. Antibiotic-antimycotic was added to the buffer (10 mL/L). The following solutions were prepared fresh daily, adjusted to pH 7.4 and gassed continually with O_2:CO_2 (19:1) for 10 min prior to use. Solution A was prepared by adding 24 mmol/L sodium bicarbonate to the calcium-free Krebs-Ringer HEPES buffer. Solution B was prepared by adding 3 g/L calcium-free albumin and 5 mmol/L EDTA to solution A. Solution C was prepared by adding 10 g/L calcium-free albumin and 5 mmol/L DL-dithiothreitol to solution A. RPMI 1640 culture media (calcium-containing) was supplemented with 25 mmol/L HEPES, 10 g/L calcium-free albumin, and 10 mL/L antibiotic-antimycotic.

Animals were killed by CO_2 inhalation followed by cervical dislocation. Colons were removed, rinsed of luminal contents with solution A and weighed. Segments were ligated

distally, inverted, distended with solution A and ligated proximally. Distended segments were placed in plastic containers with 40 mL of solution B and gassed continually with O_2:CO_2 (19:1) in a shaking water bath (50 oscillations/min) at 37 °C for 20 min. Segments were then transferred to plastic containers with 50 mL of cold solution C and agitated constantly with a disposable plastic pipette for 15 min to free cells. Cells were kept on ice from this step onward. The distended intestinal segment was then removed and the cell suspension centrifuged for 5 min at 600 x g. Cells were washed with supplemented culture media and pressed through a 100 mesh screen. A discontinuous percoll density gradient (40% and 15% percoll by volume with 0.15 mol/L NaCl; 700 x g for 15 min) was used to purify epithelial cells, according to a modification of Lundqvist et al. (1992). Isolated cells were washed and re-suspended in supplemented culture media at concentrations of 50 - 100 mg DNA/L (10 - 20 x 10^9 cells/L). Cell removal from underlying musculature was previously confirmed by histological examination of segments after cell removal.

The incubation media used for cell proliferation analysis and oxygen consumption measurements was prepared by supplementation of RPMI 1640 media with 10 g/L calcium-free bovine serum albumin, 25 mmol/L HEPES buffer and 10 mL/L antibiotic-antimycotic. The following substrates were present in the incubation media, unless otherwise stated: 1 mmol/L glutamine, 11 mmol/L glucose, 5 mmol/L acetate, 5 mmol/L propionate, and 5 mmol/L butyrate. The concentrations given are the final solution concentrations after the aliquot of cell suspension was added to the incubation media. Incubation media were adjusted to pH 7.4 prior to use.

Oxygen Consumption

Oxygen consumption by isolated colonocytes was determined using the YSI Model 53 Oxygen Monitor System (Yellow Springs Instruments, OH) as per Wu et al. (1995) with modification. Four oxygen probes were used in one heating block. In each probe chambers, 3.0 mL of air-saturated incubation media were used (composition outlined

above, including 15 mmol/L mixed short-chain fatty acids (5 mmol/L each of acetate, propionate and butyrate), 11 mmol/L glucose, and 1 mmol/L glutamine) were heated to 37°C and agitated with a magnetic stirrer. Solutions were gassed with O_2:CO_2 (19:1) for 2 minutes prior to use. An aliquot of cell suspension (100 µL) containing approximately 8 µg DNA was added to each probe chamber. Oxygen consumption increased linearly with cell concentration up to and including 50 µg DNA per chamber. Oxygen probes were then inserted into the chambers, given 5 minutes to stabilize the monitor readings, and oxygen consumption was monitored for the next 15 min, during which oxygen uptake was linear with time. Probe membranes were replaced after each determination. The probes used to monitor oxygen uptake were randomized each day for colonocyte suspensions from each diet treatment.

Cell Proliferation

^{3}H-Thymidine incorporation into isolated colonocytes was used to determine relative rate of cell proliferation as per Ryder et al. (1994) with modification. An aliquot of cell suspension (25 µL) containing approximately 2 µg DNA was added to wells in a 96-well plate. Cells were incubated with one of two variations on the incubation media outlined above: either 0 mmol/L or 15 mmol/L short-chain fatty acids (5 mmol/L each of acetate, propionate and butyrate). Both variations included 11 mmol/L glucose and 1 mmol/L glutamine. ^{3}H-Thymidine was added to each of the variations for a final level of 92 MBq/L and at a specific activity of 3.07 TBq/mmol. This resulted in a thymidine concentration of 30 nmol/L.

An aliquot (175 µL) of incubation media was added to each sample well and mixed with cells. Blank wells (containing no cells) were also prepared. Plates were then incubated at 37°C for 2 h in a humidified metabolic incubator with a continuous gas flow of air:CO_2 (19:1). At the end of the incubation period, cells were harvested from the plates using a semiautomatic cell harvester (Skatron Instruments, Lier, Norway). Filter discs containing the washed harvested cells were placed in scintillation vials with 4.0 mL EcoLite

scintillation fluid. ^{3}H was counted using a Beckman LS 5801 counter (Beckman Instruments, Irvine, CA). The incorporation of thymidine into cells was expressed as pmol/(g DNA · h).

Protein Synthesis

^{3}H-Phenylalanine incorporation into isolated colonocytes was used to determine relative rate of protein synthesis as described by Higashiguchi et al. (1993). Cells were isolated and after the final wash were re-suspended in supplemented culture media. Cells were washed twice in an albumin-free culture media solution containing RPMI 1640, 1% antibiotic-antimycotic and 25 mmol/L HEPES. The final cellular DNA concentration was approximately 80 mg DNA/L.

For the protein synthesis assay, the incubation media contained RPMI 1640 culture media, 100 mU/L insulin, 0.5 mmol/L phenylalanine, 74 MBq/L (2.0 mCi/L) of ^{3}H-phenylalanine, 1% antibiotic-antimycotic, 11 mmol/L glucose, and 25 mmol/L HEPES. Cells (0.3 mL) were incubated in 2.0 mL of one of the four following variations on the incubation media: 1) 0 mmol/L short-chain fatty acids and 0 mmol/L glutamine, 2) 0 mmol/L short-chain fatty acids and 1 mmol/L glutamine, 3) 15 mmol/L mixed short-chain fatty acids (5 mmol/L each of acetate, propionate and butyrate) and 0 mmol/L glutamine, 4) 15 mmol/L mixed short-chain fatty acids and 1 mmol/L glutamine.

Incubations were carried out in siliconized glass tubes for 30 minutes at 37°C in a shaking water bath. During the incubation, tubes were gassed continuously with O_2:CO_2 (19:1). Metabolism was stopped by the addition of 5 mL of ice-cold 10% trichloroacetic acid (TCAA) to each tube. Blanks were prepared by adding cells after the addition of the 10% TCAA. The pellets were obtained and washed once with ice-cold 10% TCAA, once with 10% TCAA containing 5 mmol/L phenylalanine, and once again with cold 10% TCAA.

The pellets were then extracted with 5 mL of ethanol:ether (1:1), and were dried overnight in a fume-hood. The dried pellet was re-suspended in 1.0 mL of Soluene and 0.1 mL water and counted with 4 mL of Hionic Fluor. The incorporation of phenylalanine into protein was expressed as nmol/(mg DNA·h). Intracellular precursor specific activity of phenylalanine was assessed as per Higashiguchi et al. (1993) to ensure that no differences existed between the labeling of the intercellular free amino acid pools between diet treatments and solutions. Intracellular phenylalanine concentrations and specific activities were used to determine fractional protein synthesis rates (%/day).

Other Analytical Procedures

The DNA content of the cell suspensions was determined fluorometrically using the method of Prasad et al. (1972) with calf thymus DNA as a standard. Alkaline phosphatase activity of the cell suspension was determined spectrophotometrically using kit #245 (Sigma Chemical, St. Louis, MO). Lactate dehydrogenase activity of the cell suspension supernatant was measured to determine release of the enzyme into the incubation media as an indicator of cell viability. Activity was determined spectrophotometrically using kit # 228 (Sigma Chemical, St. Louis, MO).

Statistics

One observation was determined as the mean of triplicate measurements except for oxygen consumption measurements which were determined using 2 replicates. For all parameters, one observation from each diet was obtained on each test day. The general linear model procedure in SAS (Version 6.04, SAS Institute, Cary, NC) was used for all statistical analysis. Because of animal variability between observations, differences between diets or within a diet group were determined using a three-way ANOVA model (diet, incubation solution and animal) followed by Duncan's test for multiple comparisons where required. Statistical significance was defined as $P \leq 0.05$. Values given are means ± SEM, for n = 8 or 11, as indicated.

RESULTS

Although weight gain and diet consumption were slightly higher for the 15% fiber group, this was not reflected in a similar change in digestible energy intake (Table 4-2). Including 15% fiber in the diet resulted in a 23% larger colonic mass (Table 4-2). Because we have previously shown that DNA content correlates highly ($P \leq 0.0001$) with cell number, regardless of diet (Chapter 3), DNA content was used as the denominator for all measurements. Initial colonocyte viability and alkaline phosphatase activity did not differ between diets (Table 4-3). Colonocyte viability was decreased to 88% after a 30 minute incubation at 37 °C and to 80% after a 2 h incubation at 37 °C. Oxygen consumption rate did not differ between diets (Table 4-3).

Cell proliferation, as assessed by ^{3}H-thymidine incorporation, was higher in cells from the 15% fiber group regardless of the presence or absence of 15 mmol/L mixed hort-chain fatty acids in the incubation media (Figure 4-1). In the absence of short-chain fatty acids, the fiber-containing diet resulted in a 20% higher rate of cell proliferation. In the presence of 15 mmol/L short-chain fatty acids, the fiber-containing diet resulted in a 27% higher rate of cell proliferation as compared to the fiber-free diet. Within a diet treatment, the addition of short-chain fatty acids to the incubation media significantly reduced ^{3}H-thymidine incorporation by 52% for the fiber-free diet and by 43% for the fiber-containing diet.

Intracellular specific activities of 3H phenylalanine were approximately 150 MBq/mmol and did not vary between treatments or incubation solutions. Thus, the intracellular precursor pool of labeled phenylalanine equilibrated with that of the incubation media, consistent with the findings of Higashiguchi et al. (1993). Protein synthesis, as assessed by rate of ^{3}H-phenylalanine incorporation into colonocytes, did not differ between diet treatments regardless of solution composition (Figure 4-2). The addition of 15 mmol/L short-chain fatty acids to the incubation media did not affect the rate of ^{3}H-phenylalanine incorporation into protein regardless of diet, unless 1 mmol/L glutamine was present in the

incubation media (Figure 4-2). In the presence of glutamine, short-chain fatty acids decreased ^{3}H-phenylalanine incorporation into protein by 18% for the fiber-free diet and by 20% for the fiber-containing diet. Increasing the glutamine concentration in the incubation media from 0 to 1 mmol/L reduced ^{3}H-phenylalanine incorporation into protein, but only when short-chain fatty acids were present in the incubation media.

DISCUSSION

This is the first report of the influence of dietary fiber on both in vitro cell proliferation and protein synthesis in isolated colonocytes. Including 15% fiber in an elemental diet resulted in an increase in cell proliferation. These observations agree with studies assessing the effect of fiber supplementation on cell proliferation (most commonly measured histologically by bromodeoxyuridine or ^{3}H-thymidine incorporation in vivo) which have found that dietary fibers, specifically fermentable fibers, increase proliferation and/or crypt proliferation zone in rats (Boffa et al., 1992; Jacobs, 1983; Jacobs et al., 1983; Jacobs and Lupton, 1984; Jacobs and Schneeman, 1981; Lee et al., 1993; Lupton and Jacobs, 1987; Lupton and Kurtz, 1993; Palacio et al., 1989; Zhang and Lupton, 1994). Edwards et al. (1992) showed that after 18 months of high-fiber ingestion, cell proliferation increased in the proximal colon due to a higher percentage of cells in s-phase, and that prolif rating cells were expanded to higher regions of the crypts. Expanded proliferative zones were also found by Lupton and Jacobs (1987) in rats after 4 weeks of feeding.

Previous studies have established strong correlations between luminal short-chain fatty acid concentration (derived from fiber fermentation) and in vivo proliferation rates (Boffa et al., 1992; Lupton and Kurtz, 1993; Zhang and Lupton, 1994). In our study, isolated cells were not affected by the direct exposure to 15 mmol/L short-chain fatty acids in culture media. However, others have found that the products of fiber fermentation, not the fiber per se, are stimulating proliferation and that short-chain fatty acids can have trophic effects in regions of the gut not directly exposed to fermentation (Goodlad et al., 1989). It has been suggested that indirect means such as a signalling or neural mechanism

may be responsible for the stimulatory effect of short-chain fatty acids on colonocyte proliferation (Frankel et al., 1994b; Sakata and Von Engelhardt, 1983). Additionally, indirect effects of short-chain fatty acids such as the increase in colonic blood flow (Kvietys and Granger, 1981), dilation of colonic resistance arteries (Mortensen et al., 1990) and increased colonic absorption rates (Demigné and Rémésy, 1985) help to explain the differences between in vivo and in vitro effects of short-chain fatty acids on cell proliferation, since isolated cells would not be exposed to the same signals as the intact gut.

In the current study, the results indicate that in vitro incubation with short-chain fatty acids decreased cell proliferation rate in isolated rat colonocytes. Short-chain fatty acids have been shown to inhibit in vitro proliferation of sheep rumen epithelium (Sakata et al., 1980) and rat cecal tissue (Sakata, 1987) although Scheppach et al. (1992) found in vitro concentrations up to 100 mmol/L of mixed short-chain fatty acids increased proliferation indices in human cecal mucosa and Frankel et al. (1994a) found no change with in vitro provision of butyrate to human colonocytes. The inhibitory effect of short-chain fatty acids, particularly butyrate, on proliferation of human colon carcinoma cell lines has also been characterized and supports our findings (Dexter et al., 1984; Gamet et al., 1992; Niles et al., 1988).

Viability was not altered by diet and although there was a trend toward increased alkaline phosphatase activity with the inclusion of fiber in the diet (suggesting an increase in cell maturity) this difference was not statistically significant. It is possible that discrepancies in weight gain due to diet contributed to the changes in cell proliferation, although the magnitude of change in colon mass was similar to that observed in Chapters 2 and 3, when no increase in animal weight gain was seen with high-fiber feeding.

The addition of SCFA to the incubation media affected the rate of ^{3}H-phenylalanine incorporation into protein only in the presence of 1 mmol/L glutamine. Higashiguchi et al.

(1993) reported that 0.67 mmol/L glutamine stimulated protein synthesis in isolated enterocytes but this effect was not seen when an amino acid mixture was used in place of glutamine. Similarly, in this study the addition of glutamine to a complete media did not stimulate protein synthesis. However, when short-chain fatty acids were present, 1 mmol/L glutamine inhibited protein synthesis. Through cell volume regulation, Häussinger et al. (1993) has established that physiological glutamine concentrations increase hepatocyte protein synthesis. Interactions between extracellular glutamine and short-chain fatty acid concentrations have not been assessed. A decreased protein synthesis rate due to osmotic cell shrinkage has been reported by Stoll et al. (1992) who found that the addition of 60 mmol/L NaCl was associated with a lower rate of protein synthesis in hepatocytes. Adding 15 mmol/L mixed short-chain fatty acids may also have changed cell volume, although only when glutamine was present in the incubation media was a significant decrease in protein synthesis observed. The fractional protein synthesis rate observed in this study indicates that 100% protein synthesis in these cells would occur in less than one day. This is consistent with the findings of Stein et al. (1994) which indicated that colonic fractional protein synthesis rates were 153 and 157 %/day in proximal and distal colon segments, respectively. These rates were reduced by up to 70% when nutrients were delivered intravenously.

Frankel et al. (1994a) reported that 10 mmol/L butyrate increased protein synthesis in human ulcerative colitis tissue but not in normal tissue. The change in protein synthesis observed was independent of extracellular osmotic changes. Munks and Turner (1994) found a reduction in synthesis of heat shock proteins in Drosophila cells cultured in the presence of either propionate or butyrate. The addition of short-chain fatty acids to the medium increases extracellular osmolarity, which may result in cell shrinkage. Additionally, physiological concentrations of neutralized short-chain fatty acids cause an immediate reduction in intracellular pH of colonocytes due to influx of propionate into the cells (Desoignie and Sellin, 1994). It appears that interactions may exist between intracellular pH, extracellular osmolarity and cell volume.

Although the rate of proliferation was greater for colonocytes from the animals fed the 15% fiber diet versus the 0% fiber diet, this did not translate into an increase in oxygen uptake or alterations in protein synthesis. Our data also suggests that in vitro, short-chain fatty acids decrease cell proliferation and, in the presence of glutamine, decrease protein synthesis in isolated colonocytes. Future work must be done to establish the mechanism through which these changes arise.

TABLE 4-1

Composition of the experimental diets

Ingredient	0% Fiber	15% Fiber
	g/kg diet	
Amino Acid Mix[1]	200.0	179.3
Fat[2]	100.0	89.7
Glucose	427.2	352.0
Sucrose	213.6	176.0
Alphacel	--	25.0
Fibrad[3]	--	125.0
Mineral Mix[4]	44.1	39.5
Vitamin Mix[5]	12.6	11.3
Choline Chloride	2.5	2.2
Digestible Energy[6] (*MJ/kg*)	17.9	16.0
Amino Acid Content (*g/MJ*)	11.2	11.2
Nitrogen Content (*g/MJ*)	1.72	1.72

[1]Supplied (g/kg mixture): L-arginine · HCl, 67.5; L-histidine, 22.5; L-isoleucine, 41; L-leucine, 67.5; L-lysine HCl, 90; L-methionine, 41; L-phenylalanine, 58; L-threonine, 41; L-tryptophan, 13; L-valine, 47.5; L-alanine, 17.5; L-aspartic acid, 17.5; L-glutamic acid, 239.5; glycine, 116.5; L-proline, 27.5; L-cystine, 22.5; L-serine, 17.5; L-tyrosine, 22.5; L-asparagine, 30.

Continued...

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[2]Contained (g/kg mixture): hydrogenated beef tallow, 392; linseed oil 20; and safflower oil, 588. Polyunsaturated:saturated fatty acid ratio was 1.0, as determined chromatographically.

[3]Ross Laboratories, Columbus, OH. Contained a mixture of pea fiber, oat fiber, sugar-beet fiber, xanthan gum and soy lecithin.

[4]Supplied (g/kg mixture): calcium phosphate dibasic, 500; potassium citrate monohydrate, 220; sodium chloride, 74; potassium sulfate, 52; magnesium oxide, 24; ferric citrate pentahydrate, 6.0; manganous carbonate, 3.5; zinc carbonate, 1.6; potassium iodate 0.01; cupric carbonate, 0.3; sodium selenite, 0.01; chromium potassium sulfate, 0.55; sucrose, 118.

[5]Supplied (per kg mixture): thiamine hydrochloride, 600 mg; riboflavin, 600 mg; pyridoxine hydrochloride, 700 mg; nicotinic acid, 3.0 g; D-calcium pantothenate, 1.6 g; folic acid, 200 mg; D-biotin, 20 mg; cyanocobalamin, 1.0 mg; retinyl palmitate (250,000 IU/g), 1.6 g; DL-α-tocopherol acetate (250 IU/g), 20 g; cholecalciferol (400,000 IU/g), 250 mg; menaquinone, 5.0 mg; sucrose, 972.9 g.

[6]Digestible energy was calculated from gross energy measurements of dietary components and in vivo digestible energy measurements for the mixed fiber source.

TABLE 4-2

Growth, Diet Consumption and Colonic Mass[1]

		0% Fiber	15% Fiber
Weight Gain	*g/d*	4.4 ± 0.3^{a}	5.6 ± 0.3^{b}
Diet Consumption	*g/d*	23.1 ± 0.7^{a}	26.6 ± 0.7^{b}
Digestible Energy	*kJ/d*	413 ± 11	425 ± 12
Colonic Mass	*g*	1.75 ± 0.05^{a}	2.15 ± 0.05^{b}

[1] Values are means ± SEM, n=19. Within a row, means with different letters differ significantly ($P \leq 0.05$).

TABLE 4-3

Colonocyte Characteristics and Oxygen Uptake[1]

	0% Fiber	15% Fiber
Initial Cell Viability *%*	94.7 ± 1.3	95.0 ± 1.2
Alkaline Phosphatase *U/g DNA*	17.4 ± 3.3	23.5 ± 3.0
Oxygen Consumed *nmol/(μg DNA · h)*	45.6 ± 4.2	49.3 ± 3.1

[1] Values are means ± SEM, n=19. For oxygen consumption, n=11. Within rows, no values were significantly different ($P \leq 0.05$).

Figure 4-1 The effect of dietary fiber and short-chain fatty acids on the cell proliferation rate of isolated colonocytes. Values are means ± SEM, n=11. ^{3}H-Thymidine incorporation into colonocytes was measured in incubation media with or without 15 mmol/L short-chain fatty acids (5 mmol/L each of acetate, propionate and butyrate). Incubation media contained 1 mmol/L glutamine and 11 mmol/L glucose; complete incubation media composition is outlined in the methods section. Within a diet, bars with different letters are significantly different ($P \leq 0.05$). *Indicates means that are different from the 0% fiber diet ($P \leq 0.05$).

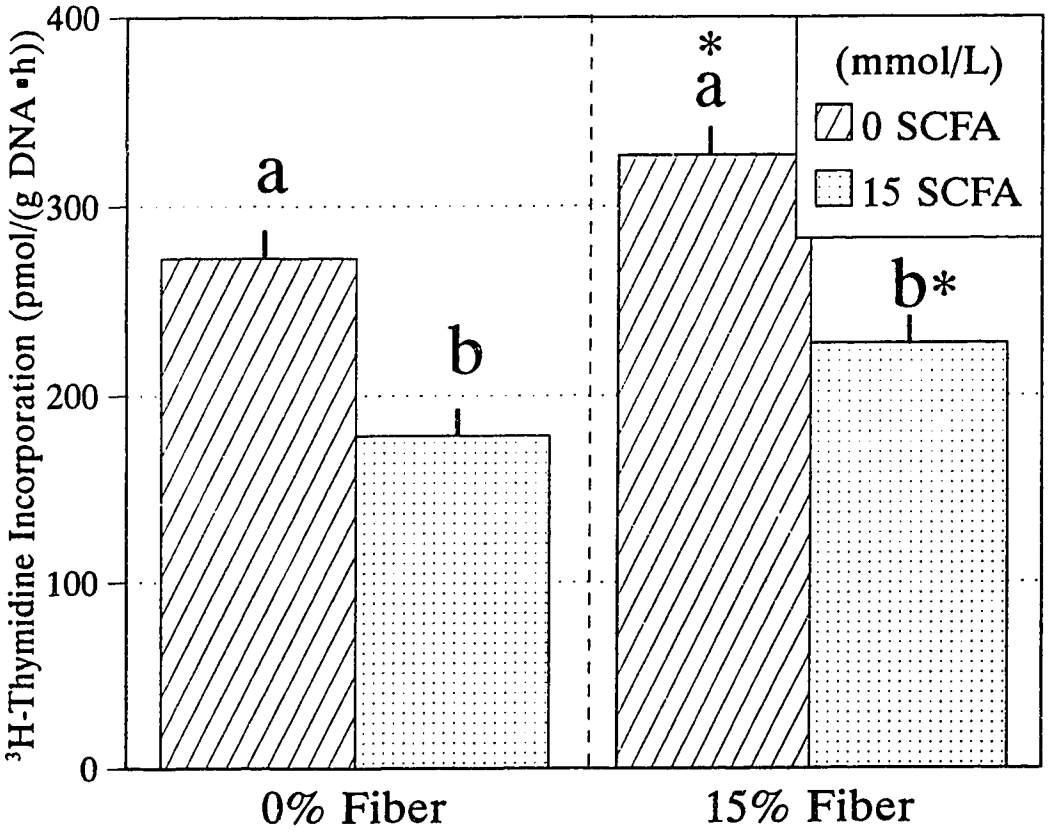
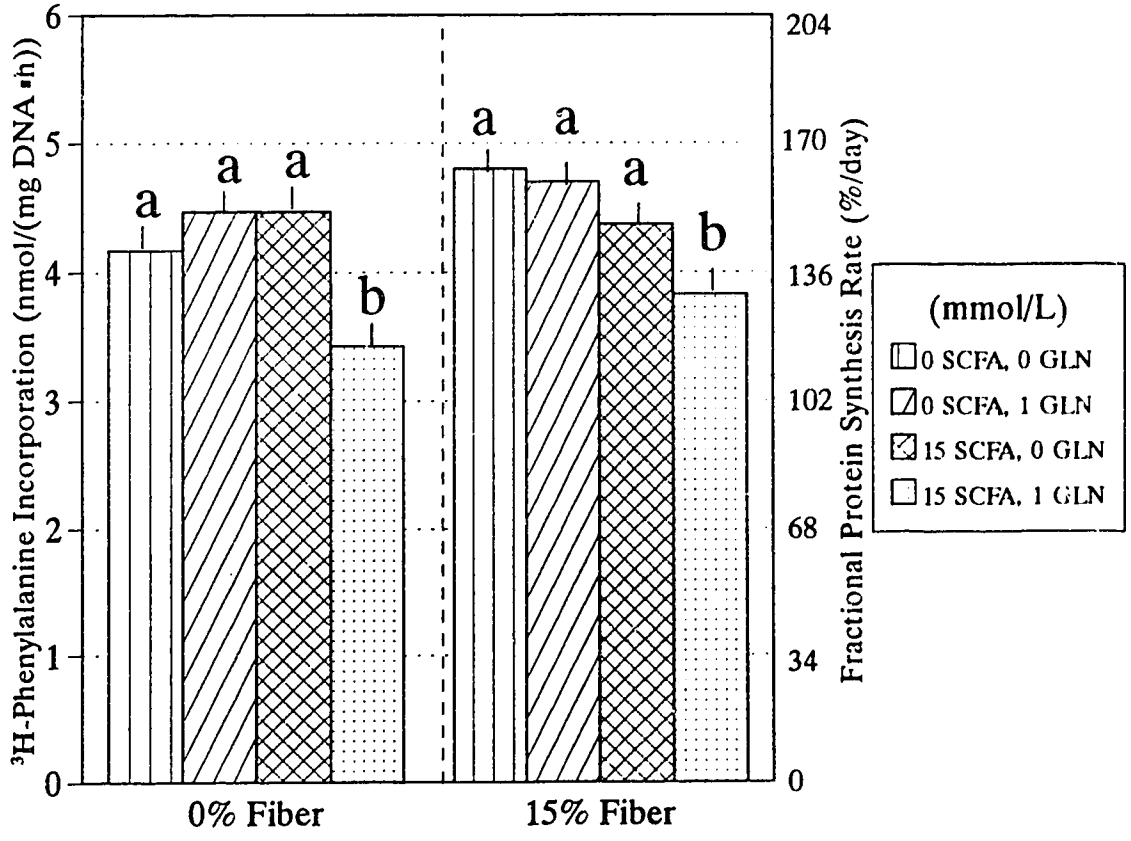


Figure 4-2 The effect of dietary fiber and incubation media composition on protein synthesis in isolated colonocytes. Values are means ± SEM, n=8. ^{3}H-Phenylalanine incorporation into colonocytes was measured in incubation media with or without 15 mmol/L short-chain fatty acids (5 mmol/L each of acetate, propionate and butyrate) and containing either 0 or 1 mmol/L glutamine. Fractional rate of protein synthesis was calculated based on intracellular specific activity and concentration of phenylalanine. Complete incubation media composition is outlined in the methods section. Within a diet, bars with different letters are significantly different ($P \leq 0.05$). *Indicates means that are different from the 0% fiber diet ($P \leq 0.05$).



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CHAPTER 5

SHORT-CHAIN FATTY ACIDS DECREASE CELL PROLIFERATION AND PROTEIN SYNTHESIS IN EPITHELIAL CELLS FROM RAT COLON[1]

INTRODUCTION

Short-chain fatty acids, the main organic products of fiber fermentation in the large intestine (Bugaut, 1987), are readily oxidized by cecal and colonic epithelial cells (Fleming et al., 1991; Roediger, 1982). Not only do these fatty acids supply the colon with metabolic energy, research has demonstrated other metabolic effects on the colon such as increased blood flow (Kvietys and Granger, 1981), increased luminal absorption rate (Demigné and Rémésy, 1985) and enhanced in vivo cell proliferation rates (Boffa et al., 1992; Zhang and Lupton, 1994).

It has been suggested that short-chain fatty acids act as indirect metabolic signals to up-regulate colonic metabolism; particularly cell proliferation (Frankel et al., 1994b; Sakata and VonEngelhardt, 1983). When colonic epithelial cells are isolated, short-chain fatty acids have the effect of decreasing cell proliferation (Sakata, 1987). This has also been observed in human colon cancer cell lines (Dexter et al., 1984; Gamet et al., 1992; Niles et al., 1988) and in isolated rumen epithelial cells (Sakata et al., 1980). Short-chain fatty acids affect protein synthesis in human ulcerative colitis tissue (Frankel et al., 1994a) and can inhibit heat shock protein synthesis in cultured drosophila cells (Munks and Turner, 1994).

In vitro studies with hepatocytes have established that the osmolarity of the incubation solutions alters protein synthesis rates (Häussinger et al., 1993; Stoll et al., 1992).

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[1]A version of this chapter has been submitted for publication. Kathleen E. Marsman and Michael I. McBurney. Submitted to *J. Nutr.*, October 1995.

Osmotic regulation of cell cycle kinetics (Pellicciari et al., 1995) and of cell proliferation and protein synthesis (Petronini et al., 1992) in cultured cell lines has also been demonstrated.

The aim of this study was to investigate whether short-chain fatty acids supplemented into incubation media influenced cell proliferation, protein synthesis or anaplerotic flux through TCA cycle metabolism in isolated rat colonocytes and whether the alterations found were reflected in a concurrent change in cellular oxygen utilization. Additionally, we investigated whether the effect of short-chain fatty acids could be attributed mainly to butyric acid and if these parameters were influenced by extracellular osmolarity.

MATERIALS AND METHODS

Animals

Twenty-four male Sprague-Dawley rats weighing 220 - 320 g were obtained from the University of Alberta Biological Sciences colony and housed in group cages in a temperature and humidity controlled room with a 12 h light/dark cycle. The experimental protocol was approved by the Faculty Animal Welfare Policy Committee at the University of Alberta. Animals consumed a nonpurified diet (Rodent Laboratory Diet PMI #5001, PMI Feeds, Inc. St. Louis, MO) and had free access to the diet and to water.

Chemicals

L-[2,6-^{3}H]-Phenylalanine and methyl-[^{3}H]-thymidine were obtained from Amersham (Arlington Heights, IL). [2-^{14}C]-Sodium propionate was obtained from ICN Biomedicals Inc. (Irvine, CA). [1-^{14}C]-Sodium propionate was obtained from DuPont NEN Research Products (Wilmington, DE). Sodium acetate, sodium propionate, sodium *n*-butyrate, L-glutamine, calcium-free bovine albumin (fraction V), DL-dithiothreitol, benzethonium hydroxide, HEPES, EDTA, Percoll, insulin, and DNA (sodium salt from calf thymus) were obtained from Sigma Chemical (St. Louis, MO). RPMI 1640 cell culture media (without glutamine and HEPES) and broad-spectrum antibiotic-antimycotic (10.0×10^{6}

U/L penicillin C sodium, 10 g/L streptomycin sulfate, 25 mg/L amphotericin B and 8.5 g/L NaCl) were obtained from GIBCO Life Technologies (Grand Island, NY). Soluene and Hionic Fluor were obtained from Packard (Meriden, CT) and EcoLite was obtained from ICN (Costa Mesa, CA).

Colonic Epithelial Cell Isolation

Colonocytes were isolated using EDTA and mechanical stirring methods according to Roediger and Truelove (1979) with modifications (Marsman and McBurney 1995). Calcium-free Krebs-Ringer HEPES buffer (Whitesell and Abumrad 1985) was suction filtered for sterility using 0.22 micron AcetatePlus filter discs (MSI, Westboro, MA) and was adjusted to pH 7.4. Antibiotic-antimycotic was added to the buffer (10 mL/L). The following solutions were prepared daily, adjusted to pH 7.4 and gassed with O_2:CO_2 (19:1) for 10 min prior to use. Solution A contained 24 mmol/L sodium bicarbonate in calcium-free Krebs-Ringer HEPES buffer. Solution B contained 3 g/L calcium-free albumin and 5 mmol/L EDTA in solution A. Solution C contained 10 g/L calcium-free albumin and 5 mmol/L DL-dithiothreitol in solution A. Supplemented RPMI 1640 culture media (includes calcium) containing 25 mmol/L HEPES, 10 g/L calcium-free albumin, and 10 mL/L antibiotic-antimycotic was used for the final cell suspension.

Animals were killed by CO_2 inhalation followed by cervical dislocation. Colons were removed and rinsed of luminal contents with solution A. Colons were then ligated distally, inverted, distended with solution A and ligated proximally. Distended segments were placed in plastic containers with 40 mL of solution B and gassed continually with O_2:CO_2 (19:1) in a shaking water bath (50 oscillations/min) at 37 °C for 20 min. Segments were then transferred to plastic containers with 50 mL of cold solution C and agitated constantly with a disposable plastic pipette for 15 min to free cells. Isolated epithelial cells were kept on ice from this step onward. The distended intestinal segment was then removed and the cell suspension centrifuged for 5 min at 600 x g. Cell removal from underlying musculature was confirmed by histological examination of segments after cell

removal (Marsman & McBurney, unpublished observation). Cells were washed with supplemented culture media and pressed through a 100 mesh screen. A discontinuous percoll density gradient (40% and 15% percoll by volume with 0.15 mol/L NaCl; 700 x g for 15 min) was used to purify epithelial cells, according to a modification of Lundqvist et al. (1992). Isolated cells were washed and re-suspended in supplemented culture media at a concentration of approximately 80 mg DNA/L (16 x 10^9 cells/L).

The incubation media used for oxygen consumption measurements, cell proliferation analysis, and propionate oxidation determination was prepared by supplementation of RPMI 1640 media with 10 g/L calcium-free bovine serum albumin, 25 mmol/L HEPES buffer and 10 mL/L antibiotic-antimycotic. The following substrates were present in this incubation media at the levels indicated, unless otherwise stated: 1 mmol/L glutamine, 11 mmol/L glucose, 5 mmol/L acetate, 5 mmol/L propionate, and 5 mmol/L butyrate. The concentrations given are the final solution concentrations after the aliquot of cell suspension was added to the incubation media. All incubation media were adjusted to pH 7.4 prior to use.

Oxygen Consumption

Oxygen consumption by isolated colonocytes was assessed polarographically using the YSI Model 53 Oxygen Monitor System (Yellow Springs Instruments, OH) as per Wu et al. (1995) with modification. Four oxygen probes were used in one heating block. In each probe chamber, 3.0 mL of oxygen-saturated incubation media (composition outlined above, including 11 mmol/L glucose, 1 mmol/L glutamine and either no short-chain fatty acids or 15 mmol/L mixed short-chain fatty acids, 5 mmol/L each of acetic, propionic and butyric acids) were heated to 37°C and agitated rapidly with a magnetic stirrer to ensure movement of incubation solution around probe membrane. Solutions were gassed with O_2:CO_2 (19:1) for 2 minutes prior to use. An aliquot of cell suspension (100 µL) containing approximately 8 µg DNA was added to each probe chamber. Oxygen consumption increased linearly with cell concentration up to and including 50 µg DNA per

chamber. Oxygen probes were then inserted into the chambers, given a 5 min equilibration period and oxygen consumption was monitored for 15 min, during which oxygen uptake was linear with time. Probe membranes were replaced after each determination.

Cell Proliferation

The rates of ^{3}H-thymidine incorporation into isolated colonocytes were used to determine relative rate of cell proliferation (Ryder et al., 1994). Aliquots of cell suspension (30 µL) containing approximately 2 µg DNA were added to wells in a 96-well plate. Cells were incubated with one of seven variations on the incubation media outlined in Table 5-1. ^{3}H-Thymidine was added to each of the variations for a final level of 92 MBq/L, at a final specific activity of 3.07 TBq/mmol and a final concentration of 30 nmol/L.

An aliquot (200 µL) of each of the seven different incubation media was added to sample wells and mixed with cells. Blank wells, containing no cells, were also prepared. Plates were then incubated at 37°C for 2 h in a humidified metabolic incubator with a continuous gas flow of air:CO_2 (19:1). At the end of the incubation period, cells were harvested from the plates using a Semiautomatic Cell Harvester (Skatron Instruments, Lier, Norway). Filter discs containing the washed harvested cells were placed in scintillation vials with 4.0 mL EcoLite scintillation fluid. ^{3}H was counted using a Beckman LS 5801 counter (Beckman Instruments, Irvine, CA).

Propionate Oxidation and Anaplerotic Flux

Rates of $^{14}CO_2$ release from oxidation of either [1-^{14}C]-propionate or [2-^{14}C]-propionate were assessed to determine anaplerotic flux and propionic acid oxidation rate. Isotopes were present at 32 MBq/L and at a specific activity of 2 GBq/mmol, leading to a propionate concentration of 16 µmol/L in the incubation solutions.

Incubations were carried out in siliconized glass tubes (Vacutainer, Becton Dickson,

Rutherford, NJ) containing polypropylene microcentrifuge tubes (Fisher Scientific, Ottawa, ON, Canada) as center wells for CO_2 collection. Aliquots of cell suspension (100 µL) were added to 1.0 mL of incubation solution. Incubation tubes were gassed with O_2:CO_2 (19:1) for 5 s, sealed with a rubber stopper and incubated for 40 min at 37 °C in a shaking water bath. Incubations were stopped by injecting 100 µL of citric acid (1.5 mol/L, pH 5.0) into sealed tubes. Blanks were prepared by adding 100 µL of cell suspension and citric acid simultaneously after the incubation period.

To collect $^{14}CO_2$, 300 µL of benzethonium hydroxide were injected through the rubber stoppers and into the center wells of the incubation tubes. $^{14}CO_2$ was collected for 1 h at 37 °C in a shaking water bath. Center wells were then removed, placed in scintillation vials with 4.0 mL of Hionic Fluor and ^{14}C was counted using a Beckman LS 5801 counter (Beckman Instruments, Irvine, CA). To calculate $^{14}CO_2$ release, the activity of blank tubes was subtracted from that of sample tubes.

The propionate CO_2 ratio illustrates the ratio of carbons from different positions in the molecule (in this case, C-1:C-2 from propionate) which are converted to CO_2. This parameter represents the likelihood of a molecule leaving as another intermediate product once it has entered TCA cycle metabolism (Kelleher, 1985). Because C2 and C3 of propionate are theoretically oxidized at the same rate in TCA cycle metabolism, the total CO_2 formation from propionate in this system can be calculated as ($^{14}CO_2$ formation from [1-^{14}C]-propionate)+2($^{14}CO_2$ formation from [2-^{14}C]-propionate).

The A+T ratio, also a measurement of anaplerotic flux (Kelleher, 1985) represents the probability that a compound will complete one turn of the TCA cycle by either remaining in the cycle ("T") or leaving as a 4-carbon intermediate and re-entering via acetyl-CoA ("A").

Protein Synthesis

^{3}H-Phenylalanine incorporation into isolated colonocytes was used to determine relative rate of protein synthesis as described by Higashiguchi et al. (1993). Cells were isolated and after the final wash and re-suspension in supplemented culture media, cells were washed twice in an albumin-free culture media solution containing RPMI 1640, 1% antibiotic-antimycotic and 25 mmol/L HEPES. Final cellular DNA concentration was approximately 80 mg DNA/L.

For the protein synthesis assay, the incubation media contained RPMI 1640 culture media, 100 mU/L insulin, 0.5 mmol/L phenylalanine, 74 MBq/L (2.0 μCi/mL) of ^{3}H-phenylalanine, 1% antibiotic-antimycotic, and 25 mmol/L HEPES. Cells (0.3 mL) were incubated in 2.0 mL of one of the seven variations on the incubation media outlined in Table 5-1.

Incubations were carried out in siliconized glass tubes (Vacutainer, Beckton Dickson, Rutherford, NJ) for 30 minutes at 37°C in a shaking water bath. During the incubation, tubes were gassed continuously with O_2:CO_2 (19:1). Metabolism was stopped by the addition of 5 mL of ice-cold 10% trichloroacetic acid (TCAA) to each tube. Blanks were prepared by adding cell aliquots after the addition of the 10% TCAA. Cell pellets were obtained and washed once with ice-cold 10% TCAA, once with 10% TCAA containing 5 mmol/L phenylalanine, and once again with cold 10% TCAA.

The pellets were then extracted with 5 mL of ethanol:ether (1:1) and were dried overnight in a fume-hood. The dried pellet was re-suspended in 1.0 mL of Soluene and 0.1 mL water and counted with 4 mL of Hionic Fluor. The incorporation of phenylalanine into protein was expressed as nmol/(mg DNA · h). Intracellular specific activity of phenylalanine was assessed as per Higashiguchi et al. (1993) to ensure that the intracellular precursor free amino acid pool was equivalent to the specific activity of phenylalanine in the incubation solutions. Intracellular phenylalanine concentrations and

specific activities were used to determine fractional protein synthesis rates (%/day).

Other Analytical Procedures

The DNA content of the cell suspensions was determined fluorometrically using the method of Prasad et al. (1972) with calf thymus DNA as a standard. Colonocyte viability was assessed over time in the different incubation solutions. Lactate dehydrogenase activity released into the cell suspension supernatant was measured as an indicator of cell viability (Del Castillo et al., 1991). Activity was determined spectrophotometrically using kit # 228 (Sigma Chemical, St. Louis, MO). Alkaline phosphatase activity of the cell suspensions was measured using kit #245 (Sigma Chemical, St. Louis, MO).

Statistics

Two colons were pooled each experimental day for one observation. One observation was determined as the mean of triplicate measurements except oxygen consumption measurements which were determined using 2 replicates. The general linear model procedure in SAS (Version 6.04, SAS Institute, Cary, NC) was used for all statistical analysis. Because of animal variability between observations, differences were determined using a two-way ANOVA model (incubation solution and experimental day) followed by Duncan's test for multiple comparisons where required. Statistical significance was defined as $P \leq 0.05$. Values given are means ± SEM, for n = 12.

RESULTS

We have previously shown that DNA content correlates highly ($P \leq 0.0001$) with cell number (Chapter 3) and thus DNA content was used as the denominator for all measurements. Colon mass was 2.1 ± 0.2 g. Alkaline phosphatase activity in the cell suspensions averaged 24.5 ± 2.1 U/g DNA. Colonocyte viability prior to analysis of metabolic parameters was 94.2 ± 0.6 %. Evaluation of the viability of colonocytes in the different solutions revealed an overall decrease of 4.2 ± 1.4 % viability in the first 30 minutes, with no differences between solutions. After the 2 h incubation period, the

overall decrease in viability was 13.4 ± 1.4%. After 2 h, the solutions containing either 30 mmol/L butyrate or 10 mmol/L mixed short-chain fatty acids (corresponding to solutions 5 and 6 in Table 1) showed no significant decrease in cell viability from time zero. Thus, for the 2 h viability measurement, cells incubated in these 2 solutions were significantly more viable than cells in all other solutions ($P \leq 0.05$). No other viability differences between solutions were detected. Oxygen consumption rate did not differ due to the presence or absence of 15 mmol/L short-chain fatty acids in the incubation media (Table 5-2).

Cell proliferation, as assessed by ^{3}H-thymidine incorporation, was affected by incubation solution composition (Figure 5-1). Solutions containing NaCl at either 15 mmol/L or 30 mmol/L were used as iso-osmotic controls for the addition of either 15 mmol/L or 30 mmol/L mixed short-chain fatty acids (which were included in the sodium salt form). The addition of NaCl to the incubation media did not significantly alter proliferation rate. However, the addition of short-chain fatty acids, either at 15 mmol/L or 30 mmol/L drastically reduced proliferation rates by 47% and 56%, respectively. The magnitude of this effect was not observed by the addition of butyric acid alone at either the 5 or 10 mmol/L level, although proliferation was reduced by 19% and 29%, respectively, when compared to the solution without butyrate. The decrease observed was dose-dependent in that proliferation tended to decrease further when the butyrate or short-chain fatty acid concentrations were increased from 5 to 10 mmol/L or from 15 to 30 mmol/L but this trend was not significant.

The oxidation rates of C-1 and C-2 from propionate as well as overall propionate oxidation rates are shown in Table 5-3. The presence of 5 mmol/L butyric acid in the incubation media drastically reduced CO_2 formation from both labeled propionate molecules. Simiiarly, the A + T ratio decreased, indicating more flux through TCA cycle metabolism (Table 5-3). The presence of butyrate in the incubation media also increased

the propionate CO_2 ratio, indicating that in the presence of 5 mmol/L butyric, more molecules are entering and leaving TCA cycle metabolism.

The addition of either 15 mmol/L NaCl or 15 mmol/L short-chain fatty acids did not alter protein synthesis in this system although there was a non-significant trend toward a decrease in ^{3}H-phenylalanine incorporation (Figure 5-2). However, the addition of 30 mmol/L NaCl and 30 mmol/L short-chain fatty acids reduced protein synthesis by 23% and 25%, respectively. This effect was not different from the addition of 10 mmol/L butyric acid alone. Nor did the addition of 5 mmol/L butyrate result in a different rate of protein synthesis that the 15 mmol/L NaCl or the 15 mmol/L short-chain fatty acids. The rates of protein synthesis with short-chain fatty acid containing solutions as compared to the corresponding iso-osmotic NaCl controls were not different.

DISCUSSION

This data shows that in vitro short-chain fatty acids decrease both proliferation and protein synthesis rates in isolated colonocytes. Although short-chain fatty acids did not influence oxygen uptake in colonocytes, anaplerotic flux through TCA cycle pathways was increased by the addition of 5 mmol/L butyrate in vitro.

Cell Proliferation

The observation that changes in cell proliferation observed with the addition of short-chain fatty acids in vitro are not due to extracellular osmolarity is a novel finding in this study. Short-chain fatty acids reduced colonocyte proliferation rate regardless of the osmotic pressure of the medium. Although previous work assessing the effect of short-chain fatty acids on cell proliferation has found that butyrate was largely responsible for the reduced rate in vitro (Gamet et al., 1992) and that luminal butyrate concentrations most closely correlated to the enhanced proliferation rate in vivo (Boffa et al., 1992), our results indicate that butyrate is not solely responsible for the antiproliferative effects of mixed short-chain fatty acids in vitro. Similarly, Frankel et al. (1994a) recently reported that

changes in proliferation of colonic mucosa obtained from humans with ulcerative colitis, Crohn's disease, and diverticulitis were independent of medium osmolarity.

Protein Synthesis

Protein synthesis was inhibited both by 30 mmol/L short-chain fatty acids and isomolar NaCl. This finding is consistent with the work of Stoll et al. (1992) which established that the rate of protein synthesis in isolated hepatocytes was osmotically regulated regardless of whether raffinose or NaCl was used to adjust incubation media osmolarity. Häussinger et al. (1994, review) has established that hepatocyte proliferation is regulated by cellular hydration state and that osmotic conditions which induce cell shrinkage result in reduced protein synthesis.

The fractional rate of protein synthesis found in this study indicates that 100% protein synthesis would occur in less than one day for these cells. This is consistent with the in vivo work of Stein et al. (1994) which established a fractional synthesis rate of approximately 150 %/day in both proximal and distal colon segments in rats. Frankel et al. (1994a) found 10 mmol/L butyrate did not alter colonocyte protein synthesis in vitro (independent of osmolarity) for normal, Crohn's or diverticulitis mucosa. However butyrate inhibited protein synthesis in mucosa from humans with ulcerative colitis. It has been suggested that intracellular changes in pH induced by short-chain fatty acid absorption may be linked to the osmotically regulated alterations in cell function, due to the influx of sodium required to remove hydrogen ions once the protonated form of a short-chain fatty acid is absorbed (DeSoignie and Sellin, 1994).

Oxygen Uptake

The observation that 15 mmol/L of mixed short-chain fatty acids does not reduce oxygen uptake by colonocytes is supported by the work of Fleming et al. (1991) and Roediger (1982) which showed that adding butyrate to a medium containing glucose did not reduce oxygen uptake by isolated colonocytes but either maintained or increased oxygen uptake.

Thus, although short-chain fatty acids do alter cell proliferation and protein synthesis (especially at the 30 mmol/L level), overall metabolic energy expenditure was unchanged.

Anaplerotic Flux

The addition of butyric acid to a medium already containing other metabolic fuels such as glutamine and glucose showed a shift in TCA cycle metabolism in colonocytes. The propionate CO_2 ratio and the A+T probability both indicate that, in the presence of butyrate, substrates entering TCA cycle metabolism are less completely oxidized to CO_2 before leaving TCA cycle metabolism as other intermediate products. Because butyric acid is so readily oxidized by colonocytes (Fleming et al., 1991; Marsman and McBurney, 1995; Roediger, 1982) and enters oxidative metabolism as acetyl-CoA, it may necessitate the entry of other molecules into TCA cycle metabolism to provide oxaloacetate. An increased flux through the TCA cycle would allow for the intermediate molecules to be removed from oxidative metabolism to other cellular purposes, without increasing overall oxygen consumption.

When butyrate was present in the incubation media, CO_2 formation from propionic acid was drastically reduced, indicating that butyrate either displaces propionate prior to complete oxidation or exerts a non-competitive inhibitory effect on propionate oxidation. Transport of short-chain fatty acids is thought to be through a bicarbonate- and pH-dependent active transport, carrier mediated mechanism (Reynolds et al., 1993). It could be that propionate and butyrate are competitively absorbed into the cell or that they compete for co-acylation, a required step prior to TCA cycle oxidation, once inside the cell. Neither hypothesis has been confirmed.

Final Conclusion

Butyrate altered TCA cycle metabolism, leading to less complete oxidation of substrates, but oxygen uptake was unchanged. Mixed short-chain fatty acids reduced colonocyte proliferation, independent of osmotic regulation and the reduction in protein synthesis

seems to be attributable to changes in the osmotic pressure of the extracellular media. Further investigation is required to determine the signals whereby short-chain fatty acids influence cell proliferation and protein synthesis.

TABLE 5-1

Composition of Incubation Solutions[1]

Solution #	Acetate	Propionate	Butyrate	NaCl
	mmol/L			
1	.	.	.	.
2	.	.	5	.
3	5	5	5	.
4	.	.	.	15
5	.	.	10	.
6	10	10	10	.
7	.	.	.	30

Incubation solutions also contained 11 mmol/L glucose, 1 mmol/L glutamine, 10 g/L bovine serum albumin, 25 mmol/L HEPES buffer, 10 g/L antibiotic-antimycotic and were made up with RPMI 1640 complete culture media. Acetate, propionate, and butyrate were added to incubation media as the sodium salt form. Concentrations shown here are the final concentrations of the solutions once the cell aliquots were added to the incubation media. For cell proliferation analysis only, thymidine was added at a level of 30 nmol/L. For protein synthesis determination only, the albumin was omitted and 0.5 mmol/L phenylalanine and 100 mU/L insulin were included.

TABLE 5-2

The effect of short-chain fatty acids on oxygen consumption by isolated colonocytes[1]

	Short-Chain Fatty Acid Concentration *mmol/L*	
	0	15
Oxygen Consumed *nmol/(μg DNA · h)*	44.6 ± 5.6	46.0 ± 5.6

[1] Values are means ± SEM, n=12. Values were not significantly different ($P \leq 0.05$). Solutions contained either 0 or 15 mmol/L mixed short-chain fatty acids (SCFA). Complete solution compositions are outlined in Table 5-1 for solution #1 and #3 for 0 and 15 mmol/L SCFA, respectively.

TABLE 5-3

The effect of butyrate

on anaplerotic flux and propionate oxidation by isolated colonocytes[1]

	Butyrate concentration *mmol/L*	
	0	5
[1-^{14}C]:[2-^{14}C]-propionate CO_2 ratio	1.78 ± 0.08[a]	2.15 ± 0.09[b]
A+T Probability	0.72 ± .02[a]	0.63 ± 0.02[b]
$^{14}CO_2$ from [1-^{14}C]-propionate *µmol/(g DNA · h)*	95.5 ± 4.2[a]	14.4 ± 4.2[b]
$^{14}CO_2$ from [2-^{14}C]-propionate *µmol/(g DNA · h)*	53.7 ± 2.1[a]	6.6 ± 2.1[b]
Total CO_2 formation from propionate *µmol/(g DNA · h)*	203 ± 8[a]	27.6 ± 8[b]

[1] Values are means ± SEM, n=12. Within a row, values with different letters are significantly different ($P \leq 0.05$). Complete solution compositions are outlined in Table 5-1 for solution #1 and #2 for 0 and 5 mmol/L butyric acid, respectively.

Figure 5-1 The effect of incubation media composition on the proliferation rate of isolated colonocytes. Values are means ± SEM, n=12. ^{3}H-Thymidine incorporation into colonocytes was measured in incubation media containing 0, 5 or 10 mmol/L butyrate in the presence and absence of 10 or 20 mmol/L of both acetate and propionate (Ac+Prop, equimolar amounts of each). These values were compared to values obtained in solutions containing 0, 15 or 30 mmol/L NaCl. Complete composition of the incubation media is outlined in the Table 5-1. Means with different letters are significantly different ($P \leq 0.05$).

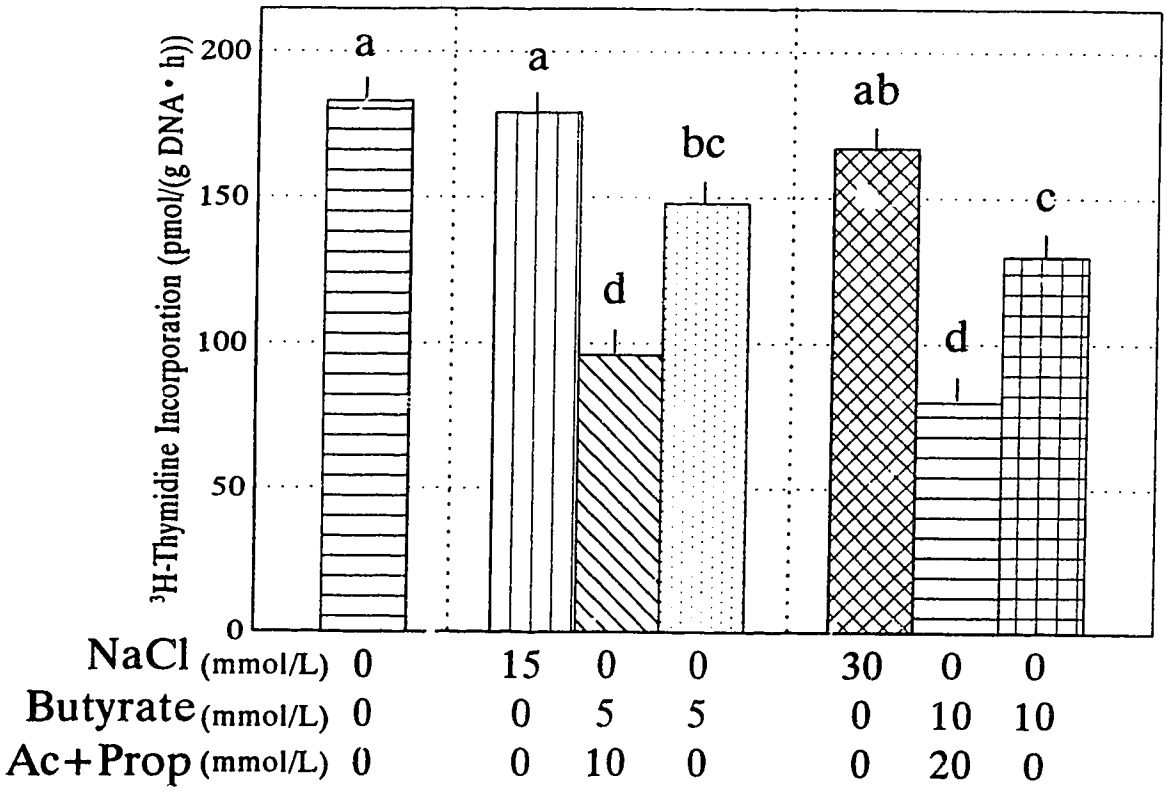
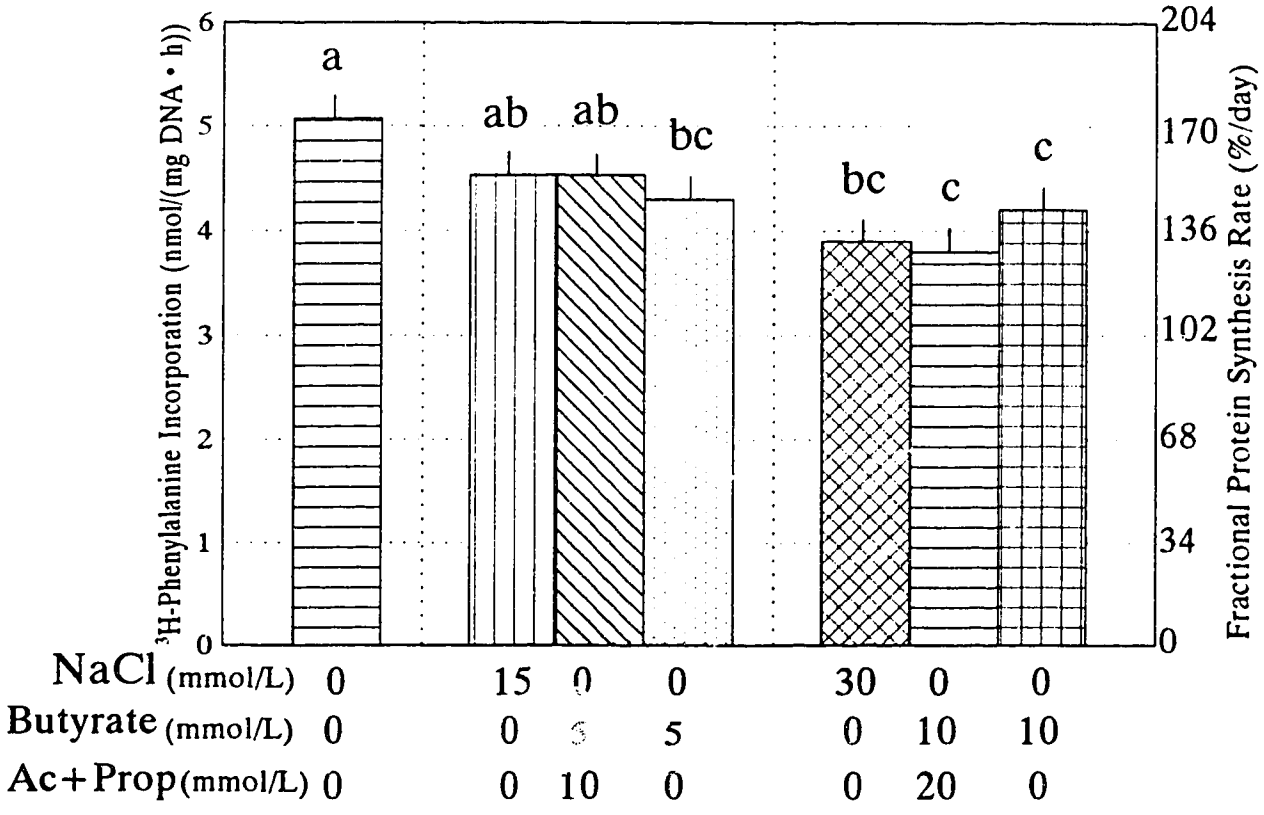


Figure 5-2 The effect of incubation media composition on protein synthesis in isolated colonocytes. Values are means ± SEM, n=12. ^{3}H-Phenylalanine incorporation and fractional protein synthesis rates in colonocytes were measured in incubation media containing 0, 5 or 10 mmol/L butyrate in the presence and absence of 10 or 20 mmol/L of both acetate and propionate (Ac+Prop, equimolar amounts of each). These values were compared to values obtained in solutions containing 0, 15 or 30 mmol/L NaCl. Complete composition of the incubation media is outlined in the Table 5-1. Means with different letters are significantly different ($P \leq 0.05$).



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CHAPTER 6

GENERAL DISCUSSION AND CONCLUSIONS

Hypothesis 1

Colonocytes isolated from rats fed high-fiber vs. fiber-free diets will have:

- increased oxidative utilization of short-chain fatty acids, glucose and glutamine
- increased rates of oxygen uptake, cell proliferation and protein synthesis

Increased substrate oxidation rates for glucose, glutamine and short-chain fatty acids were observed with fiber supplementation in Chapter 2. However, in Chapter 3 only the increase in glutamine oxidation was replicated. Thus, consumption of fiber resulted only in increased glutamine oxidation. It was observed that fiber consumption increased colonocyte proliferation rate but not protein synthesis or oxygen uptake (Chapter 4).

Hypothesis 2

In isolated colonocytes, the addition of short-chain fatty acids to incubation media will:

- inhibit glucose and glutamine oxidation
- increase oxygen uptake, cell proliferation and protein synthesis

Short-chain fatty acids moderated colonocyte fuel metabolism. Providing short-chain fatty acids did not change glucose or glutamine oxidation but butyric acid reduced the amount of propionic acid converted to CO_2. Anaplerotic flux increased when butyric acid was provided to colonocytes in vitro but oxygen uptake was unaffected. Short-chain fatty acids reduced colonocyte protein synthesis and cell proliferation. Protein synthesis but not cell proliferation was regulated by extracellular osmolarity.

The supplementation of fiber into an elemental diet achieved the desired effect of increasing colonic mass after 2 weeks of consumption, resulting in an increase of 20% for each 15% increment of fiber in the diet. This effect was consistent with the work of others (Maxton et al., 1987).

Alterations in metabolism of colonocytes were seen with intestinal hypertrophy induced by fiber supplementation. The rate at which glutamine was converted to CO_2 was consistently greater for cells from fiber-fed animals than for those from animals consuming fiber-free diets (Figure 2-2 and Figure 3-1). Together with the observed decrease in net glutamate formation in colonocytes from the high-fiber group (Figure 2-3 and Figure 3-2) and the trend toward increased anaplerotic flux (Table 3-6), it can be concluded that fiber increased glutamine oxidation and encouraged the entry of glutamate into TCA cycle metabolism.

Consistent with the work of others, substrate oxidation was ultimately unaffected by fiber consumption. Darcy-Vrillon et al. (1993) recently assessed the effect of low fiber (4%) vs. high fiber (16%) and found a non-significant increase in colonocyte butyrate oxidation of approximately 25%. Clausen and Mortensen (1994) examined the effect of 10% pectin supplementation on oxidation kinetics in colonocytes and found no major alterations due to diet. The magnitude of the changes found in the current work (Figure 2-1) was similar to that of Darcy-Vrillon et al. (1993). ATP production rates from glucose, glutamine, and short-chain fatty acids were not changed with level of fiber in the diet (Table 3-7).

Differences existed between the oxidation results in Chapters 3 and 3. These discrepancies may be attributable to differences in diet composition and consumption. The diets in Chapter 2 were not matched in nutrient to energy ratio, and the high fiber diet contained more nutrients per unit energy. Animals also consumed fewer calories and gained less weight on the fiber-containing diet; a trend which was reversed in subsequent chapters. Although oxidative differences between short-chain fatty acids were not found

in Chapter 3, the same differences in glutamine oxidation were observed in both Chapters 2 and 3.

Previous reports using buffer/salt solutions containing one or two oxidizable substrates concluded that short-chain fatty acids competitively reduced the amount of glucose and glutamine converted to CO_2 (Fleming et al., 1991; Roediger, 1982). In the complete culture media system used in the current experiments, the addition of short-chain fatty acids would only marginally change the availability of substrates for TCA cycle metabolism, relative to their addition in a single substrate system. Thus, in this model, short-chain fatty acid availability does not influence metabolism of other oxidizable substrates. Nevertheless, butyrate inhibited propionate oxidation in complete media (Table 5-3) as observed in a simple substrate system by Clausen and Mortensen (1994). It may be that these molecules compete for absorption into the cell, since there is evidence to support a common transport mechanism (Mascolo et al., 1991).

It is also possible that, once inside the colonocyte, propionate and butyrate compete for activation by co-acylation enzymes. Butyrate enters TCA cycle metabolism as acetyl-CoA and propionate enters as succinyl-CoA which can replenish 4-carbon TCA cycle intermediates. Thus, it is likely that the inhibition of propionate oxidation by butyrate occurs prior to the entry of these substrates into oxidative metabolism, since propionate and butyrate do not directly compete for entry into TCA cycle metabolism.

Previous work in single-substrate systems found that the addition of butyrate increased oxygen consumption (Fleming et al., 1991; Roediger, 1982) but this was not supported by the oxygen consumption data obtained in the current system under study. In complete media, colonocytes may utilize oxygen at an optimal rate that cannot be further increased by the addition of substrate to the media. The use of culture media reduces the likelihood that the results obtained reflect only substrate availability.

Some of the oxidation data generated in these studies can be compared to the previous work of others though the use of conversion factors to change denominators. Thus, converting the work of others to units used in this thesis, the comparisons in Table 6-1 can be made.

TABLE 6-1

Converted Data From Previous Oxidation Studies[1]

	CO_2 production from different substrates			Oxygen consumed
	10 or 11 mmol/L glucose	5 mmol/L glutamine	5 or 10 mmol/L butyrate	nmol/(μg DNA · h)
Reference	nmol/(μg DNA · h)			
Darcy-Vrillon et al. 1993	14.4	10.8	24.0	- -
Firmansyah et al. 1989	1.3	1.7	3.5	- -
Fleming et al. 1991	11.3	11.3	22.6	32 - 53
Roediger 1982	9.4	1.9	4.5	24 - 38
Current Data	3.0	2.5	5.0	45 - 50

[1]Values approximated based on the conversion factors of 1 g dry weight of cells = 31.8 mg DNA (Firmansyah et al., 1989) and 1 x 10^6 cells = 5 μg DNA (Chapter 3).

The values in Table 6-1 are based on the work of others, done in single-substrate incubation systems as compared to the current data, which was assessed in complete culture media. Although some discrepancies exist, it is evident that the current data is in an acceptable range. This conversion of the work of thers also indicates the difficulties

involved in comparing oxidation values between different lab groups. The high rate of oxygen consumption in the current data, as compared to that of thers indicates that our cells were at least as viable as the cells used by others.

The increase in anaplerotic flux when butyrate is added to complete culture media (Table 5-3) is indicative of the affinity of colonocytes for butyrate oxidation (Chapter 3). The increased entry of acetyl-CoA into TCA cycle metabolism from butyrate metabolism increases the availability of 4-carbon TCA cycle intermediates for citrate formation. Although some butyrate is completely oxidized to CO_2, increased anaplerosis indicates that proportionately more TCA cycle intermediates are entering and leaving oxidative metabolism pathways when butyrate is present. This observation, together with the lack of increase in oxygen consumption when short-chain fatty acids are supplied (Table 5-2), illustrates that the rate of fuel oxidation is not substrate-dependent in this system. The decrease in net glutamate and aspartate production from glutamine when short-chain fatty acids are present in the media (Figure 2-3) is consistent with altered anaplerotic flux. Both glutamate and aspartate may undergo further TCA cycle metabolism to provide more 4-carbon TCA cycle intermediates for oxaloacetate formation.

The increase in colonocyte proliferation rate in cells isolated from rats fed the high-fiber vs. the fiber-free diet (Figure 4-1) is consistent with the work of others (Boffa et al., 1992; Jacobs and Lupton, 1984; Zhang and Lupton, 1994) which established that the ingestion of dietary fiber increases in vivo colonic proliferation rates. Ahnen et al. (1988) found that more mature populations of colonocytes isolated from the tip of the villous-crypt axis had a decreased rate of ^{3}H-thymidine incorporation. Also, rats fed high fiber diets have enhanced proliferative zones (Lupton and Jacobs, 1987). Thus, the current results suggest that a greater number of immature cells are present in colonocyte suspensions from the fiber group.

Short-chain fatty acids inhibited proliferation in isolated colonocytes (Figure 4-1 and

Figure 5-1). Although in vivo perfusion of short-chain fatty acids stimulated proliferation (Frankel et al., 1994), it was found that the nervous system was an integral part of this response. In vitro studies have the effect of removing cells from other physiological effects of short-chain fatty acids, such as increased regional blood flow (Mortensen et al., 1990; Nutting et al., 1991). Thus, the only effects of short-chain fatty acids on proliferation rates which can be observed in vitro are the direct local effects. Although short-chain fatty acids did not increase cell proliferation in this model, the possibility has not been precluded that the indirect in vivo response to short-chain fatty acids is responsible for enhanced cell proliferation with fiber supplementation.

In Chapter 5 butyrate was found to be important but not solely responsible for the short-chain fatty acid effects on protein synthesis. Moreover, osmolarity did not regulate cell proliferation (Figure 5-1). Others have observed an antiproliferative effect of butyrate on cultured cells (Dexter et al., 1984; Gamet et al., 1992; Niles et al., 1988) and our data is consistent with theirs. The results suggest that short-chain fatty acids, particularly butyrate, provide a metabolic signal to cells to reduce cell proliferation.

Fiber supplementation did not significantly increase protein synthesis in isolated colonocytes. Ahnen et al. (1988) found an increase in protein synthesis for immature colonocytes from crypts vs. more mature cells from the villous. Our data showed a similar although non-significant trend for high vs. low fiber treatments. The measurement of alkaline phosphatase, an indicator of cell maturity, in the colonocyte population showed a non-significant trend toward increased cell differentiation with the fiber-supplemented diet (Table 3-3 and Table 4-3). These two observations are not necessarily contradictory since enhanced proliferation in the crypts (or larger proliferative zones) and more mature or differentiated cells remaining on the villous structure can occur concurrently. Both of these possibilities would be consistent with the increase in colonic mass induced with fiber-supplementation. Exposure to butyrate in vitro stimulates cellular differentiation in human colonic carcinoma cells (Niles et al., 1988). Luminal in vivo exposure to butyrate

may also increase differentiation.

Others have established that protein synthesis can be regulated osmotically in isolated hepatocytes (Häussinger et al., 1994; Stoll et al., 1992). Similarly, the addition of short-chain fatty acids reduces protein synthesis in isolated colonocytes and this effect can be attributed to osmotic regulation (Figure 5-2). The fractional rates of protein synthesis found in this study indicate that 100% synthesis of protein would occur in less than one day. This is consistent with in vivo protein synthesis rates determined in whole colon by Stein et al. (1994) which found approximately 150 %/day fractional synthesis rate in the colon. Our rate is also consistent with the work of Yoshida et al. (1992) which found that in vivo fractional protein synthesis rates in the colons of rats fed by TPN were between 40 - 70 %/day. This rate increased with sepsis. Stein et al. (1994) found a dramatic decrease (up to 70%) in colonic fractional synthesis rate when animals were fed intravenously vs. oral feeding. Although this suggests that increased luminal nutrient supply increases fractional synthesis rate, our data showed that including fiber in the diet (which would increase luminal short-chain fatty acid supply) did not have a similar effect.

Final Conclusion

Short-term fiber supplementation increases colonic mass due in part to enhanced proliferation, as was confirmed by in vitro assessment in Chapter 4. An up-regulation of colonocyte glutamine oxidation but not glucose or short-chain fatty acid oxidation was observed with fiber supplementation. Although this increase in glutamine utilization did not result in an increase in overall ATP generation, it may be important to colonocytes for other synthetic purposes, such as polyamineand amino acid synthesis, proliferation, or for urea synthesis. The fiber content of the diet did not alter overall ATP yield from oxidative metabolism nor, ultimately, oxygen uptake. This indicates that although colon mass may be increased, the oxidative metabolism of individual cells remains unchanged. Dietary fiber and luminal nutrient supply do not change cell function.

Oxidation of glucose and glutamine by colonocytes was unaffected when these cells were exposed to short-chain fatty acids in a complete culture media substrate system in vitro, indicating that these substrates do not competitively inhibit the oxidative metabolism of the other, as had previously been observed using simple substrate systems. Butyrate inhibits propionate oxidation, an effect which may be regulated prior to the entry of either substrate into TCA cycle metabolism, such as through competitive transport into the cell or by competition for co-acylation enzymes prior to oxidation. Butyrate modified anaplerotic flux through TCA cycle metabolism, decreasing the likelihood that any molecule, once entering TCA cycle metabolism, will be completely oxidized to CO_2. This illustrates that butyrate metabolism can alter TCA cycle flux without significantly changing overall oxidation of other substrates.

The decrease in colonocyte protein synthesis but not proliferation induced by short-chain fatty acids was attributable to alterations in extracellular osmolarity. It is possible that the in vitro effects of short-chain fatty acids on intracellular colonocyte pH and osmolarity may be contributing to the reduced biosynthesis observed in these studies because of an increased energy expenditure to maintain the intracellular environment. In vivo, absorption of short-chain fatty acids is accompanied by serosal removal of cell products and ions which may assist the cell in maintenance of a constant intracellular pH. The direct exposure of colonocytes to short-chain fatty acids does not appear to be the mechanism whereby dietary fiber exerts trophic effects on the colon. However, the in vitro model of assessment removes the other possible effects short-chain fatty acids may exert (through neural or hormonal signalling or increased regional blood flow and nutrient delivery) to increase colonic mass and proliferation rate. Thus, it should not be concluded that short-chain fatty acids lack a trophic effect on colon cells per se, but rather it can be concluded that direct exposure of colon cells to these compounds is an inadequate model with which to assess the mechanism for observed in vivo effects.

In summary, this thesis confirms that the ingestion of a high-fiber diet results in a greater

colonic mucosal mass but this does not induce physiologically significant changes in cellular metabolism. Using a complete culture media substrate system which mimics serosal nutrient availability, short-chain fatty acids do not influence the oxidative metabolism of glucose or glutamine. Butyrate does seem to inhibit propionate oxidation and decreases the proability that any molecule entering the TCA cycle is completely oxidized. In vitro colonocyte protein synthesis and cell proliferation are influenced by short-chain fatty acid concentration. The influence of short-chain fatty acids on intracellular pH and osmolarity remains to be better elucidated. Clearly, however, in terms of whole-body energetics, dietary fiber increases colonic energy demand via increased colon mass but not through increased cellular oxidative metabolism. Short-chain fatty acids cannot replace the cellular requirement for oxidation of glucose and glutamine.

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APPENDIX 1

DETERMINATION OF THE DIGESTIBLE ENERGY CONTENT OF THE FIBER SOURCE USED

In monogastric animals, dietary fiber moves through the upper intestinal tract undigested[1]. When the fiber arrives at the large bowel, bacterial fermentation degrades the fiber and the main fermentation products (acetate, propionate, and butyrate) are absorbed by cecal and colonic mucosa[2]. The amount of these short-chain fatty acids absorbed depends on the fermentability of the fiber source[3]. Thus, although no upper intestinal energy is derived from fiber, a certain amount of energy is derived from absorption of fermentation products. Since the amount of digestible energy provided by a fiber source varies according to the fermentability, this value must be determined for each particular fiber source in order to accurately account for this digestible energy value in diet formulations.

Objective

To determine the digestible energy value of the fiber source containing (in a ratio of 5:1) Fibrad (a mixture of pea, oat, and sugar-beet fibers, xanthan gum and soy lecithin), provided by Ross Laboratories (Columbus, Ohio) and Alphacel (ICN Biomedicals, Mississauga, Canada).

Methods

30 female Sprague-Dawley rats (180 - 200 g) were fed one of the five diets outlined in Table A-1. The diets formulation used an elemental defined formula diet as a base and

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[1]Bugaut, M. Occurrence, absorption and metabolism of short chain fatty acids in the digestive tract of mammals. *Comp. Biochem. Physiol.* 1987; **86B**: 439 - 472.

[2]Fleming, S.E. Influence of dietary fiber on the production, absorption, or excretion of short chain fatty acids in humans. In: *CRC Handbook of Dietary Fiber in Human Nutrition* (Spiller, G.A., ed.) CRC Press, Boca Raton, FL. Vol. 2, 1993: pp. 387 - 412.

[3]McBurney, M.I. Potential water-holding capacity and short-chain fatty acid production from purified fiber sources in a fecal incubation system. *Nutrition* 1991; 7: 421 - 424.

supplemented the fiber source into the diet without removing other nutrients to compensate for energy or volume. This resulted in diets containing either 0%, 10%, 20%, 30%, or 40% fiber on a mass basis. Daily, the animals were provided access to a certain amount of diet that provided basic requirements were met[1]. The amount provided to each animal contained the same amount of base diet (0% fiber), so that only the daily fiber consumption varied between groups. This ensured that no differences existed between intakes of protein, fat, minerals or vitamins among diet groups. Intakes were checked daily to ensure that actual diet consumption was in keeping with this design.

After 10 days of diet consumption, feces were collected for 5 days. Feces were freeze-dried and finely ground. Portions of the feces (1.0 g) were subsampled and gross energy was determined using bomb calorimetry. Gross energy values for the diet formulations were also determined and digestible energy for the 0% fiber diet was calculated. The gross energy intake minus gross fecal energy was assessed to determine digestible energy[2]. This was plotted against fiber content to determine digestible energy attributable to fiber.

Results

The amount of protein, fat, minerals and vitamins consumed by animals did not vary among diet treatments. The regression of digestible energy versus fiber content of the diets is shown in Figure A-1. The regression equation for this increment was used to determine digestible energy of the base diet and the fiber source. The digestible energy of the 0% fiber diet (base diet) was determined to be 17.5 kJ/kg.

Final Conclusion

In this model, the digestible energy content of Fibrad:Alphacel (5:1) is 5 kJ/g (1.2 kcal/g).

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[1]Canadian Council on Animal Care. *Guide to the Care and Use of Experimental Animals*. (Olfert, E.D., Cross, B.M. and McWilliam, A.A. eds.) Bradda Printing, Ottawa, Canada. 2nd Edition, Vol.1 1993.

[2]McBurney, M.I. and Sauer, W.C. Fiber and large bowel energy absorpiton: validation of the integrated ileostomy-fermentation model using pigs. *J. Nutr.* 1993; **123**: 721 - 727.

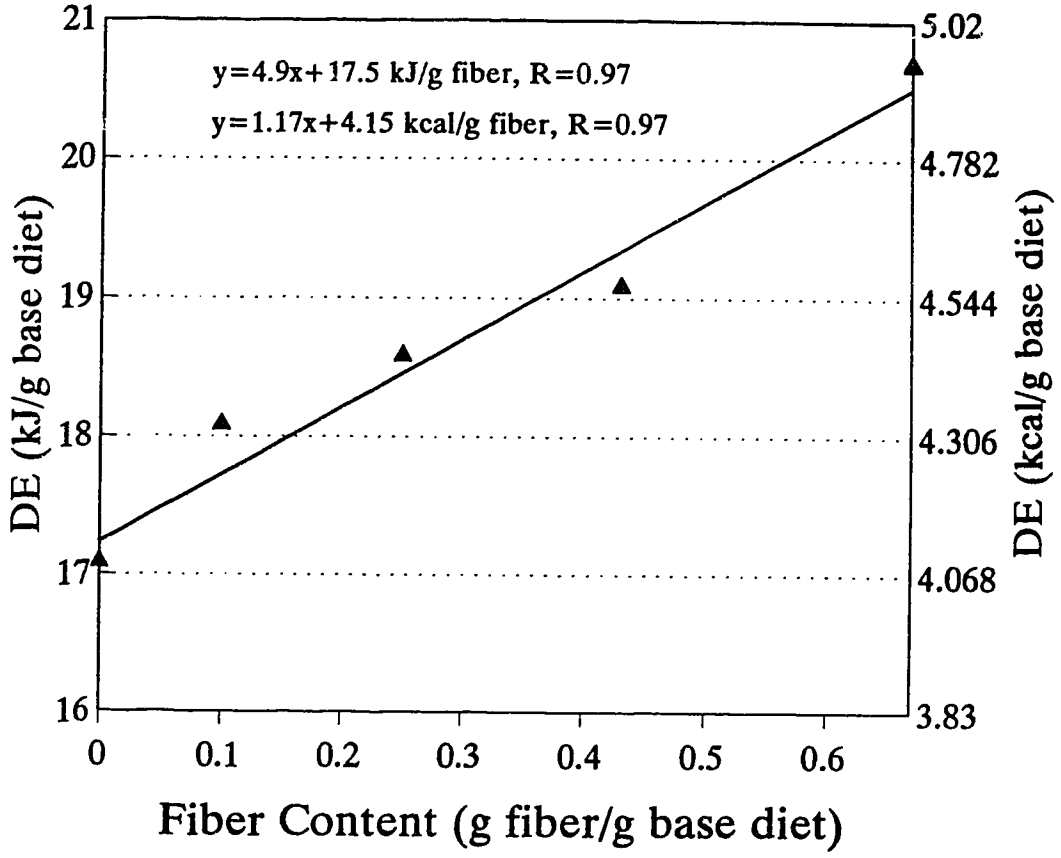
TABLE A-1

Composition of Diets Used for Determination of Digestible Energy Content of a Fiber Source

	Fiber Content of Diets				
	0%	10%	20%	30%	40%
	Base Diet				
Ingredient	*g/kg*				
Amino acid mix[1]	178.5	160.6	142.8	124.9	107.1
Corn oil	100.0	90.0	80.0	70.0	60.0
Glucose	443.0	398.7	354.4	310.1	265.8
Sucrose	221.5	199.4	177.2	155.0	132.9
Fibrad	0	83.0	166.0	249.1	332.0
Alphacel	0	17.0	34.0	51.0	68.0
Mineral mix[1]	50.0	45.0	40.0	35.0	30.0
Vitamin Mix[1]	7.0	6.3	5.6	4.9	4.2
Diet Consumed (*g/d*)	10	11	13	14	17
Amino Acids Consumed (*g/d*)	1.8	1.8	1.8	1.8	1.8

[1]Complete composition can be found in **Chapter 2, Table 2-1**.

Figure A-1 The digestible energy attributable to a fiber source supplemented in an elemental diet.



APPENDIX 2

CALCULATIONS

1. *Conversion of ^{14}C Labeled Molecules to CO_2 and Other Products*

^{14}C-labeled substrates can be used to quantitate oxidative metabolism of the molecules so labeled. The total amount of CO_2 formed by cells from a particular substrate (for U-labeled molecules), or from a certain carbon location (for C1 or C2 labeled molecules) can provide a good estimate of relative differences in oxidative metabolism between treatment groups. Also, product formation from U-labeled molecules can give a good estimate of the metabolic fate of a substrate. Differences in the type of isotopically labeled molecules may be unavoidable due to cost and availability of ^{14}C-labeled molecules.

Where:

A = activity of product formed per incubation tube (*DPM*)

B = activity of product formed per blank tube (*DPM*)

C = activity present in incubation tube (*DPM*)

D = concentration of substrate in final incubation volume (*nmol/L*)

E = final incubation volume (*L*)

F = number of cell in incubation tube ($x10^6$ *cells*)

G = amount of DNA per incubation tube (μg)

N = number of carbons per molecule of substrate

P = number of carbons per molecule of product

T = incubation time (*h*)

a) *Oxidation from [1-^{14}C]- or [2-^{14}C]- or [6-^{14}C]-Labeled Molecules*

The quantity of substrate for which at least the labeled carbon was converted to $^{14}CO_2$ (**Y**) can be calculated as follows. Because carbons from different locations in a

molecule are metabolized at different rates in different systems[1], this value can not be converted to total CO_2 production unless the ratio of CO_2 formation for different carbons in the molecule (propionate CO_2 ratio) has been established in the system used. Units are either (*nmol substrate/(10^6 cells · h)*) or (*nmol substrate/(μg DNA · h)*).

$$\mathbf{Y} = \frac{\mathbf{(A - B) \times D \times E}}{\mathbf{C \times (F \text{ or } G) \times T}}$$

b) *Oxidation and Product Formation from [U-^{14}C]-Labeled Molecules*

Because [U-^{14}C]-labeled molecules represent all carbons in the molecule equally, the $^{14}CO_2$ or ^{14}C-labeled product formed from the oxidation of a U-labeled molecule will be directly proportional to the total CO_2 or other product formed, and thus CO_2 production or product formation can be calculated directly (**Z**). Units are either (*nmol product/(10^6 cells · h)*) or (*nmol product/(μg DNA · h)*).

$$\mathbf{Z} = \frac{\mathbf{(A - B) \times D \times E \times N}}{\mathbf{C \times (F \text{ or } G) \times T \times P}}$$

2. *Anaplerotic Flux*

Anaplerotic flux measurements indicate the completeness with which a molecule is oxidized through TCA cycle metabolism[2]. The ratios of carbons being oxidized from different positions in a molecule, and the probability that a molecule, once it enters TCA cycle metabolism, will stay in for further oxidation can be estimated with these equations.

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[1]Kight, C.E. and Fleming, S.E. Nutrient oxidation by rat intestinal epithelial cells is concentration dependent. *J. Nutr.* 1993; **123**: 876 - 882.

[2]Kelleher, J.K. Analysis of tricarboxylic acid cycle using [^{14}C]citrate specific activity ratios. *Am. J. Physiol.* 1985; **248**: E252 - E260.

For complete explanation of the meaning of these measurements, see Chapters 3 and 5.

a) *Glucose (Pyruvate) CO_2 Ratio*

$$Q = \frac{Y[2\text{-}^{14}C]\text{-glucose}}{Y[6\text{-}^{14}C]\text{-glucose}}$$

b) *Propionate (Acetate) CO_2 Ratio*

$$R = \frac{Y[1\text{-}^{14}C]\text{-propionate}}{Y[2\text{-}^{14}C]\text{-propionate}}$$

c) *"A + T" Probability*

$$S = \frac{2(Y[2\text{-}^{14}C]\text{-propionate})}{Y[1\text{-}^{14}C]\text{-propionate} + Y[2\text{-}^{14}C]\text{-propionate}}$$

3. *Theoretical ATP Yield*

Based on the amount of $^{14}CO_2$ formed from either [U-^{14}C]-labeled substrates or [1-^{14}C]- or [2-^{14}C]-labeled substrates (converted with the appropriate propionate CO_2 ratio), the theoretical production of ATP formed from substrate oxidation can be determined. For some molecules, the formation of other intermediate products is also considered. Units used are (*nmol ATP/(μg DNA · h)*).

Where: **L** = Lactate formation rate (*nmol lactate/(μg DNA · h)*)

M = Aspartate formation rate (*nmol aspartate/(μg DNA · h)*)

R = Propionate CO_2 ratio

Y = Substrate oxidation rate from a [1-^{14}C]-labeled molecule (*nmol substrate/(mg DNA · h)*)

Z = CO_2 formation rate from a [U-^{14}C]-labeled molecule (*nmol CO_2/(μg DNA · h)*)

a) *Glucose*

Complete oxidation of 1 mole of glucose to CO_2 yields 38 ATP, and formation of 2 lactate molecules yields 2 ATP.

$$\mathbf{H} = 38(\mathbf{Z}/6) + \mathbf{L}$$

b) *Glutamine*

Complete oxidation of 1 mole of glutamine to CO_2 yields 24 moles of ATP[1]. Total CO_2 produced from glutamine oxidation was calculated as total moles of CO_2 produced from glutamine minus one mole of CO_2 for every mole of aspartate formed from glutamine, due to the stoichiometric production of 1 mole of CO_2 for every mole of aspartate formed from glutamine. Assuming that glutamate metabolism proceeds mainly through aminotransferase, an additional 9 moles of ATP are generated for every mole of glutamine converted to aspartate[2].

$$\mathbf{I} = 24((\mathbf{Z} - \mathbf{M})/5) + 9\mathbf{M}$$

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[1]Newsholme, P. and Newsholme, E.A. Rates of utilization of glucose, glutamine and oleate and formation of end-products by mouse peritoneal macrophages in culture. *Biochem. J.* 1989; **261**: 211 - 218.

[2]Okine, E.K., Glimm, D.R., Thompson, J.R. and Kennelly, J.J. Influence of stage of lactation on glucose and glutamine metabolism in isolated enterocytes from dairy cattle. *Metabolism* 1995; **44**: 1 - 7.

c) *Propionate*

ATP from propionate was calculated for propionate entering TCA cycle metabolism as succinyl-CoA and forming three moles of CO_2 from complete oxidation, producing 18 moles of ATP[1]. Based on the number of moles of propionate for which at least C1 was converted to CO_2, the propionate CO_2 ratio can then be used to calculate total ATP yield.

$$\mathbf{J} = \frac{(\mathbf{Y}[1\text{-}^{14}C]\text{-propionate} \times \mathbf{R}) + 2(\mathbf{Y}[1\text{-}^{14}C]\text{-propionate})}{3} \times 18$$

d) *Butyrate*

ATP from butyrate was calculated for butyrate metabolized through 2 acetyl-CoA, forming four moles of CO_2 from complete oxidation, producing 27 moles of ATP. Based on the number of moles of propionate for which at least C1 was converted to CO_2, the propionate CO_2 ratio can then be used to calculate total ATP yield.

$$\mathbf{K} = \frac{2(\mathbf{R} \times \mathbf{Y}[1\text{-}^{14}C]\text{-butyrate} + \mathbf{Y}[1\text{-}^{14}C]\text{-butyrate})}{4} \times 27$$

[1]Martin, D.W., Mayes, P.A., Rodwell, V.W. and Granner, D.K. *Harper's Review of Biochemistry*. Lange Medical Publications, Los Altos, CA. 20th Edition 1983.