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Ketogenesis from Butyrate and Acetate by the Caecum and the Colon of Rabbits

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1. When studied *in vitro*, tissue from the caecum and the proximal colon of rabbits converted butyrate into ketone bodies. The conversion was similar to that observed with liver slices. The ketogenic activity was associated with the mucosa rather than the muscle of the gut wall and, in the colon, diminished as the distance from the caecal–colonic junction increased. 2. Tissue from the wall of the ileum, caecum, proximal colon and distal colon was also shown to metabolize [1-^{14}C]butyrate to carbon dioxide. 3. Enzyme assays showed that in both liver tissue and caecal mucosa the activity of hydroxymethylglutaryl-CoA synthase was more than ten times that of acetoacetyl-CoA deacylase. Labelling experiments *in vitro* gave confirmation of the hydroxymethylglutaryl-CoA pathway. 4. The significance of the conversion of butyrate into ketone bodies is discussed.

The caecum and the colon of rabbits have been shown to have high concentrations of volatile fatty acids and it has been suggested that these two organs are functionally analogous to the rumen and omasum of the ruminant (Henning & Hird, 1972*a*). For sheep, the molar proportions of the individual volatile fatty acids in the ruminal vein are not the same as in the rumen fluid, indicating that acetate is substantially transported to the blood stream intact, whereas butyrate is almost wholly metabolized by the rumen epithelium (Annison *et al.*, 1957). Studies *in vitro* have confirmed that butyrate is converted into ketone bodies by rumen epithelium (Pennington, 1952, 1954; Hird & Symons, 1959). Epithelial tissue from the fermentative organs of kangaroos and guinea pigs has also been shown to have ketogenic activity in the presence of physiological concentrations of butyrate (Henning & Hird, 1970).

The aim of the present investigation was to examine more closely the analogies between the caecum/colon system of the rabbit and the rumen/omasum system of the ruminant. The major volatile fatty acids in digesta from the hind-gut of rabbits are acetic acid and butyric acid (Henning & Hird, 1972*a*) and, accordingly, the metabolism of these two compounds has been studied.

Materials and Methods

Materials

Chemicals. Materials used in the study of ketogenesis *in vitro* were obtained and/or prepared as described by Henning & Hird (1970). For assays of ketogenic enzymes, acetoacetyl-CoA (grade 2, sodium salt), acetyl phosphate (lithium salt), coenzyme A (grade 1, free acid, from yeast) and phosphotransacetylase (from *Clostridium kluyveri*, 1000 units/mg of protein) were all obtained from the Sigma Chemical Co., St. Louis, Mo., U.S.A.

Animals. The animals used for these experiments were wild rabbits (*Oryctolagus cuniculus*), which had been kept in captivity for 1–6 months. They were given fresh lucerne each day together with a standard commercial diet (Barastoc layers' pellets; Barastoc, Melbourne, Vic., Australia) and were allowed to feed *ad libitum* until the time of killing.

Incubations

Incubations in vitro. The preparation of tissue and subsequent incubation were done as previously described (Henning & Hird, 1970). In some experiments the whole gut wall was incubated and in others the muscle was stripped off and the mucosa was incubated alone. For the colon, stripping was facilitated by the insertion of a glass rod through the lumen as done by Parsons & Paterson (1960). The proximal colon was defined as the sacculated region adjacent to the caecum. The rest of the large intestine, from the end of the sacculations to the anus, was defined as the distal colon plus rectum. The first discrete faecal pellets were always observed in the distal colon close to the end of the sacculations.

At the end of the incubation period (1–2h) the medium was removed and deproteinized by the $Ba(OH)_2 - ZnSO_4$ method of Weichselbaum & Somogyi (1941). Dry weights of tissues were determined by heating at 105°C for 16h. It was sometimes

considered to be more relevant to express the results on the basis of the total oxygen consumption of the tissue rather than on the basis of tissue dry weight, as oxygen consumption is closely related to oxidative metabolism of the tissue.

Radioactivity in carbon dioxide and at C-1 and C-3 of acetoacetate was measured by the methods given earlier (Henning & Hird, 1970), except that Hyamine hydroxide (10X; Packard Instrument Co., Warrenville, Downers Grove, Ill., U.S.A.) was used as the terminal absorbant for $^{14}CO_2$ and that the enzymic method of Williamson *et al.* (1962) was used for determination of acetoacetate. 3-Hydroxybutyrate was measured (enzymically) in all experiments and was assumed to have the same specific radioactivity as acetoacetate.

Presentation of results of incubations in vitro. The term 'total ketone bodies' refers to acetone plus acetoacetate plus 3-hydroxybutyrate. Since acetone is a derived product, the fraction acetone plus acetoacetate is referred to simply as acetoacetate.

Assay of ketogenic enzymes

3-Hydroxy-3-methylglutaryl-CoA synthase (EC 4.1.3.5) and acetoacetyl-CoA deacylase (EC 3.1.2.–) were assayed by the methods described by Williamson *et al.* (1968). The synthase assay measures the overall formation of acetoacetate, which includes the lyase. In tissues examined by Williamson *et al.* (1968) the lyase was present in excess and this has been assumed to be true in the current work. If this is not so, the values given for hydroxymethylglutaryl-CoA synthase would be underestimated. More extensive homogenization and sonication procedures were necessary, however, to achieve maximal release of the synthase from the mucosal tissues. The treatments used were as follows. All tissues were minced finely with scissors and transferred to a Sorvall Omn-Mixer with 5vol. of the homogenization medium. For liver the homogenization time (at half maximal speed) was 15s and for mucosal tissues it was 120s. The homogenate was then exposed to ultrasonic vibration for 5min at 16kcyc./s and centrifuged for 30min at 30000*g*. The supernatant fluid was considered (Williamson *et al.*, 1968) to contain the total soluble protein of the cell (i.e. cytoplasm and mitochondrial matrix). The enzyme activities measured with this fluid are taken as the activities of the whole homogenate.

Results

Metabolism of acetate and butyrate in vitro

In the present work concentrations of acetate, propionate and butyrate (80mM, 5mM and 10mM respectively) have been regarded as physiological (Henning & Hird, 1972*a*) and have therefore been used whenever feasible.

Tissue from the caecum and the proximal colon of wild rabbits was found to form ketone bodies in the presence of butyrate; the distribution of activity between the muscle and mucosa of the wall of the caecum and proximal colon is shown in Table 1. Most of the ketogenic activity and oxygen consumption was found to be associated with the mucosa. In the caecum of the rabbit, the mucosal surface is thrown into a fold (about 1cm in height), which spirals along the organ. When this fold was excised and incubated separately, it was found to have the same ketogenic activity as the mucosa from the main

Table 1. *Ketogenesis in various tissues from rabbits*

The tissues were incubated in the presence and absence of 10mM-butyrate as substrate. Results are expressed as μmol/h per 50mg dry wt. and are given as means±S.E.M.

Tissues	No. of animals	10mM-Butyrate as substrate	Total ketone bodies (μmol)	3-Hydroxybutyrate (% of total)	Oxygen consumption (μmol)
Caecal mucosa	10	–	0.7±0.09	41±3.2	15±0.7
Caecal mucosa	16	+	2.7±0.23	47±1.4	17±0.5
Caecal muscle	2	–	0.2±0.07	15±5.0	3±0.9
Caecal muscle	2	+	0.3±0.07	28±7.6	4±0.6
Colonic mucosa*	11	–	0.4±0.09	43±2.3	12±1.0
Colonic mucosa*	15	+	2.4±0.20	45±2.3	14±0.9
Colonic muscle*	2	–	0.2±0.10	7±3.0	6±0.5
Colonic muscle	2	+	0.3±0.10	19±1.0	5±0.7
Liver slices	5	–	1.1±0.06	6±1.5	7±0.2
Liver slices	9	+	1.4±0.10	10±1.3	8±0.3

* From the proximal region of the colon.

wall of the caecum. The ketogenic activity of liver slices is similar to that of caecal and colonic mucosa (Table 1) but the ability of butyrate to stimulate ketone-body production above the endogenous value was confined to the intestinal tissues. There was considerably less 3-hydroxybutyrate produced by liver.

As the concentration of butyrate in contents from the rectum is very much lower than in those from the proximal colon (Henning & Hird, 1972*a*), ketogenesis along the length of the proximal and distal colon and the rectum was examined for a possible relationship between concentration of butyrate in the gut contents and ketogenic activity. Intact tissue was used to lessen the time of preparation. To compensate for the varying thickness of the muscle wall the results have been expressed in terms of the oxygen consumption. Fig. 1 shows results obtained when tissue from various regions of the hind-gut was incubated with [1-^{14}C]-butyrate. The capacity to oxidize butyrate to carbon dioxide was approximately constant from the ileum to the rectum. In contrast, the conversion of butyrate into ketone bodies occurred almost entirely in the caecum and the proximal colon, the extent of conversion falling off steeply with increasing distance along the proximal colon. At each region of the hind-gut (Fig. 1) the specific radioactivity of the acetoacetate formed was 70–76% that of the added [1-^{14}C]butyrate, indicating a precursor–product relationship.

Since the mucosa of the caecum and proximal colon is normally bathed in a mixture of volatile fatty acids, the effect of acetate and propionate on the metabolism of [1-^{14}C]butyrate was investigated. In the presence of physiological concentrations of acetate and propionate, both separately and in combination, there was no observed effect on the oxidation of butyrate either to carbon dioxide or to ketone bodies. The experiment also showed that increasing the osmolarity of the incubation from 320 to 490 mosmolar had no significant effect on the metabolism of butyrate.

It has been argued (Hird & Symons, 1961, 1962) that preferential incorporation of [1-^{14}C]acetate into C-1 of ketone bodies indicates the operation of the 3-hydroxy-3-methylglutaryl-CoA pathway of ketogenesis. Table 2 shows that, as with rumen epithelium (Hird & Symons, 1961) from both gut tissues and liver slices from rabbits gave unequal labelling of the two acetyl moieties of acetoacetate formed. In each case approximately twice as much ^{14}C radioactivity was located at C-1 as at C-3. These results suggest that acetoacetate was formed at least in part by the 3-hydroxy-3-methylglutarate-CoA pathway. The specific radioactivities of the [^{14}C]acetoacetate

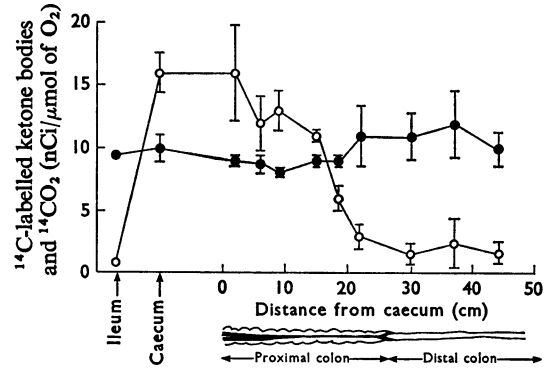

Fig. 1. *Oxidation of* 10 mM-[1-^{14}C]*butyrate* (*sp. radioactivity* 140 *nCi*/μ*mol*) to ^{14}*C-labelled ketone bodies* (○) *and to* $^{14}CO_2$ (●) *by pieces of intestinal wall*

Results are expressed on the basis of the amount of oxygen consumed during the incubation (1 h), i.e. as nCi/μmol of oxygen consumed. Each point represents the mean value from four animals and the bars show S.E.M., except for the ileum, where only one animal was used. The proximal and distal colon are shown in diagrammatic form. The longitudinal muscle bands of the proximal colon were avoided.

Table 2. *Incorporation of* [1-^{14}C]*acetate into ketone bodies formed from butyrate by tissues from rabbits*

The tissues indicated were incubated in the presence of 10 mM-butyrate together with 3 mM-[1-^{14}C]acetate (1 μCi/μmol). Results are given as means ± S.E.M. for three animals.

		[^{14}C]Acetoacetate formed		
		Specific radioactivity	Distribution of ^{14}C (%)	
Tissue	Total ketone bodies (μmol/h per 50 mg dry wt.)	(% of that of ^{14}C-labelled substrate)	C-1	C-3
Caecal mucosa	1.8 ± 0.37	3.0 ± 0.30	66 ± 1.7	34 ± 1.7
Colonic mucosa*	2.8 ± 0.46	4.1 ± 0.07	67 ± 1.2	33 ± 1.2
Liver slices	1.3 ± 0.20	10.2 ± 3.2	63 ± 1.9	37 ± 1.9

* From the proximal region of the colon.

Table 3. *Activities of 3-hydroxy-3-methylglutaryl-CoA synthase and acetoacetyl-CoA deacylase in tissues from rabbits*

Assays were done on the whole homogenate. Mean values from three animals are given together with S.E.M. The activities of both enzymes are expressed as μmol/min per g of tissue at 25°C.

Tissue	3-Hydroxy-3-methylglutaryl-CoA synthase (*a*)	Acetoacetyl-CoA deacylase (*b*)	Ratio (*a*)/(*b*)
Liver	0.84±0.08	0.08±0.01	11±1.5
Caecal mucosa	0.12±0.01	0.01±0.005	12±2.0

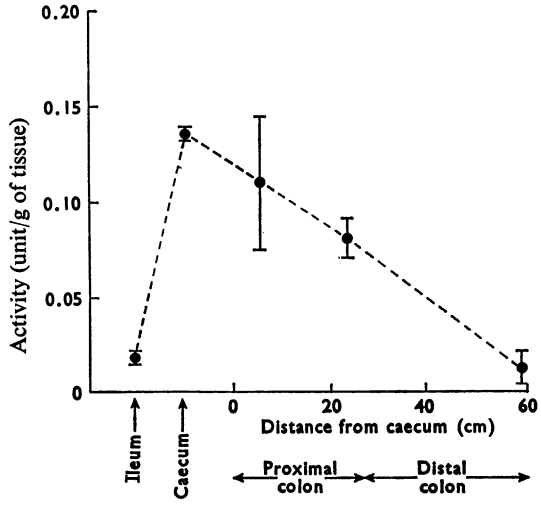

Fig. 2. *Activity of 3-hydroxy-3-methylglutaryl-CoA synthase in mucosal tissue from five regions of the hind-gut of rabbits*

The units of activity are μmol/min at 25°C. Each point represents the mean value from three animals and the bars show the S.E.M.

formed were low in comparison with the [1-^{14}C]-acetate added, but this is to be expected in terms of the large amount of unlabelled butyrate present in each incubation.

Enzymes of ketogenesis

There are generally believed to be two possible pathways for the formation of acetoacetate from acetoacetyl-CoA. The first is direct hydrolysis catalysed by a specific deacylase enzyme (Drummond & Stern, 1960) and the second is via the intermediate 3-hydroxy-3-methylglutaryl-CoA as described by Lynen *et al.* (1958). The labelling studies given above are consistent with the operation of the 3-hydroxy-3-methylglutaryl-CoA pathway in the various ketogenic tissues from rabbits. To confirm this conclusion, the activities of the key enzymes of the two pathways, namely acetoacetyl-CoA deacylase (EC 3.1.2.–) and 3-hydroxy-3-methylglutaryl-CoA synthase (EC 4.1.3.5), have been assayed. The results in Table 3 show that for both the liver and the caecal mucosa the activity of the synthase was more than ten times that of the deacylase. On the basis of tissue weight, the activities of both enzymes were considerably lower in the caecal mucosa than in the liver. This observation is similar to that reported for bovine rumen epithelium compared with bovine liver (Baird *et al.*, 1970).

The activity of 3-hydroxy-3-methylglutaryl-CoA synthase in mucosal tissue from various regions of the hind-gut of rabbits is shown in Fig. 2. The profile of this activity is very similar to the profile given above for the conversion of butyrate into ketone bodies during incubations *in vitro* (Fig. 1).

Discussion

The gut tissues which showed high ketogenic activity during incubations *in vitro* were from regions whose contents are characterized by high concentrations of volatile fatty acids (namely the caecum and the proximal colon; Henning & Hird, 1972*a*). Correspondingly, where the contents are low in volatile fatty acids (as in the ileum and the distal colon; Henning & Hird, 1972*a*), the gut tissues showed little or no ketogenic activity. Similar correlations between ketogenic activity (Pennington, 1952) of mucosal tissues of the digestive tract and concentrations of volatile fatty acids in the lumen (Phillipson & McAnally, 1942) are also apparent for sheep and for kangaroos (Henning & Hird, 1970). These observations suggest that all mucosal tissues which are normally bathed in high concentrations of volatile fatty acids (butyrate) may possess the enzymes required for the formation of ketone bodies. However, the conversion of butyrate into ketone bodies *in vivo* during transport across the caecum is not extensive (Henning & Hird, 1972*b*).

Both acetate and butyrate were also oxidized to carbon dioxide by mucosa from the hind-gut of rabbits. However, in contrast to ketone-body production, the oxidation of butyrate to carbon dioxide showed no progressive fall along the length of the colon and therefore, unlike ketogenesis, showed no correlation with the concentrations of volatile fatty acids in the lumen.

Pathway of ketone-body formation

When acetoacetate is formed from acetoacetyl-CoA via 3-hydroxy-3-methylglutaryl-CoA, C-1 of acetyl-CoA is specifically incorporated into C-1 of acetoacetate (Lynen *et al.*, 1958; Rudney & Ferguson, 1959). Taking this fact and assuming that acetoacetyl-CoA formed under biological conditions by the thiolase reaction represents a random recombination of acetyl units [see, however, the results obtained with partly purified pig heart thiolase (Beinert & Stansly, 1953)], then it can be predicted that the operation of the 3-hydroxy-3-methylglutaryl-CoA pathway in intact cells in the presence of [1-^{14}C]acetyl-CoA will give rise to ketone bodies preferentially labelled in the C-1 position. Labelling results consistent with the operation of the 3-hydroxy-3-methylglutaryl-CoA pathway have been obtained in the present work for the ketogenic tissues of rabbits. The close correlation between 3-hydroxy-3-methylglutaryl-CoA synthase activity and ketogenic capacity along the colon makes this conclusion firmer.

For mammalian liver there are now ample data to show that the major enzymes of ketogenesis are also those of the 3-hydroxy-3-methylglutaryl-CoA pathway (Caldwell & Drummond, 1963; Williamson *et al.*, 1968), although genuine acetoacetyl-CoA deacylase activity is thought to exist (Burch & Curran, 1969). The enzymes of the 3-hydroxy-3-methylglutaryl-CoA pathway have also been found in rumen epithelium (Baird *et al.*, 1970). An alternative pathway has been proposed for this tissue (Bush & Milligan, 1971); however, the low activity recorded for 3-hydroxy-3-methylglutaryl-CoA by these authors could be due to their use of low concentrations of magnesium in the assay mixture without reference to the effect of this on the extinction coefficient of acetoacetyl-CoA (Stern, 1956).

Physiological role of ketogenesis

The metabolic conversion of butyrate into ketone bodies is a feature which is common to the epithelia of all fermentative organs so far studied, but the significance of this metabolism, or of ketogenesis in the liver for that matter, is not yet understood.

A consequence of the pathway of acetoacetate synthesis is the liberation of coenzyme A, and this may be one of its important functions. Tissues such as liver and caecal epithelium are presented with high concentrations of fatty acids, which could result in over-esterification of coenzyme A. Under these conditions the citrate synthase reaction would be the only source of free coenzyme A. Since high concentrations of fatty acids may cause inhibition of the citrate synthase reaction (Williamson & Hems, 1970) the pathway of ketogenesis would become an important extra system for the maintenance of a steady concentration of free coenzyme A. There is some evidence to show that in rumen epithelium *in vitro* respiration is inhibited in the presence of propionate unless there is sufficient carbon dioxide to facilitate the oxidation of propionate (Pennington, 1954). These results are consistent with immobilization of coenzyme A as propionyl-CoA. Furthermore, in rat and sheep liver homogenates the inhibition of acetate oxidation by propionate can be partly relieved by the addition of coenzyme A (Pennington & Appleton, 1958).

As well as providing an extra route for the regeneration of coenzyme A, the conversion of butyrate into ketone bodies may also be important in maintaining a constant supply of energy for the mucosal cells in the manner that has been suggested for liver. When oxidation is rapid the diversion of acetoacetyl-CoA to ketone bodies is an effective means of conserving the major portion of the energy of the original fatty acid (Mayes & Felts, 1967). Since ketone bodies enter the circulatory system the carbon skeleton can be utilized by other tissues.

One further way in which ketogenesis could play a special role in the mucosal cells is by facilitating the transport of butyrate carbon from the lumen to the capillaries. Conversion of butyrate into ketone bodies in the mucosal cells would have a favourable effect on the concentration gradient and would enhance absorption if the absorptive process were dependent on diffusion, as it is in the rumen (Dobson, 1961). In addition, formation of ketone bodies in the rumen wall effectively prevents a large part of the butyrate that is produced in the rumen from entering the circulatory system, where it may disturb glucose homeostasis (Ash *et al.*, 1964; Anand & Black, 1970).

Apart from having a special role in the mucosal cells, the significance of ketogenesis could lie in the ketone bodies themselves. There is now evidence to show that, in both ruminants and non-ruminants, ketone bodies can serve as efficient substrates for oxidation in tissues such as skeletal muscle, cardiac muscle and kidney (Leng & Annison, 1964; Little *et al.*, 1970; Houghton & Ruderman, 1971) and cerebral tissue both *in vitro* (Leng & Annison, 1964; Rolleston & Newsholme, 1967; Ide *et al.*, 1969; Itoh & Quastel, 1970) and *in vivo* (Owen *et al.*, 1967; Hawkins *et al.*, 1971).

Since the ketogenic pathway appears to have evolved in all fermentative organs (post-gastric and

pre-gastric) it might be expected that it has a common role in all fermentative organs. If this is true then it seems most likely that the importance of ketogenesis would be associated with the necessity to metabolize butyrate in the mucosal cells rather than with a necessity for the utilization of ketone bodies by other tissues.

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