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Oxidation of short and medium chain C_2-C_8 fatty acids in Sprague-Dawley rat colonocytes

J R Jørgensen, M R Clausen, P B Mortensen

Abstract

***Background*—The predominant colonic short chain fatty acids, acetate, propionate, and butyrate, are oxidised into CO_2 in colonocytes from rat and humans in the preferred order of butyrate (C_4) > propionate (C_3) > acetate (C_2) – hence butyrate is considered to be the principal oxidative substrate for colonocytes.**

***Aims*—To compare colonocyte oxidation of valerate (C_5), hexanoate (C_6), and octanoate (C_8) with that of butyrate.**

***Methods*—Isolated rat colonocytes were incubated in the presence of a concentration range of 1-^{14}C labelled C_2-C_8 fatty acids. Oxidation rates were obtained by quantifying the production of $^{14}CO_2$, and V_{max} (maximum velocity) and K_m (Michaelis-Menten constant) were calculated by computer fitting of the data to a Michaelis-Menten plot.**

***Results*—The K_m value of acetate (0·56 (SEM 0·02) mmol/l) was about fourfold higher than the K_m of butyrate (0·13 (0·01) mmol/l), whereas the K_m values of valerate (0·19 (0·01) mmol/l), hexanoate (0·19 (0·01) mmol/l), and octanoate (0·16 (0·01) mmol/l) were of the same order of magnitude as the K_m of butyrate. Acetate did not influence butyrate oxidation, whereas butyrate strongly inhibited the oxidation of acetate. By contrast, valerate, hexanoate, and octanoate inhibited colonocyte oxidation of butyrate equally or more than the reverse inhibitory effect of butyrate on valerate, hexanoate, and octanoate oxidation. The maximum rates of ATP production were in the order of valerate > octanoate = hexanoate > butyrate > acetate (28·47 (0·70), 21·78 (0·75), 21·33 (0·78), 16·12 (0·49), 9·09 (0·34) (μmol/min/g) respectively).**

***Conclusions*—Valerate, hexanoate, and octanoate seem to be excellent substrates for colonocyte oxidation, similar to butyrate. These results may influence the choice of fatty acid composition in enemas used for treatment of patients in whom deficient colonocyte oxidation is suspected – for example, patients with ulcerative colitis and diversion colitis.**

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Keywords: short chain fatty acids, kinetic constants, fatty acid oxidation, colon, ulcerative colitis.

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Short chain fatty acids (SCFAs) are present in high concentrations of 100–150 mmol/l in colonic contents. They are formed by bacterial fermentation of carbohydrates and proteins. SCFAs are readily absorbed by the colonic mucosa and are considered to be important substrates for oxidation in the colonocyte. Earlier studies have shown that butyrate is the most important substrate for the nourishment of the colonocyte and previous metabolic studies on isolated colonocytes from rats and humans have indicated that colonocytes use SCFAs as respiratory fuel in the preferential order of butyrate > propionate > acetate.[1 2] These studies found the Michaelis-Menten constant (K_m) of butyrate oxidation to CO_2 to be considerably lower than the K_m of propionate and acetate. The simultaneous incubation of the same colonocyte suspension with butyrate and acetate or butyrate and propionate indicated that butyrate inhibited the oxidation of both acetate and propionate, whereas the effect of acetate and propionate on butyrate oxidation was much less pronounced – that is, if presented for the option of having the choice between butyrate and acetate or propionate, colonocytes preferred to oxidise butyrate.[1]

In suspensions of isolated rat and human colonocytes Roediger has previously shown that 70% to 80% of oxygen consumption is due to butyrate oxidation.[3 4] Large bowels diverted of their faecal stream and therefore deprived of luminal production of SCFAs have been shown to develop diversion colitis, which has been treated successfully with SCFA irrigation.[5]

Various clinical studies treating distal ulcerative colitis with enemas of SCFAs or butyrate have been promising,[6–8] whereas a recent trial has shown disappointing results.[9] Some studies on isolated colonocytes and biopsy specimens from patients with ulcerative colitis have indicated that the disease is associated with an inhibited oxidation of butyrate,[10 11] whereas other studies have been unable to show such a defect.[2 12] A recent study, including measurements of maximum velocity (V_{max}) and K_m, has suggested that butyrate is equally well qualified as an excellent substrate for oxidation in colonocytes from both patients with ulcerative colitis as well as control patients resected for colonic cancer.[2]

Acetate, propionate and butyrate constitute approximately 90% of the SCFAs present in the colonic lumen. SCFAs present in lower concentrations are isobutyrate, valerate, isovalerate, and hexanoate.[13] The aim of the present study was to evaluate the preference among these SCFAs present in smaller proportions (and octanoate) for oxidation in isolated rat colonocytes and to compare these results with the colonocyte affinity for butyrate oxidation – that is, to answer the question whether or not butyrate is as unique a substrate

for colonocyte oxidation when compared with the oxidation of C_5-C_8 fatty acids, as earlier shown in comparison with the oxidation of C_2-C_3 fatty acids (acetate and propionate).

Methods

Animals and diet

Male Sprague-Dawley rats were used in all experiments (Møllegaard Breeding Centre, Skensved, Denmark). Animals were fed a standard diet consisting of crude protein 19%, crude fat 4%, crude fibre 6%, ash 7%, moisture 13·5%, and nitrogen free extract 50·5% (Altromin International, Lage, Germany). Animals were adapted to the diet for at least 14 days before use. Food and water were provided ad libitum.

Materials

Bovine serum albumin (fraction V), dithiothreitol (DTT), unlabelled hexanoate, and radioactively labelled compounds [1-^{14}C]-acetate, [1-^{14}C]-valerate, and [1-^{14}C]-hexanoate were obtained from Sigma Chemical Co (St Louis, MO, USA). Unlabelled butyrate was obtained from BDH Limited (Dorset, UK). Labelled compounds [1-14C]-butyrate and [1-14C]-octanoate were obtained from Du Pont-NEN Research Products (Boston, MA, USA). EDTA, unlabelled isobutyrate, valerate, isovalerate, and octanoate were obtained from Merck Chemical Co (Darmstadt, Germany).

Isolation and incubation of colonocytes

Animals were killed by stunning and cervical fracture. The entire colon (from the caecum to the rectal ampulla) was rapidly removed and flushed clear of luminal contents with isotonic sodium chloride. Isolated cells were prepared as previously described,[14] with the exception that DTT was used at 1 mmol/l throughout. In each of the experiments cells from eight to 10 donor animals were pooled providing cell suspensions containing 6·1–12·3 mg dry weight of epithelial cells/ml.

A freshly prepared cell suspension (1 ml) was incubated in 25 ml conical flasks with 1 ml of Krebs-Henseleit saline (pH 7·4),[15] containing 2·5% (w/v) bovine serum albumin and the appropriate substrates. Flasks were gassed with O_2:CO_2 (19:1, v/v) for 15 seconds, stoppered immediately, and shaken at 90 oscillations/minute in a 37°C water bath. Each incubation flask was equipped with a glass centre well. Incubations were terminated at 40 minutes by injecting 0·5 ml of 10% perchloric acid through the stopper into the suspension. In a similar manner, the centre wells were filled with 0·5 ml of 10 mol/l NaOH to adsorb CO_2. The flasks were shaken for a further two hours on ice to allow the labelled $^{14}CO_2$ to diffuse and be trapped, after which 0·1 ml adsorbent was transferred in duplicate to counting vials and mixed with 10 ml scintillation liquid (Ultra Gold, Packard Instrument, Chemical Operations, Groningen, The Netherlands).

Labelled substrates (sodium salts, pH 7·4) were evaluated at concentrations ranging from 0·125 to 5·0 mmol/l for acetate, butyrate, valerate, hexanoate, and octanoate if there was no addition of unlabelled substrate, and concentrations ranging from 0·125 to 16·0 mmol/l if unlabelled substrate was added. When tests for competition among the substrates were performed, the concentration of unlabelled substrate was 1 mmol/l. In each experiment, identical control incubations were run in parallel from which exogenous substrate was omitted, to correct for the non-specific radioactivity.

Analytical methods

Radioactivity was counted in a model 4530 Packard TriCarb liquid scintillation spectrometer (Packard Instrument, IL, USA) and counts were corrected for non-specific activity as mentioned. After protein precipitation of the incubation medium, supernatants were neutralised to pH 7·4 with 20% KOH. The amounts of β-hydroxybutyrate and acetoacetate were determined spectrophotometrically (LKB Biochrom 4050 spectrophotometer, Pharmacia Biosystems, Copenhagen, Denmark). Absorbance changes were corrected for parallel changes in reagent blanks. Duplicate measurements of dry weights were obtained by drying 1 ml cell suspension overnight at 65°C and subtracting the dry weight of 1 ml albumin containing medium.

Kinetic variables and calculations

Rates of oxidation by isolated rat colonocytes, obtained for a range of concentrations, were plotted, resulting in curves obeying Michaelis-Menten kinetics for all substrates, allowing the determination of the kinetic constants V_{max} and K_m. The apparent values were calculated by computer fitting of the data to a Michaelis-Menten plot (Enzfitter, RJ Leatherbarrow, Elsevier, Amsterdam, The Netherlands).

All results are expressed as μmol/min/g dry weight of epithelial cells. Generation of CO_2 from ^{14}C-labelled SCFAs was calculated from the specific activities and trapped $^{14}CO_2$ in sodium hydroxide.

Statistics

Results are expressed as means (SEM). Comparisons between acids (groups) were performed by the one way analysis of variance (ANOVA) on repeated measures. If a statistically significant result was obtained ($p<0·05$), group differences were tested pairwise by the Student-Newman-Keuls method (significant difference at $p<0·05$).

Results

CO_2 production from C_2-C_8 fatty acids in colonocytes

Figure 1 shows the rate values of $^{14}CO_2$ production from the ^{14}C labelled C_2-C_8 fatty acids

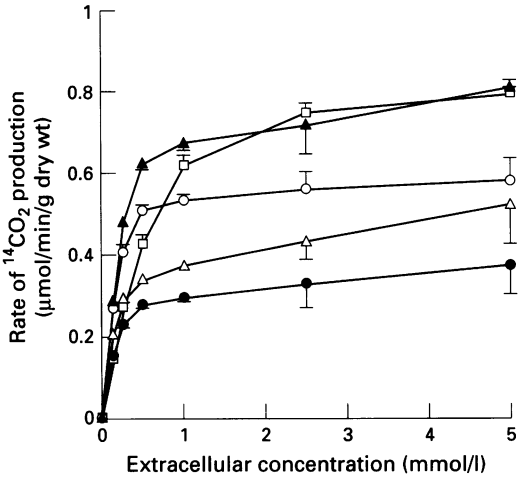

Figure 1: Production rates of $^{14}CO_2$ (mean (SEM)) versus substrate concentrations for oxidation of ^{14}C labelled acetate (□), butyrate (○), valerate (▲), hexanoate (△), and octanoate (●) in isolated rat colonocytes. Table I shows V_{max} and K_m.

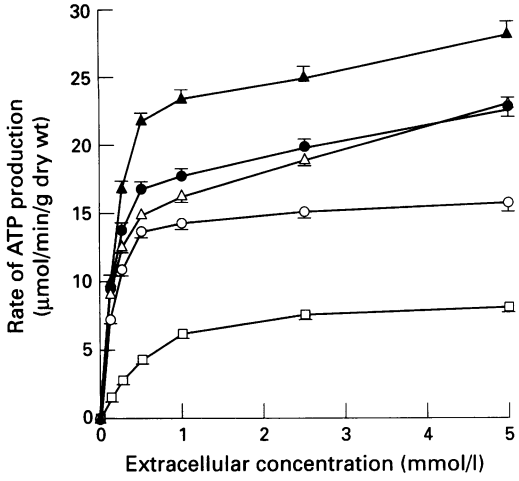

Figure 2: Production rates of ATP (mean (SEM)) versus substrate concentrations for oxidation of acetate (□), butyrate (○), valerate (▲), hexanoate (△), and octanoate (●) in isolated rat colonocytes.

as a function of substrate concentrations, and Table I shows the corresponding kinetic constants, V_{max} and K_m. All five fatty acids were incubated separately in cell suspensions originating from the same batch of colonocytes and experiments were repeated in 10 different batches. The apparent values of V_{max} were all significantly different in the order of acetate > valerate > butyrate > hexanoate > octanoate. However, complete oxidation of 1 mol acetate, butyrate, valerate, hexanoate, and octanoate provides 10, 27, 35, 44, and 61 mol adenosine triphosphate (ATP) respectively,[16] which were multiplied by the $^{14}CO_2$ production to calculate the ATP production from the specific acids. Therefore, the decreasing V_{max} with increasing carbon length of the fatty acids does not reflect the energy produced, measured as ATP, and is probably, at least in part, due to all acids being labelled in the 1-C carbon atom only.

TABLE I *Kinetic constants for production of $^{14}CO_2$ from 1-^{14}C labelled C_2-C_8 fatty acids*

Substrate (n=10)	*V_{max} (µmol/min/g)*	*ATP_{max} (µmol/min/g)*	*K_m (mmol/l)*
Acetate	0·91 (0.12)[a]	9·09 (0·34)[a]	0·56 (0·02)[a]
Butyrate	0·60 (0.06)[b]	16·12 (0·49)[b]	0·13 (0·01)[b]
Valerate	0·81 (0·06)[c]	28·47 (0·70)[c]	0·19 (0·02)[c]
Hexanoate	0·49 (0·06)[d]	21·33 (0·78)[d]	0·19 (0.01)[c]
Octanoate	0·34 (0·04)[e]	21·78 (0·75)[d]	0·16 (0.01)[bc]
ANOVA (p value)	<0·001	<0·001	<0·001

Values are means (SEM); n=number of experiments. In each experiment the same batch was used to assess the kinetic constants of all five fatty acids. Means not sharing the same superscript letter within a column are significantly different at p<0·05 (Student-Newman-Keuls method).

TABLE II *Changes in kinetic constants for production of $^{14}CO_2$ from 1-^{14}C labelled C_2-C_8 fatty acids by isolated colonocytes when incubated in the presence of different unlabelled fatty acids (1 mmol/l)*

Substrate	*V_{max} (µmol/min/g)*	*K_m (mmol/l)*	*Inhibition*
Series 1 (n=5):			
^{14}C labelled butyrate	0·74 (0·07)[a]	0·23 (0·02)[a]	–
^{14}C labelled butyrate and acetate	0·73 (0·04)[a]	0·26 (0·04)[a]	None
^{14}C labelled acetate	1·05 (0.28)[b]	0·64 (0·03)[b]	–
^{14}C labelled acetate and butyrate	0·23 (0·03)[c]	0·39 (0·02)[c]	Non-competitive
ANOVA (p value)	<0·001	<0·001	
Series 2 (n=5):			
^{14}C labelled butyrate	0·81 (0·04)[a]	0·26 (0·02)[a]	–
^{14}C labelled butyrate and valerate	0·86 (0·06)[a]	1·32 (0·23)[b]	Competitive
^{14}C labelled valerate	1·01 (0·03)[b]	0·29 (0·04)[a]	–
^{14}C labelled valerate and butyrate	1·20 (0·16)[c]	1·95 (0·31)[c]	Competitive
ANOVA (p value)	<0·001	<0·001	
Series 3 (n=5):			
^{14}C labelled butyrate	0·69 (0·05)[a]	0·22 (0·04)[a]	–
^{14}C labelled butyrate and hexanoate	0·68 (0·02)[a]	1·65 (0.05)[b]	Competitive
^{14}C labelled hexanoate	0·50 (0·03)[b]	0·20 (0·02)[a]	–
^{14}C labelled hexanoate and butyrate	0·67 (0·04)[a]	2·13 (0·29)[c]	Competitive
ANOVA (p value)	<0·001	<0·001	
Series 4 (n=5):			
^{14}C labelled butyrate	0·60 (0·03)[a]	0·17 (0·03)[a]	–
^{14}C labelled butyrate and octanoate	0·58 (0·03)[a]	4·43 (0·38)[b]	Competitive
^{14}C labelled octanoate	0·37 (0·02)[b]	0·17 (0·02)[a]	–
^{14}C labelled octanoate and butyrate	0·45 (0·03)[c]	1·04 (0·12)[c]	Competitive
ANOVA (p value)	<0·001	<0·001	

Values are means (SEM); n=number of experiments. A series consists of n experiments. In each experiment the same batch was used to assess the kinetic constants. Means not sharing the same superscript letter within a column are significantly different at p<0·05 (Student-Newman-Keuls method).

Figure 2 and Table I show that ATP production from even chained fatty acids was doubled when the substrate of oxidation was changed from acetate to butyrate. However, a further 30% increase was registered from butyrate to hexanoate, before ATP production levelled off with identical ATP productions from hexanoate and octanoate. An even higher ATP production was achieved from valerate, but the comparison between the odd chained valerate and the even chained fatty acids is impeded by the fact that ^{14}C labelled valerate only produces one ^{14}C labelled acetyl-CoA, whereas the remaining unlabelled propionyl-CoA is metabolised by a separate pathway. Hence, the calculated rate of ATP production from valerate implies that the propionyl-CoA produced is oxidised at similar rates as the acetyl-CoA produced, which may not be the case. The K_m of acetate was about fourfold higher than the K_m of butyrate (Table I). However, the K_m values of valerate, hexanoate, and octanoate were comparable with the K_m value of butyrate (Table I). Small differences were found between the K_m of hexanoate and valerate in comparison with the K_m of butyrate (Table I), but the pairwise comparisons between butyrate and valerate, hexanoate, and octanoate in additional experiments did not substantiate the existence of significant

TABLE III *Changes in kinetic constants for production of $^{14}CO_2$ from 1-^{14}C labelled butyrate by isolated rat colonocytes when incubated in the presence of isobutyrate or isovalerate (1 mmol/l)*

Substrate	*V_{max} (µmol/min/g)*	*K_m (mmol/l)*	*Inhibition*
Series 1 (n=5):			
^{14}C labelled butyrate	0·74 (0·04)	0·24 (0·03)[a]	-
^{14}C labelled butyrate and isobutyrate	0·76 (0·04)	0·25 (0·03)[a]	None
^{14}C labelled butyrate and isovalerate	0·76 (0·05)	0·94 (0·08)[b]	Competitive
ANOVA (p value)	0·54	<0·001	

Values are means (SEM); n=number of experiments. A series consists of n experiments. In each experiment the same batch was used to assess the kinetic constants. Means not sharing the same superscript letter within a column are significantly different at p<0·05 (Student-Newman-Keuls method).

differences in K_m values between the C_4-C_8 fatty acids (Table II).

Substrate competition among C_2-C_8 fatty acids in colonocytes

The strong and non-competitive inhibitory effect of butyrate on acetate oxidation and the lack of acetate inhibiting butyrate oxidation (Table II) indicate that colonocytes prefer butyrate to acetate for oxidation, as also suggested by the higher K_m of acetate. The impression that butyrate was equally accepted for colonocyte oxidation in comparison with valerate, hexanoate and octanoate, and by contrast with acetate, was further substantiated by pairwise and simultaneous incubation of butyrate and one of the other fatty acids (Table II). The addition of valerate to cell suspensions incubated with labelled butyrate increased the K_m value about fivefold without changing V_{max}, which indicates competitive inhibition of butyrate oxidation by valerate. Likewise, butyrate increased the K_m of valerate oxidation by the same order of magnitude. Similarly, hexanoate and octanoate inhibit butyrate oxidation to the same extent or more than butyrate inhibited the oxidation of hexanoate and octanoate. The mutual inhibitory effect was competitive in all cases – that is, it was overcome by increasing substrate concentrations as indicated by the nearly unaffected V_{max} values (Table II).

Table III shows the effects of the branched chained SCFAs isobutyrate and isovalerate on butyrate oxidation to obtain the complete picture of how colonic SCFAs influence butyrate oxidation. Isobutyrate had no effect on V_{max} or K_m of butyrate oxidation. Isovalerate – like valerate, hexanoate, and octanoate – showed a competitive inhibition of colonocyte oxidation of butyrate, although the more moderate increase in K_m may indicate the effect to be weaker.

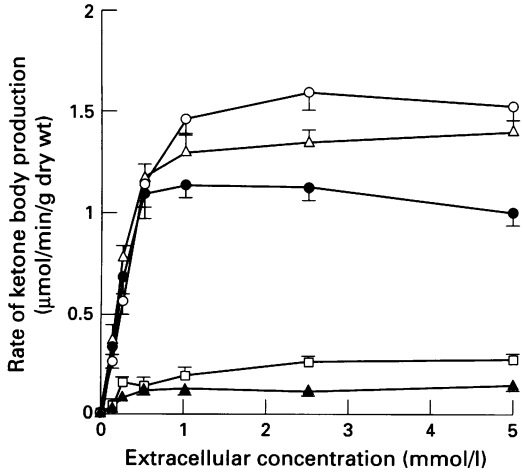

Figure 3: Production rates of ketone bodies (acetoacetate plus β-hydroxybutyrate) (mean (SEM)) for oxidation of acetate (□), butyrate (○), valerate (▲), hexanoate (△), and octanoate (●) in isolated rat colonocytes.

Ketogenesis from C_2-C_8 fatty acids in colonocytes

Figure 3 shows the production rates of ketone bodies (acetoacetate plus β-hydroxybutyrate) (mean (SEM)) as a function of added C_2-C_8 fatty acid concentrations. Colonocyte ketogenesis from butyrate, hexanoate, and octanoate was considerably higher than ketogenesis from acetate and valerate. V_{max} values of ketone body production were 1·80 (0·16), 1·50 (0·00), and 1·23 (0·10) µmol/min/g dry wt) for butyrate, hexanoate, and octanoate, and the K_m values were 0·56 (0·15), 0·25 (0·00), and 0·26 (0·08) mmol/l, respectively. Ketogenesis from acetate and valerate was too sparse for meaningful calculations of kinetic constants, but maximal ketone body production was 5–10 times lower than the production from butyrate, hexanoate, and octanoate.

Discussion

Recent investigations have made it clear that certain substrates play a key part in the nourishment of the mucosal cells in the gastrointestinal tract. Much attention has been focused on glutamine and its effects on the enterocytes in the small intestine, and butyrate seems to have a similar role in the nutrition of the colonic mucosa. Recent studies on isolated colonocytes have indicated that the other two SCFAs found in high concentrations in the lumen of the large bowel – acetate and propionate – may also be suitable substrates, but K_m values of oxidation and inhibitory studies exposing colonocytes to acetate, propionate, and butyrate have indicated that given the choice, butyrate is the preferred substrate for colonocytes both in rats[1] and humans.[2] The finding of acetate having a K_m about fourfold higher than the K_m of butyrate (Table I) and the doubling of the ATP production from even chained fatty acids when the substrate of oxidation was changed from acetate to butyrate (Table I; Fig 2) is in accordance with earlier findings.[1]

The metabolic fate of fatty acids of four carbon atoms or more is β-oxidation to acetyl-CoA, which is oxidised in the tricarboxylic acid cycle or converted to ketone bodies if the cell possesses the enzymes needed for ketogenesis as colonocytes and hepatocytes do. Acetate is not β-oxidised, but directly converted to acetyl-CoA, by contrast with the C_4-C_8 fatty acids, which are oxidised in the mitochondria. Although the end product, acetyl-CoA, is the same for all the even chained C_2, C_4, C_6 and C_8 fatty acids, it seemed to play a major role in the K_m of oxidation (CO_2 production) and the ketogenecity of the acids, whether acetyl-CoA was

produced as a result of an intramitochondrial β-oxidation (butyrate, hexanoate, and octanoate) or by a direct, possibly cytosolic, activation of acetate. The ketogenic response was far less in the second case and the K_m of oxidation was three to four times higher for acetate than for butyrate, hexanoate, and octanoate.

The odd chained fatty acids – for example, valerate – differ from the even chained by their production of propionyl-CoA, which is gluconeogenic due to its conversion to succinyl-CoA and oxaloacetate. Therefore, the much lower ketogenic effect of acetyl-CoA produced by the β-oxidation of valerate, compared with the ketone body production from butyrate, hexanoate, and octanoate, is probably due to the antiketogenic glucose-like effects of the propionyl-CoA simultaneously produced.

On the other hand, the K_m of valerate oxidation was in the low range as seen for the even chained C_4-C_8 fatty acids, indicating that the 1-^{14}C labelled acetyl-CoA produced from mitochondrial β-oxidation of 1-^{14}C labelled valeryl-CoA shared its biochemical fate with intramitochondrial acetyl-CoA produced from mitochondrial oxidation of butyrate, hexanoate, and octanoate rather than acetyl-CoA directly activated from acetate.

In conclusion, fatty acids, even or odd chained, are preferred for oxidation (low K_m values of CO_2 production) if their oxidation involves the intramitochondrial production of acetyl-CoA caused by the necessity of β-oxidation. The rapid oxidation of the C_4-C_8 fatty acids may, furthermore, relate to their independence of carnitine acyltransferase for transport into the mitochondria,[17] which is needed for long chain fatty acid transport. The rapid intramitochondrial production of acetyl-CoA was also decisive for colonocyte ketogenesis, which could be inhibited if the gluconeogenic propionyl-CoA was produced (or glucose added, not shown). Hence, colonocyte preference for butyrate as an oxidative substrate in comparison with the other main colonic SCFAs, acetate and propionate, is probably rooted in the metabolic fate it shares with hexanoate and octanoate – that is, free access to mitochondrial β-oxidation and rapid conversion to intramitochondrial acetyl-CoA. As a consequence, the "unique" oxidative properties of butyrate in colonocytes are apparently shared with hexanoate and octanoate. Experiments with simultaneous incubation of butyrate with valerate, hexanoate, or octanoate further substantiated that these acids are equally qualified for colonocyte oxidation (Table II) by contrast with propionate and especially acetate (Table II).[1]

Butyrate has been shown to exhibit other effects on the large bowel besides being oxidised. Various cellular effects have been associated with its inhibitory effects on tumour growth and possible beneficial effect on colonic cancer,[18–22] and previous studies have indicated that some of these non-oxidative cellular events caused by butyrate are not always shared with fatty acids of either shorter or longer chain length or are more effective.[19 21] Thus chain length specificity of fatty acids on cell differentiation differs, and the effects on cell differentiation by hexanoate and octanoate and possibly longer chained fatty acids are not to be regarded as equal to the effects of butyrate.

Pharmacological nourishment of the sigmoid and rectal mucosa has been a key idea in the treatment of patients with distal ulcerative colitis with enemas of SCFAs or butyrate.[6–9] From a nutritional point of view the present study suggests that enemas containing hexanoate or octanoate may be as efficacious as butyrate, provided that results can be extrapolated from rat colonocytes to human colonocytes and from in vitro conditions to the in vivo situation. Calories applied per volume of hexanoate or octanoate enemas in isoosmolar solutions might then be correspondingly higher compared with butyrate enemas in proportion to the higher chain length of hexanoate and octanoate. Similar arguments may apply to treatment of patients with diversion colitis by SCFA irrigation. By contrast, the antineoplastic effect of butyrate is probably not associated with its oxidation in colonocytes, but rather related to its effects on cell differentiation. Hexanoate and octanoate should, therefore, not be considered as equivalents to butyrate when it comes to antineoplastic properties on colonic neoplasia.

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