Concentrations and metabolism of volatile fatty acids in the fermentative organs of two species of kangaroo and the guinea-pig

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(*Received* 5 *May* 1969—*Accepted* 10 *July* 1969)

1. Contents from the fermentative organs of the kangaroo and guinea-pig were found to have concentrations of total volatile fatty acids ranging from 50 to 140 mM.

2. In each instance acetic was the most abundant acid, followed by propionic, then n-butyric. Trace amounts of isobutyric, n-valeric and isovaleric acids were present.

3. When studied in vitro, tissue from the wall of each fermentative organ was shown to metabolize butyrate to ketone bodies. Acetoacetate was the major ketone body. The presence of acetate and propionate did not affect ketogenesis from butyrate.

4. In the guinea-pig caecum most of the ketogenic activity resided in the mucosa.

5. The upper colon of the guinea-pig was as active as the caecum in metabolizing butyrate to ketone bodies.

6. For both the guinea-pig caecum and the kangaroo fermentative stomach, incubations with ^{14}C-labelled butyrate showed that the proportion oxidized to CO_2 was considerably less than that metabolized to ketone bodies.

Herbivores have an expansion of the alimentary tract in which the passage of food is delayed and digestion by micro-organisms takes place but, apart from ruminants, little attention has been given to the biochemistry of digestion in such animals.

For the wallaby, it has been reported that the principal site of microbial fermentation is the forestomach as in the ruminant (Moir, Somers & Waring, 1956), and it has been inferred that the situation is similar in the kangaroo (Moir, 1968). However, the fermentative stomach of the kangaroo differs from that of the sheep both in its gross morphology and in the nature of its epithelial lining. Whereas the whole of the rumen, reticulum and omasum of the sheep has a stratified squamous epithelium, the kangaroo forestomach is predominantly lined with simple columnar epithelium which is highly glandular. Likewise, in the guinea-pig, the fermentative organ (the caecum) has a columnar, glandular epithelium.

The occurrence of volatile fatty acids (VFA) in the fermentative stomach of the small wallaby *Setonix brachyurus* has been reported by Moir *et al.* (1956) and in the caecum of the guinea-pig by Hagen & Robinson (1953). In both of these reports the absorption of VFA from the fermentative organ was shown. In this paper, we present the results of quantitative analyses of the individual VFA in the contents of fermentative organs of two species of kangaroo and the guinea-pig, together with some aspects of the metabolism of VFA by the walls of these organs.

MATERIALS AND METHODS

Chemicals

Sebacic acid (British Drug Houses Ltd) for chromatography was recrystallized twice from acetone. Acid-washed Chromosorb W, 80–100 mesh (Varian Pty. Ltd, Crows Nest, NSW, Australia) was coated with sebacic acid (20%) by the method of Jackson (1964). The packing was sieved (80–100 mesh) immediately before use. Standard solutions of VFA and their salts were prepared from redistilled acids (Analytical Reagent; Hopkin and Williams).

Carbon tetrachloride (Analytical Reagent; May and Baker Ltd) and 2,4-dinitrophenyl-hydrazine (analytical reagent, British Drug Houses Ltd), used in acetone analyses, were purified as described by Hird & Weidemann (1964).

3-Hydroxybutyrate dehydrogenase was prepared by the method of Williamson, Mellanby & Krebs (1962) as modified by D. H. Williamson (1967, private communication). The enzyme preparation showed dehydrogenase activity towards malate at only 2% of the rate observed with 3-hydroxybutyrate. There was no detectable lactate dehydrogenase activity. β-NAD (Grade III from yeast) was obtained from the Sigma Chemical Co., St Louis, Missouri, USA.

Sodium [1-^{14}C]butyrate was obtained from the Radiochemical Centre, Amersham, England, and sodium [3-^{14}C]butyrate was obtained from Commissariat à l'Energie Atomique, Gif-sur-Yvette, Seine-et-Oise, France.

Experimental animals

Two kangaroos were the grey, *Macropus gigantus* (Shaw), and four were the red, *Megaleia rufa* (Desmarest). The grey kangaroos were caught wild near Melbourne and the nature of their diet is unknown. They were starved overnight between capture and slaughter. The red kangaroos had been kept in captivity at the CSIRO Division of Wildlife Research, Canberra, on improved pasture (consisting mainly of perennial ryegrass and phalaris, and supplemented with lucerne) and were allowed free access to the pasture until the time of slaughter.

The guinea-pigs were randomly bred adults. They received a standard commercial diet (poultry layer's pellets; Barastoc, Melbourne) supplemented with cabbage or cauliflower leaves.

Experimental procedures

All animals were stunned and bled and the various regions of the digestive tract were ligated and removed. The contents were collected from each region and stored at $-15°$ in sealed jars until analysed. Selected parts of the wall of the fermentative organs were washed free of contents with 0·9% (w/v) NaCl and immediately placed in oxygenated Krebs–Ringer–phosphate solution.

Unless otherwise stated, tissue from the guinea-pig caecum was used intact without removal of the circular muscle layer, but the longitudinal muscle bands of this organ were avoided. With the kangaroo it was found possible to strip the muscle layers away from the mucosa and this was routinely done. The tissue was cut into small pieces

(approximately 0·5 cm × 0·5 cm) and 200–400 mg fresh weight were added to each flask.

Incubations were performed in a Warburg apparatus at 38° for guinea-pig, and 37° for kangaroo. The incubation medium in each instance consisted of 120 mM-NaCl, 6·0 mM-KCl, 1·2 mM-$MgSO_4$, 6·6 mM-NaH_2PO_4 and 10·6 mM-Na_2HPO_4. The pH was 7·4. Calcium was omitted from the medium since this ion had been shown by Ontko (1967) to inhibit endogenous ketogenesis in rat liver homogenates. The total incubation volume was 3·0 ml. Substrates, at pH 7·4, were tipped from the side-arm after a 10 min pre-incubation period. Air was used as the gas phase.

At the end of the incubation period (1–2 h), the medium was removed and deproteinized with $HClO_4$ (final concentration 2%, w/v). Dry weights of tissue were determined by drying at 105° for 16 h.

Analysis of VFA

Weighed samples of stomach and caecal contents were centrifuged at 27000*g* for 10 min. The sediment was resuspended in three volumes of water and again centrifuged. This procedure was repeated twice and the combined supernatant liquids were used for the estimation of VFA.

Total VFA were estimated, in duplicate, by steam distillation as described by Pennington (1952). A known amount of isovaleric acid was added to one distillate to act as internal standard for the gas–liquid chromatography. The sodium salts of the VFA obtained by titration to pH 9·0 were evaporated to dryness in a rotary evaporator. For gas chromatography the salts were redissolved in 0·5–5·0 ml water and 1–10 μl were injected on to the columns.

VFA were analysed in a gas chromatograph (Model 5750; F & M Scientific Division of Hewlett-Packard, Avondale, Pennsylvania) under the following conditions: flame ionization detector at 280°, a hydrogen flow of 30 ml/min, air flow of 470 ml/min, injection port temperature at 190°. Dual stainless steel columns of internal diameter 5 mm and length 122 cm were packed with 20% sebacic acid–Chromosorb W. The carrier gas (N_2) flow rate was 40 ml/min and the oven temperature was 140°. To enable aqueous solutions to be used, the injection port liners were filled with a packing modified from that recommended by Monk & Forrest (1967). The liner (stainless steel, 5 mm × 15 cm) was plugged with glass wool at the end adjoining the column; 1·2 g of a mixture of equal parts by weight of anhydrous sodium tetraborate, anhydrous sodium hydrogen sulphate and celite was packed in by vibration. This was followed by a 5:2 (w/w) mixture of anhydrous sodium hydrogen sulphate and celite respectively (approximately 0·5 g) until the liner was filled. Injection of an aqueous solution of the sodium salt of an organic acid directly into the packing leads to the protonation of the carboxyl anion and the removal of the water. Reproducible results were obtained and the packing lasted for 15–20 injections.

From standard mixtures of acetic, propionic, isobutyric, n-butyric, isovaleric and n-valeric acids, the detector response factor of each acid with respect to isovaleric was calculated. The factors were 0·23, 0·53, 0·74, 0·76, 1·0 and 0·97 respectively for the six acids listed above. They enabled the concentration of each acid in the unknown

samples to be calculated from the relative area of its peak with respect to the standard isovaleric acid peak. Since all samples contained a small amount of endogenous isovaleric acid, corrections for this amount were made. In each instance, the distillate with no added isovaleric acid was chromatographed first, and the ratio of the areas of the butyric and isovaleric acid peaks was calculated. Using this value, it was possible to correct the measured area of the isovaleric acid peak in the second distillate, for endogenous isovaleric acid, and hence to calculate accurately the concentrations of the other acids.

The above method for steam distillation followed by gas–liquid chromatography gave recoveries of 100% ± 5% for standard mixtures of VFA. The chromatography did not resolve the isomers 2-methyl butyric and 3-methyl butyric (isovaleric). The quantities of individual VFA injected on to the columns were 0·04–0·20 μmole.

Analysis of ketone bodies

Acetone and acetoacetate in the deproteinized incubation media were analysed by the colorimetric method of Hird & Symons (1959), with the modification that the conversion of acetoacetate into acetone was carried out in sealed ampoules, rather than in stoppered bottles.

The chemical method for 3-hydroxybutyrate (Hird & Symons, 1959) was not used because it was found to give variable results in the presence of other oxidizable substances such as glucose. 3-Hydroxybutyrate was, therefore, estimated by the enzymic method of Williamson *et al.* (1962). An enzymic method was not used for acetoacetate because of the appreciable decarboxylation to acetone during the incubation and subsequent procedures.

Experiments with ^{14}C-substrates

The $^{14}CO_2$ produced during incubations with [^{14}C]butyrate was collected on filter paper plus NaOH (0·2 ml, 2 M) in the centre well of the Warburg flask. The filter-paper was transferred to a graduated test tube together with distilled water washings and made up to 1·5 ml. Elution of the $Na_2{}^{14}CO_3$ was allowed to proceed for 1 h with occasional shaking. To ensure that there was no contamination by [^{14}C]butyrate, a 1·0 ml sample of each eluate was treated with $Ba(OH)_2$ in the presence of 10 μmoles of Na_2CO_3 as a carrier. The $Ba^{14}CO_3$ precipitate (on filter-paper discs) was washed several times with water and ethanol and transferred to the main compartment of a Warburg flask containing 2·7 ml water. The $Ba^{14}CO_3$ was decomposed by tipping 0·3 ml 2 M-HCl from the side-arm. The $^{14}CO_2$ released was collected in NaOH on filter-paper in the centre well and the resulting $Na_2{}^{14}CO_3$ was eluted and then counted in a liquid-scintillation spectrometer (Ansitron Inc. Wallingford, Connecticut) using the dioxane-based liquid scintillator of Bray (1960).

The specific activity at carbon one of acetoacetate was determined by the second method described by Hird & Symons (1961) with the modification that the $^{14}CO_2$ collected in the centre well was converted into $Ba^{14}CO_3$ and determined as above. It was found that without this procedure contamination by [^{14}C]butyrate occurred.

The specific activity at carbon three of acetoacetate was determined by the acetone–dinitrophenylhydrazone method of Hird & Symons (1959) with the modification that the hydrazones were extracted into toluene and counted in a liquid scintillation spectrometer using the same scintillator as for $Na_2{}^{14}CO_3$ above.

RESULTS

Presence of VFA in various regions of the digestive tract of kangaroo and guinea-pig

The kangaroo stomach consists of a large sacculated region at the anterior end which is linked by a non-sacculated region to the small, highly muscular gastric pouch or true stomach. In the adult animal, the contents of the first two regions are characterized by a neutral pH (6·0–7·0), whilst those of the gastric pouch are acidic (pH 2·5–4·0). The term 'fermentative stomach' will be used to describe the first two regions. Detailed descriptions of the stomach of the red kangaroo are given by Griffiths & Barton (1966) and of the grey kangaroo by Shäfer & Williams (1876).

Table 1. *Total volatile fatty acids in contents from different regions of the digestive tract of six kangaroos*

Animal no.	Species of kangaroo	Total VFA (μmoles/g contents)			Total VFA (μmoles/ml water)		
		Fermentative stomach	Gastric pouch	Caecum	Fermentative stomach	Gastric pouch	Caecum
1	*Megaleia rufa*	80	72	80	90	80	95
2		99	37	86	112	41	98
3		110	47	101	133	57	116
4		114	26	99	140	31	—
5	*Macropus giganteus*	73	14	34	90	17	39
6		67	—	—	80	—	—

The results in Table 1 show the total VFA in contents of the fermentative stomach, gastric pouch, and caecum of six kangaroos. For each animal, high VFA levels were found in the fermentative stomach with those in the caecum generally being similar. The levels in the gastric pouch were considerably lower. The results have also been calculated as concentrations in the water present in the contents. The values are similar to those of Moir *et al.* (1956) for the wallaby *Setonix brachyurus*.

Analysis of caecal contents from seven guinea-pigs showed the total VFA levels to range from 46 to 99 μmoles/g fresh weight, with a mean of 76 μmoles. This is similar to the average of 57 μmoles/g fresh weight obtained by Hagen & Robinson (1953) for three guinea-pigs.

Analyses of the individual VFA from the various regions of the kangaroo digestive tract and from the guinea-pig caecum are given in Fig. 1. There were no obvious differences between the two species of kangaroo used. For each organ studied, acetic was by far the most abundant acid, followed by propionic and butyric respectively: the longer-chain and branched-chain acids were present in only small amounts. This

general pattern is the same as that found in sheep rumen (El-Shazly, 1952) and sheep caecum (Williams, 1965). In the kangaroos, as in sheep, the percentage of acetic acid was always greater in the caecum than in the fermentative stomach and the percentages of propionic and butyric acids were correspondingly lower. Although the levels of total VFA were lower in the gastric pouch than in the fermentative stomach, the proportions of the individual acids were essentially the same.

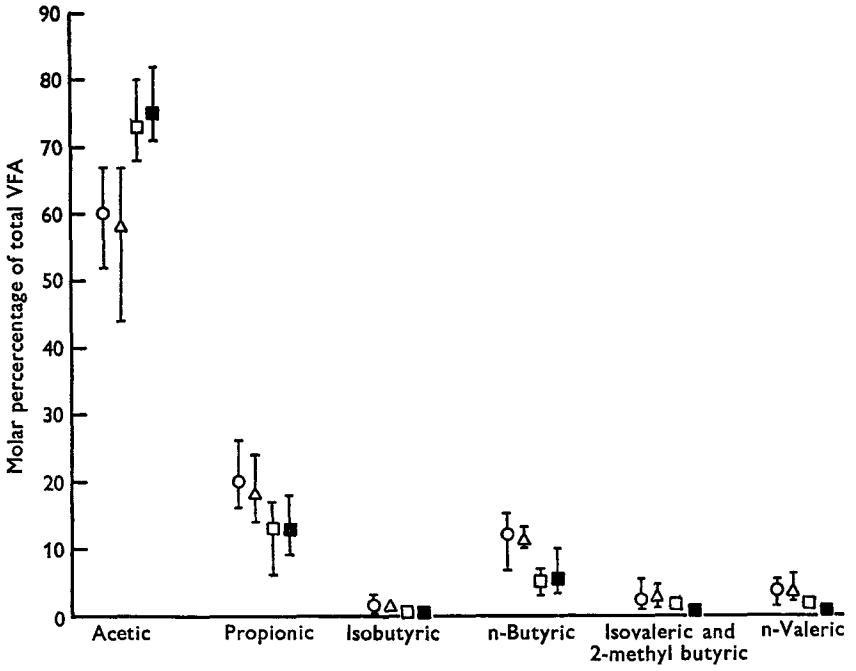

Fig. 1. Mean concentrations of volatile fatty acids in the contents of fermentative organs. Vertical lines indicate the range of values obtained. ○, kangaroo fermentative stomach; △, kangaroo gastric pouch; □, kangaroo caecum; ■, guinea-pig caecum.

Ketogenesis by tissues of the fermentative organs

Table 2 shows the oxygen consumption and ketone body synthesis in a typical experiment when guinea-pig caecal tissue was incubated in vitro with various VFA. For both butyrate and hexanoate there was a marked production of ketone bodies (predominantly as acetoacetate) and a slight stimulation of oxygen consumption. Incubation of tissue from the proximal colon of the guinea-pig gave results which were similar to those obtained with caecal tissue. For comparative purposes, guinea-pig liver slices were also incubated with butyrate and hexanoate. On a dry-weight basis the ketogenic activity of the liver slices was similar to that of the tissues of the caecum and the colon. When acetate and propionate were incubated together with butyrate, they did not affect the ketogenic activity of guinea-pig caecal tissue.

In order to determine the location of the ketogenic enzymes in the guinea-pig caecal wall, the mucosa was scraped from the underlying muscle and the two tissues were incubated separately. The results presented in Table 3 show that, in the presence of

butyrate (10 mM), the ketogenic activity of the mucosa was five times as great as that of the muscle. In both instances acetoacetate was again the predominant ketone body. Since the total ketone bodies from intact tissue approximately equalled the sum from the separated tissue, it appears that little of the ketone bodies produced by the mucosa is utilized by the associated muscle of the intact tissue.

Table 2. *Metabolism of fatty acids to ketone bodies by guinea-pig caecal tissue in vitro*

(Results are expressed as μmoles/50 mg dry weight per h)

		Ketone bodies formed	
Added substrate	O_2 consumption (μmoles)	Total (μmoles)	3-Hydroxybutyrate (% of total)
None	14	0·2	6
Acetate (10 mM)	13	0·3	4
Propionate (10 mM)	12	0·2	13
Butyrate (10 mM)	17	6·6	29
Hexanoate (5 mM)	17	6·3	33
Octanoate (5 mM)	14	0·6	12

Table 3. *Ketogenesis from butyrate in muscle and mucosa of guinea-pig caecum*

(Results expressed as μmoles/400 mg intact fresh tissue per h)

				Ketone bodies formed					
	Oxygen consumption (μmoles)			Total (μmoles)			3-hydroxybutyrate (% of total)		
Substrate	Mucosa	Muscle	Intact tissue	Mucosa	Muscle	Intact tissue	Mucosa	Muscle	Intact tissue
None	8·5	2·7	12·8	0·3	0·2	0·4	4	0	2
Butyrate (10 mM)	20·1	4·2	27·2	5·0	1·0	8·2	12	30	23

Incubations of the stomach wall of the kangaroo were made with the mucosa only, and results of a typical experiment are shown in Table 4. The mucosa from the fermentative stomach of both kangaroo species metabolized butyrate to ketone bodies, but the activity was found to be greater in *Macropus giganteus* than in *Megaleia rufa*. The results for incubations of liver slices under identical conditions are included for comparison. On a dry-weight basis, the fermentative stomach of *Macropus giganteus* had considerably higher ketogenic activity than liver slices from the same animal, whereas for *Megaleia rufa* the situation was reversed. In each instance the proportion of ketone bodies present as 3-hydroxybutyrate was very small.

In contrast to the active metabolism of butyrate by the fermentative stomach of *Macropus giganteus*, the mucosa from the gastric pouch had negligible ketogenic activity (Table 4). No incubations of gastric pouch tissue were made for *Megaleia rufa*.

The distribution of ketogenic activity over the whole area of the fermentative stomach was uniform in *Megaleia rufa* but not in *Macropus giganteus*. For the latter animal, the

upper fifth of the fermentative stomach is covered by stratified squamous rather than glandular columnar epithelium. Whereas the glandular regions had uniform ketogenic activity to the extent shown in Table 4, the stratified squamous epithelium showed negligible ketogenic activity.

Table 4. *Ketogenesis from butyrate in mucosa of kangaroo stomach*

(Results expressed as μmoles/50 mg dry weight per h)

Species of kangaroo	Tissue	Substrate	Ketone bodies formed: Total (μmoles)	Ketone bodies formed: 3-Hydroxybutyrate (% of total)
Megaleia rufa	Fermentative stomach	None	0·2	—
		Butyrate (10 mM)	0·7	4
	Liver slices	None	0·2	—
		Butyrate (10 mM)	1·6	4
Macropus giganteus	Fermentative stomach	None	0·2	—
		Butyrate (20 mM)	2·4	2
	Gastric pouch	None	0·1	—
		Butyrate (10 mM)	0·2	—
	Liver slices	None	0·5	—
		Butyrate (10 mM)	1·4	4

Table 5. *Oxidation of [^{14}C]butyrate to ketone bodies and carbon dioxide by tissues from the gut of guinea-pigs and kangaroos*

Tissue	Substrate*	Total ketone bodies (μmoles/50 mg dry wt h)	Specific activity of ketone bodies (% of specific activity of added substrate)	Percentage of metabolized ^{14}C as: [^{14}C] ketone bodies	Percentage of metabolized ^{14}C as: $^{14}CO_2$
Guinea-pig caecum	[1-^{14}C]butyrate (10 mM)	5·8	24	78	22
Guinea-pig caecum	[3-^{14}C]butyrate (10 mM)	5·5	64	94	6
Megaleia rufa fermentative stomach	[1-^{14}C]butyrate (10 mM)	0·8	41	67	33
Macropus giganteus fermentative stomach	[1-^{14}C]butyrate (10 mM)	2·9	49	75	25

* Approximately $1·6 \times 10^6$ counts/min per flask.

For both the guinea-pig caecum and the kangaroo stomach, experiments with [^{14}C]butyrate showed that butyrate is a precursor of ketone bodies. The ratio of the specific activity of the ketone bodies formed to that of the butyrate added (Table 5) indicates the extent of incorporation of butyrate carbon into ketone bodies. In guinea-pig caecal tissue, there was 23–25% incorporation of the label from [1-^{14}C]butyrate into ketone bodies, compared with 63–65% with [3-^{14}C]butyrate. For the mucosa of

kangaroo stomach [1-^{14}C]butyrate gave 39–49% incorporation. Table 5 also shows that, in each instance, some of the butyrate was oxidized to carbon dioxide. The proportion of the ^{14}C appearing in carbon dioxide was always lower than that in ketone bodies.

DISCUSSION

The results presented in this paper show that the contents from the fermentative organs of both the kangaroo and guinea-pig have concentrations of VFA which are very similar to those found in the ruminant (El-Shazly, 1952). Moir (1968) has recently reported similar values for the stomach contents of the wallaby *Setonix brachyurus*. Analysis of caecal contents from the fowl (Annison, Hill & Kenworthy, 1968) and from a marsupial possum (S. J. Henning & F. J. R. Hird, unpublished results) have shown that in these animals the VFA of the fermentative organ also follow the same pattern.

We have no information on rates of production of VFA in the kangaroo and guinea-pig, but in ruminants there is a linear relationship between the concentrations and the rate of production (Gray, Jones & Pilgrim, 1960; Leng & Brett, 1966). Although the concentrations of total VFA in the caecum of the kangaroo are only slightly lower than in the fermentative stomach, the latter is presumably of much greater significance in the nutrition of the animal because of its greater size.

With sheep, Phillipson & McAnally (1942) showed that, while VFA were present in the rumen in large quantities, only low concentrations could be detected in the abomasum. From our results with kangaroos, the differences in total VFA levels between the fermentative stomach and the gastric pouch were not as great as in the ruminant. This could be due to the presence in ruminants of the omasum, which is active in absorbing water and VFA (Gray, Pilgrim & Weller, 1954) and in metabolizing VFA (Hird & Symons, 1959). Further, as the stomach regions of the kangaroo are not separated, some mixing of the upper and lower contents would be possible in vivo and during the removal of the organ from the animal.

Since the immediate dietary history of the red and the grey kangaroos was different, the different levels of total VFA found in the fermentative stomachs of the two species may reflect dietary rather than species differences.

The metabolism of VFA by the rumen wall has been investigated in some detail (Pennington, 1952; Hird & Symons, 1961; Hird, Jackson & Weidemann, 1966). It has been shown that, under aerobic conditions, rumen epithelium metabolizes butyrate to a much greater extent than either acetate of propionate. Most of the butyrate is converted into ketone bodies, mainly acetoacetate. The present work shows that there is analogous metabolism of butyrate in the mucosa of two other fermentative organs, the guinea-pig caecum and the kangaroo stomach. In both these organs the mucosa is histologically different from that of the rumen. These observations suggest a generalization that the epithelium of all fermentative organs converts butyrate produced in the organ into ketone bodies.

The low proportions of 3-hydroxybutyrate produced by kangaroo tissues are paralleled by very low levels of 3-hydroxybutyrate in the arterial blood: 10–24% of

the total, compared with more than 90% for cows (Baird, Hibbitt, Hunter, Lund, Stubbs & Krebs, 1968). In the six kangaroos studied, the concentrations of total ketone bodies in the arterial blood ranged from 0·08 to 0·23 μmoles/ml.

For guinea-pigs the concentrations of VFA in the upper part of the colon are similar to those in the caecal contents (unpublished observation). This suggests that the metabolic properties of the upper colon, with respect to VFA, might well be similar to those of the caecum. Our results show that, for butyrate at least, the metabolic activity of the colon and the caecum is very similar. This indicates that the colon may have functions additional to its accepted role in the absorption of salts and water.

Experiments with whole tissue preparations from rat liver (Hird & Symons, 1962) and sheep rumen and omasum epithelial tissue (Hird & Symons, 1961) suggest that the 3-hydroxy-3-methylglutaryl CoA (HMG CoA) pathway for ketone body formation is operative. In guinea-pig caecal tissue the specific activity of ketone bodies was greater with [3-^{14}C]butyrate than with [1-^{14}C]butyrate. By the HMG CoA pathway the C_1–C_2 moiety would be diluted by unlabelled endogenous acetyl CoA before being re-incorporated into ketone bodies, whereas this would not happen with the C_3–C_4 moiety. Similarly, the proportion of C_1–C_2 moiety being exposed to oxidation by the citric acid cycle would be greater (Hird & Symons, 1961). The results obtained therefore are consistent with the operation of the HMG CoA pathway.

The significance of the metabolism of butyrate to ketone bodies by the epithelial tissue of fermentative organs is not yet understood. It is clear that the partial oxidation of butyrate could satisfy the energy needs of the epithelial tissue and at the same time provide a substrate (ketone bodies) to other tissues for further oxidation. Another consequence of ketogenesis is the regeneration of coenzyme A which could otherwise limit the rate of oxidation in cells heavily loaded with fatty acids.

We are grateful to Dr M. Griffiths and his staff at the CSIRO Division of Wildlife Research, Canberra, for much useful advice and assistance with the red kangaroos; and to Mr K. Dempster of the Department of Fisheries and Wildlife, Melbourne, for providing the two grey kangaroos. Financial assistance from the Australian Research Grants Committee is acknowledged.

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Printed in Great Britain