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Metabolic induction of experimental ulcerative colitis by inhibition of fatty acid oxidation

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Summary. There is some evidence that failure of fatty acid or β-oxidation in the epithelium of the colonic mucosa is associated with the development of ulcerative colitis. We tested the hypothesis that inhibition of fatty acid oxidation in the colonic mucosa of the rat reproduces the histological, clinical and biochemical lesions of acute ulcerative colitis of man. A specific inhibitor of β-oxidation, sodium 2-bromo-octanoate, was instilled rectally for 5 days or exposed to isolated colonic epithelial cells which were subsequently tested for their ability to β-oxidize *n*-butyrate. Weight loss, bloody diarrhoea and histological lesions occurred with 2-bromo-octanoate treated rats but not control animals. Ketogenesis and $^{14}CO_2$ production was inhibited by 2-bromo-octanoate. Of 12 animals mucosal ulceration developed in six out of eight surviving animals and in all four animals that died. Ulceration, mucus cell depletion, vessel dilatation and increases of inflammatory cells were the most prominent histological changes. Present observations indicate that inhibition of β-oxidation produces acute colitis and suggests that inhibition of β-oxidation is primary rather than secondary in the genesis of ulcerative colitis. A search for agents producing such biochemical lesions in man should be undertaken.

Keywords: Ulcerative colitis, β-oxidation-inhibitor, experimental colitis

The aetiology of ulcerative colitis in man is unknown and opinion divided whether immunological or infective mechanisms induce or maintain the characteristic mucosal appearances evident on histological examination (Kirsner & Shorter 1982). Recent biochemical studies of the mucosal epithelium of the colon affected with ulcerative colitis have demonstrated a biochemical lesion, in the sense that this term was defined by Sir Rudolph Peters (1969), even in the quiescent stages of ulcerative colitis. The biochemical lesion was an inhibition of β-oxidation of short chain fatty acids in the colonic epithelium contrasted with oxidation of carbohydrate (glucose) or amino acid such as glutamine which were not inhibited but rather enhanced when compared with the degree of oxidation of these substances in healthy subjects (Roediger 1980*a*). As fatty acids, chiefly *n*-butyrate (formulation Table 1), are the cardinal metabolic substrate oxidized by the healthy colonic mucosa (Henning & Hird 1972; Roediger 1980*b*;

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1982; Marty & Vernay 1984; Ardawi & Newsholme 1985) and as CO_2 of β-oxidation maintains efficient absorption of sodium in the colon (Roediger *et al.* 1986), it seemed worthwhile to assess the effect of metabolic inhibitors of β-oxidation (Fritz & Halperin 1973; Osmundsen 1978; Bremer & Osmundsen 1984) on the colonic mucosa. The aim of the present investigation was to establish whether inhibition of β-oxidation could induce the clinical, histological and biochemical lesions characteristic of ulcerative colitis in an experimental animal the rat. Observations on these lines would help to establish whether or not failure of β-oxidation is central to the disease process of ulcerative colitis.

β-Oxidation is the final pathway of energy utilization either from storage lipids which need specific activation for intra mitochondrial transport or from water soluble short chain fatty acids which readily diffuse into mitochondria where β-oxidation occurs (Bremer & Osmundsen 1984). The colonic epithelium is richly supplied with water soluble short chain fatty acids from the colonic lumen (Cummings 1981) and a suitable inhibitor of β-oxidation would need to be both water soluble and have unimpeded entry into mitochondria of colonic epithelial cells. Of the numerous inhibitors of fatty acid oxidation those affecting transport across mitochondrial membranes or those that control lipolysis seemed less suitable than inhibitors that affect enzymes involved in the first steps of β-oxidation (Osmundsen 1978; Bremer & Osmundsen 1984). With these criteria in mind the choice of inhibitors lay between 2-bromo-octanoate (Raaka & Lowenstein 1979; Table 1) and pent-4-enoate (Senior *et al.* 1968; Williamson *et al.* 1970; Osmundsen 1978) as biochemical information on the action of these chemical inhibitors in other tissues was readily available. Because pent-4-enoate affects glucose oxidation and β-oxidation of long chain fatty acids the more specific inhibitor 2-bromo-octanoate was chosen to investigate the effects that inhibition of β-oxidation had on the colonic mucosal epithelium. Sodium bromide and sodium octanoate were used as control substances together with 2-bromo-octanoate to verify that 2-bromo-octanoate had a specific action on β-oxidation in epithelial cells of the mucosa.

Materials and methods

Reagents and animals. Sodium octanoate and sodium bromide were obtained from BDH, Sydney, Australia. Bromo-octanoic acid was obtained from Tokyo Chemical Industry, Tokyo, Japan and converted to the sodium salt before use by titration to pH 7.4 with

Table 1. Formula of sodium butyrate, sodium octanoate and sodium 2-bromo-octanoate

Designation	Formula
Sodium butyrate	$\overbrace{CH_3.CH_2}.\overbrace{CH_2.CO}Na$
Sodium [1-^{14}C] butyrate	$\overbrace{CH_3.CH_2}.\overbrace{CH_2.{}^{*}CO}Na$
Sodium octanoate	$\overbrace{CH_3.CH_2}.\overbrace{CH_2.CH_2}.\overbrace{CH_2.CH_2}.\overbrace{CH_2.CO}Na$
Sodium 2-bromo-octanoate	$\overbrace{CH_3.CH_2}.\overbrace{CH_2.CH_2}.\overbrace{CH_2.CH_2}.\overbrace{\underset{\displaystyle Br}{CH}.CO}Na$

Brackets indicate carbon fragments that are metabolized to acetyl CoA, CO_2 and acetoacetate.
Carbon atoms are numbered from right to left.
* Denotes labelled carbon atom.

equimolar NaOH. Radioactively labelled [1-^{14}C]-butyrate was obtained from New England Nuclear, bovine serum albumin from Sigma Chemical Co. (St Louis, MO, USA) and purified enzymes and co-enzymes for substrate analysis were obtained from Boehringer Corporation, North Ryde, Australia. Laboratory animals, Sprague–Dawley rats were bred in our animal house from registered strains introduced in 1983.

Procedures and physical observations. Sprague–Dawley rats of either sex weighing 250–450 g were used in all experiments. Animals were fed on a commercially prepared diet (Milling Industries, Adelaide) and exposed to 12 h daylight to maintain a regular diurnal rhythm. Rectal instillation of sodium octanoate (500 mM), sodium 2-bromo-octanoate (500 mM) and sodium bromide (500 mM) was done on 5 consecutive days, twice daily at 11.00 am and 3.30 pm and animals killed at the end of day 5. A round tipped infant feeding catheter (8FG) was passed per rectum for 7 cm to the recto-sigmoid region and 2.0 ml of each substance in distilled water, was introduced from a 5 ml syringe attached to the catheter. Twelve animals were allocated to each treatment group and animals weighed at the beginning and the end of the experimental period. Stool consistency was subjectively assessed and tested for occult blood by the haemoccult test. Presence or absence of diarrhoea was noted by discolouration of the perianal fur.

Histological observations. The descending colon including rectum was rapidly removed, washed clear of luminal contents and opened along the antimesenteric border for macroscopic inspection. Sheets of colonic mucosa were rolled up and fixed in formalin-saline. Tissue rolls were embedded in paraffin wax and sections stained with haemotoxylin and eosin. Histological features specifically observed were ulceration and mucus cell depletion in the epithelium or density of inflammatory cells and dilated vessels in the lamina propria as these parameters are reliably observed in biopsies of inflammatory bowel disease (Giard *et al.* 1985). Severity of changes were subjectively graded and compared with controls, denoting – as no change and +, ++, +++ as mild, moderate or severe changes.

Biochemical studies. Isolated colonic epithelial cells were prepared as previously described (Roediger & Truelove 1979; Roediger 1980*b*; 1982). In essence segments of distended rat colon were incubated at 37°C in calcium-free Krebs Henseleit (K-H) saline containing 0.25% bovine serum albumin and 5 mM EDTA for 30 min in a Dubnoff type shaker in polythene flasks gassed with O_2 and CO_2 (19:1 v/v). Epithelial cells were disaggregated by vigorous manual shaking with a plastic stirrer and cells separated by centrifugation at 500 *g* for 1 min. Cells were twice washed in oxygenated K-H saline containing calcium and 1 mM DL dithiothreitol and resuspended in 15 ml of the same saline by being taken up several times into a 10 ml polypropylene pipette. Aliquots of 1 ml representing 5–10 mg dry weight were incubated for 60 min in conical flasks equipped with a glass centre well and stoppered with subaseals. Incubations were carried out at 37°C in 1 or 2 ml physiological saline containing 2–5% w/v bovine serum albumin, 5 mM DDT, and 5 mM sodium octanoate or 5 mM sodium 2-bromo-octanoate or 5 mM sodium bromide. Radioactively labelled [1-^{14}C]-butyrate, S.A. 1500 dpm/μmol to produce a final concentration of 10 mM was added to the cell-incubation mixture after 20 min of pre-exposure to octanoate, sodium 2-bromo-octanoate or sodium bromide. Exposure to radioactively labelled butyrate was from 20–60 min of incubation. At the end of each incubation 0.5 ml NaOH was injected into the centre well to collect $^{14}CO_2$ generated by cells and immediately afterwards 0.5 ml of 10% $HClO_4$ (v/v) was injected into the cell suspension. After 3 h samples (0.1 ml) of the alkaline solution was taken for counting in a liquid scintillation counter and metabolites measured in the incubation mix after neutra-

lization with 20% KOH. Acetoacetate was measured enzymatically and production of $^{14}CO_2$ and metabolites was calculated (Roediger & Truelove 1979) and expressed as μmol/min/g (dry wt).

Results

Of all the animals only those treated with bromo-octanoate lost weight (Fig. 1) and developed loose stools which were positive for occult blood. Animals exposed to sodium bromide increased their mean weight (±s.e.) from 360.8±9.6 g to 372.8±10.4 g ($n=12$) and those exposed to sodium octanoate from 304.2±5.8 g to 315.4±6.0 g ($n=12$). Of the 12 animals exposed to sodium bromo-octanoate 4 died (Fig. 1), one animal on the 3rd day of treatment and three animals on the 4th day of treatment. All the surviving animals lost weight from a mean of 306.3±7.1 g before instillations to 255.0±5.1 g ($n=8$) after instillation. Animals treated with bromo-octanoate had marked discolouration of perianal fur due to persistent diarrhoea.

None of the animals treated with sodium bromide or sodium octanoate developed any macroscopic or histological lesions in the colonic mucosa. In particular mucus cells and inflammatory cells were not affected in these animals. Of the eight animals that survived installation of 2-bromo-octanoate, six developed visible macroscopic changes (Table 2) of ulceration, diffuse submucosal haemorrhage (Fig. 2) and mucosal oedema. The animals that died were not examined histologically but in each case visible ulceration of the mucosa was present. In all surviving cases mucus cell depletion was readily noticed (Fig. 3) and epithelial ulceration prominent in the cases with macroscopic changes in the mucosa (Fig. 4). While vessel dilatation was also prominent (Fig. 3) only two animals consistently showed crypt abscesses in the epithelium. Laminal inflammatory cells were increased in all cases treated with bromo-octanoate.

The rate of CO_2 production from [1-^{14}C] butyrate by colonic epithelial cells pretreated with sodium chloride or sodium bromide (Table 3) was within the range

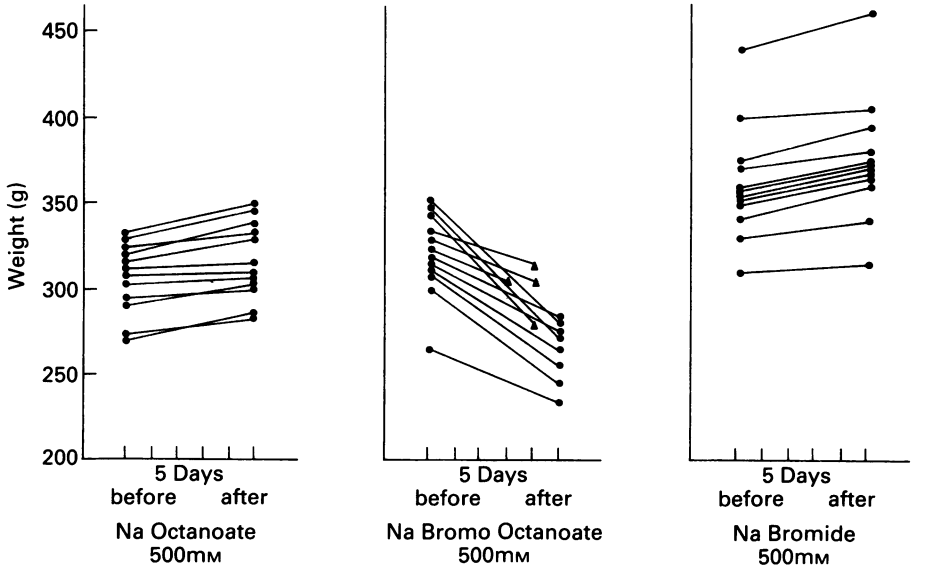

Fig. 1. Individual weight-changes of rats treated with sodium octanoate, sodium bromo-octanoate and sodium bromide: ▲, indicates death of animal.

Table 2. Subjective assessment of histological changes in the involved mucosa of the rectum

	Epithelial:			Laminal:	
Rat no.	ulcers	mucus cell depletion	crypt abscesses	dilated vessels	inflammatory cells
1	+++	++	−	++	+++
2	++	+	−	++	+++
3	−	+	−	−	+
4	+	+	−	++	++
5	−	+	−	−	+
6	+++	++	+	++	++
7	++	+	−	+	+
8	+++	++	+	++	++

Subjective assessment compared with controls: −, no change, +, ++, +++ mild, moderate or severe change.

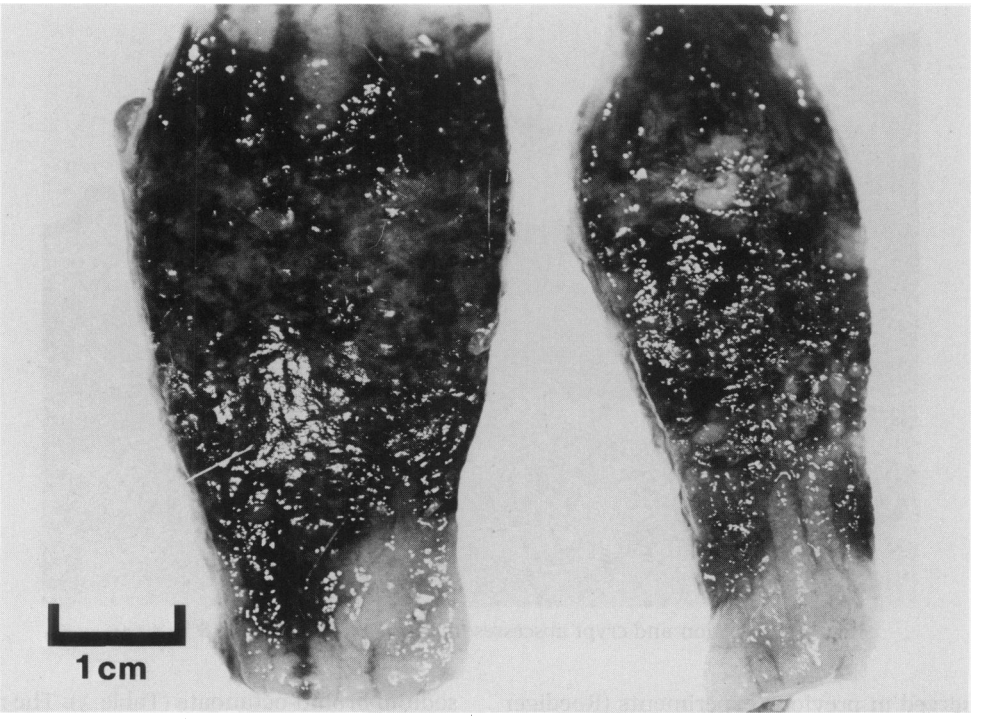

Fig. 2. Macroscopic changes in rats 6 and 8 showing severe degree of acute colitis (+++) and areas of submucosal haemorrhage.

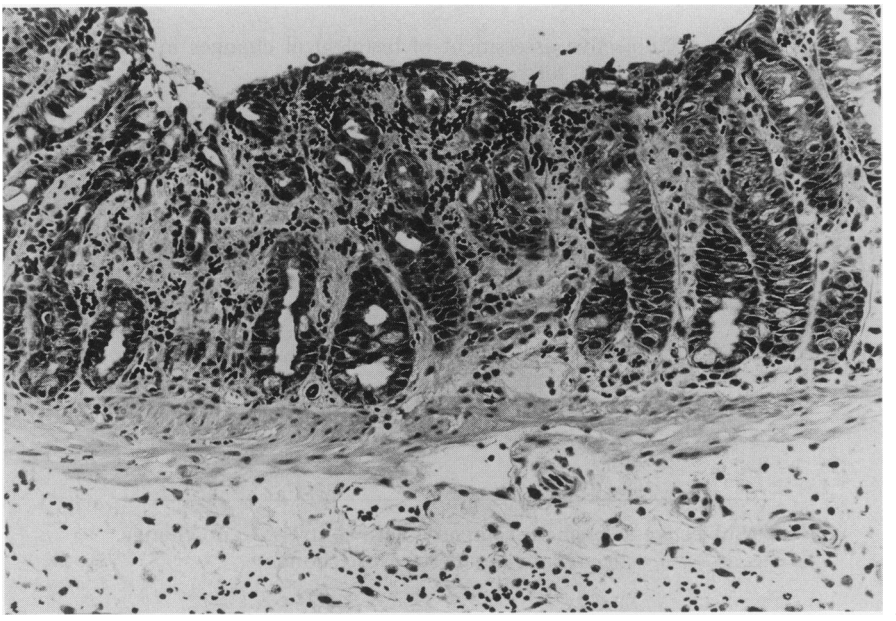

Fig. 3. Mucus cell depletion and dilatation of vessels in acute colitis. H & E, × 170.

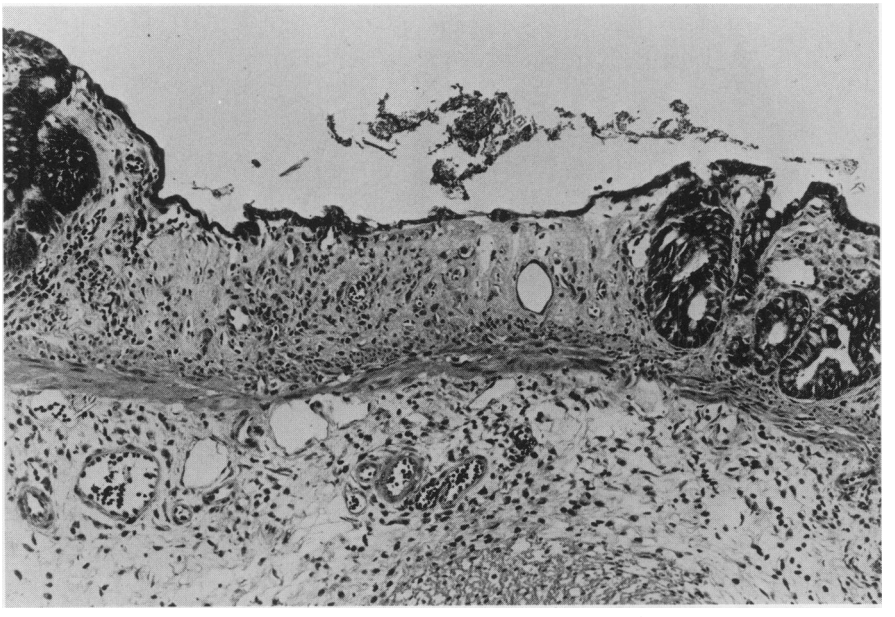

Fig. 4. Ulceration and crypt abscesses in severe acute colitis. H & E, × 140.

observed in previous experiments (Roediger 1980; 1982). The rate of CO_2 production from labelled butyrate was reduced both by pre-treatment with sodium octanoate and sodium bromo-octanoate (Table 3). The rate of ketone body production in the presence of NaCl and NaBr was non linear over 40 to 60 min (Table 4) an observation than has also

Table 3. Rates of CO_2 production from [1-^{14}C] butyrate in isolated colonic epithelial cells pre-treated with various substances (5 mM concentration)

Pre-treatment	$^{14}CO_2$ production	
0–20 min	20–40 min	40–60 min
Na chloride	1·55±0·07 (4)	1·3 ±0·04 (4)
Na bromide	1·63±0·1 (4)	1·6 ±0·1 (4)
Na octanoate	0·55±0·01 (8)*	0·44±0·01 (8)*
Na 2-bromo-octanoate	0·53±0·05 (8)*	0·35±0·04 (8)*

Values are mean±s.e. in μmoles/g (dry wt) of the number of experiments in brackets.

* Indicates $P<0.001$ (paired *t*-test) compared with NaBr values.

been previously made (Roediger 1980*b*; 1982). The rate of acetoacetate production was inhibited only in those colonic epithelial cells pre-treated with sodium 2-bromo-octanoate and the differences in ketogenesis with that observed with sodium octanoate was significantly different ($P<0.001$) (Table 4).

Discussion

The present observations on isolated cells of the colonic epithelium showed that β-oxidation and production of ketone bodies can be inhibited and that use of the same inhibitor of β-oxidation *in vivo* induced an acute colitis similar to acute ulceractive colitis (UC) observed in man (Truelove & Richards 1956). Experimental colitis can be induced by rectal instillation of numerous chemical substances for example acetic acid (MacPherson & Pfeiffer 1976; Sharon & Stenson 1985), 2-4-dinitro-chlorobenzene (DNCB) (Rabin & Rogers 1978) and formalin (Hodgson *et al.* 1978). Formalin and DNCB interact in all tissues with side-chains of complex proteins and they are therefore non-

Table 4. Rates of acetoacetate production in isolated colonic epithelial cells pre-treated with various substances (5 mM concentration)

Pre-treatment	Acetoacetate production	
0–20 min	20–40 min	40–60 min
Na chloride	2·5±0·3 (4)	1·94±0·2 (4)
Na bromide	3·8±0·4 (4)	2·56±0·3 (4)
Na octanoate	2·7±0·4 (8)	1·83±0·24 (8)
Na 2-bromo-octanoate	0·63±0·1 (8)*	0·37±0·03 (8)*

Values are mean±s.e. in μmoles/g (dry wt) of the number of experiments in brackets.

* Indicates $P<0.001$ (paired *t*-test) compared with NaBr values.

specific injuring agents. The acute colitis produced with acetic acid depended upon the concentration of hydrogen ions and as this organic acid also produces gastric and duodenal ulcers (Okabe & Pfeiffer 1972) its injurious action too is non specific. The present model of acute colitis with bromo-octanoate contrasts with these non-specific tissue damaging agents in that it has a biochemically defined level of action in the cell (Raaka & Lowenstein 1979; Bremer & Osmundsen 1984). Control experiments with sodium bromide and sodium octanoate excluded a possible non-specific action due to high concentrations of fatty acid which may also be injurious to the intestinal mucosa (Enser 1964).

That 2-bromo-octanoate is a potent and specific inhibitor of β-oxidation was first shown in the perfused liver and in isolated mitochondria of the liver (Raaka & Lowenstein 1979; Bremer & Osmundsen 1984). These initial observations showed that bromo-octanoate like sodium octanoate entered mitochondria in a carnitine-independent manner which was in keeping with diffusion rather than transport of water soluble fatty acids into mitochondria. The inclusion of labelled butyrate during the entire incubation with bromo-octanoate (0–60 min instead of 20–60 min) prevented the inhibitory action of bromo-octanoate (results not shown). This observation was also made by Raaka and Lowenstein (1979) in the liver and suggests that bromo-octanoate needs to be metabolised in the cell before it exhibits its inhibitory action. We consequently pre-incubated cells with bromo-octanoate in all our studies with isolated cells.

The duration of inhibition of β-oxidation in epithelial cells of the colon was not evaluated by long term survival studies of rats. The inhibition of bromo-octanoate in isolated mitochondria cannot be reversed by washing inhibited mitochondria (Raaka & Lowenstein 1979) and in general the inhibition of β-oxidation in liver persists long after inhibitors have been removed from the circulation (Osmundsen 1978). Further studies concerning the duration of experimental colitis, the immunological changes and liver damage associated with experimental colitis need to be carried out. Rectally instilled bromo-octanoate may cause liver changes by the observations already outlined (Bremer & Osmundsen 1984; Raaka & Lowenstein 1979). An association of liver changes and experimental colitis is analogous to human UC in which histological changes in the liver are also frequently seen (Shepherd *et al.* 1983).

The mucosa of defunctioned loops of colon, depleted of luminal *n*-butyrate, displays diminished β-oxidation (Roediger *et al.* 1986) and impaired cell turnover (Sakata & Yajima 1984) but no experimental colitis. In the present experiments inhibition of β-oxidation and the consequent colitis occurred in the presence of normal concentrations (5–20 mM) of luminal *n*-butyrate. Defunctioned loops of colon with low *n*-butyrate levels may be more sensitive to inhibitors of β-oxidation and consequently development of colitis. This proposal needs to be studied more closely in experimental animals especially as ulcerative colitis in man is not altered when the colon is defunctioned (Truelove *et al.* 1965).

The manner in which bromo-octanoate causes inhibition of β-oxidation is unresolved: two mechanisms could be involved. A product of activated bromo-octanoate could directly inhibit enzymes of β-oxidation, mainly thiolase which controls ketogenesis and entry of acetyl-CoA into the Krebs cycle (Raaka & Lowenstein 1979; Bremer & Osmundsen 1984) or alternatively irreversible sequestration of free CoA into conjugated derivatives such as bromo-octanoyl-CoA may occur (Raaka & Lowenstein 1979; Senior *et al.* 1968). This would diminish free CoA levels in cells and consequently efficient β-oxidation of fatty acids. The latter proposal has, on biochemical grounds, been challenged (Osmundsen 1978) however measurements of free and conjugated CoA levels in tissues involved with ulcerative colitis has shown that the disease condition

is associated with an increased level of conjugated derivatives of CoA and low levels of free CoA (Ellestad–Sayed *et al.* 1976). Prolonged exposure (8–10 min) of liver tissue to bromo-octanoate eventually diminished levels of free CoA and raised levels of conjugated CoA (Raaka & Lowenstein 1979). Whatever the precise mechanism of inhibition of β-oxidation may be, a ready supply of free CoA for effective β-oxidation is essential as reducing CoA levels in colonic tissue by means other than exposure to bromo-octanoate produces experimental colitis (Wintrobe *et al.* 1943; Nelson 1968) and malabsorption of ions (Nelson 1968) analogous to that seen in human ulcerative colitis.

There are clinical implications in the biochemical observations now reported. The colon in animal and man, both in health and in UC, is richly supplied with *n*-butyrate which is preferably oxidized (Henning & Hird 1972; Roediger 1980*b*; 1982; Marty & Vernay 1984; Ardawi & Newsholme 1985) by the healthy colonic mucosa but in UC there appears to be an incapacity to oxidize butyrate to CO_2 and ketone bodies (Roediger 1980*a*; 1984). Compensatory mechanisms of tissue oxidation occur in the diseased colonic mucosa so that glutamine, which is least dependent upon free CoA for oxidation, is preferably oxidized (Roediger 1980*b*). Such a compensatory oxidation of glutamine is also seen when fatty acids are withheld from actively metabolizing kidney tubules (Baverel *et al.* 1984). Enhanced glutamine oxidation is thus a compensatory mechanism observed in other tissues and a 'pathway of adjustment' when fatty acids are either not available or capable of being oxidised. Of central concern then is whether the inhibition of β-oxidation is a primary or secondary phenomenon in the disease process of ulcerative colitis. Because inhibition of β-oxidation can initiate an acute colitis the view is strengthened that inhibition of β-oxidation is one of the primary factors in the development of epithelial damage. The implications are that a local mechanism in the colonic mucosa with UC leads to the inhibition of β-oxidation and therefore colitis.

The present investigations were confined to one inhibitor of β-oxidation and the choice of the inhibitor was in disregard to whether it might occur or be produced in the human colon. It is most unlikely that bromo-octanoate is present in the human or animal colon nevertheless substances analogous in the action of bromo-octanoate on β-oxidation could possibly be found in the colon of man—for example mercaptoacetate (Bauche *et al.* 1981), mercaptobutyrate or derivatives of short chain fatty acids such as 2-methyl butyrate (Høverstadt *et al.* 1985) which are end products of bacterial fermentation in the colon. The hypothesis that an inhibitor of β-oxidation in the colonic mucosa may cause ulcerative colitis deserves further experimental attention in the light of observations now presented.

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