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The Metabolism of D(—)-β-Hydroxybutyrate in Sheep

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The association of raised blood concentrations of ketone bodies with certain pathological conditions, e.g. diabetes mellitus in man and bovine ketosis, has resulted in the intensive study of occurrence and metabolism in mammals of acetoacetate and β-hydroxybutyrate, as reviewed by Campbell & Best (1956). It is now recognized that many tissues are capable of utilizing ketone bodies as substrates for oxidative metabolism (Krebs, 1961), but much work on β-hydroxybutyrate based on the racemic mixture of the acid is difficult to interpret since there is evidence that the L(+)- and D(−)-forms are utilized by tissues at different rates (Marriott, 1914; Lehninger & Greville, 1953; McCann, 1957). In the present studies the metabolism of D(−)-β-hydroxybutyrate in sheep was investigated both *in vitro* and *in vivo*. D(−)-β-Hydroxy[^{14}C]butyrate was prepared by incubating sodium [^{14}C]butyrate with liver slices, or with sheep-rumen epithelium. The capacity of sheep tissues to oxidize D(−)-β-hydroxy [^{14}C]butyrate was examined by incubating the acid with preparations of rumen epithelium, liver, kidney, brain, muscle, lung and spleen. The metabolic interrelations of β-hydroxybutyrate with glucose and short-chain fatty acids in isolated tissues were also examined. In studies on the intact conscious sheep entry rates of β-hydroxybutyrate were measured by isotope dilution by using continuous-infusion procedures. Comparison of the specific activities of blood β-hydroxybutyrate and blood carbon dioxide during the terminal stages of β-hydroxy[^{14}C]butyrate infusions allowed rough calculation of the contribution of this substrate to total carbon dioxide production.

MATERIALS AND METHODS

Experimental animals. Merino wethers (aged 2–3 years) were housed indoors and fed on lucerne chaff (800 g./day). Animals used for entry rate measurements were trained to stand quietly in stocks.

Preparation of tissues. Liver, heart, ventricle, kidney-cortex, spleen and brain (cerebrum and cerebellum) slices were cut free-hand by the procedures described by Leng & Annison (1963). Brain tissue was removed within 2–3 min. from sheep killed by exsanguination. A mechanical saw was used to open the top of the head and provide access to the brain. A tissue mince of lung was prepared by slicing and chopping the tissue with a razor blade. Individual bundles of skeletal-muscle (sartorius) fibres were teased out with fine dissecting needles. Rumen epithelium was prepared as described by Pennington (1952), and thin (1 mm.) sheets of rumen-wall muscle were stripped from rumen epithelium and cut into 1 cm. squares.

Tissues were incubated in Warburg flasks at 39° in Krebs–Ringer phosphate (gas phase: 100% O_2) or Krebs–Ringer bicarbonate [gas phase: CO_2+O_2 (5:95)] prepared as described by Umbreit, Burris & Stauffer (1957). The flasks were shaken at 100 strokes/min. Results are expressed as μmc (or μmoles or mg.)/hr./g. of tissue.

Preparation of D(−)-β-*hydroxy*[^{14}C]*butyrate.* Liver slices (3 g.) or rumen epithelium (4 g.) were incubated with sodium [1-^{14}C]butyrate or sodium [2-^{14}C]butyrate (200 μmoles; 300 μc) in 24 ml. of Krebs–Ringer bicarbonate for 4 hr. by the incubation procedures described by Leng & Annison (1963). After removal of tissues by centrifuging, the flask contents were deproteinized with an equal volume of 6% $HClO_4$. The combined filtrates from three flasks were adjusted to pH 7·0, concentrated *in vacuo* with a rotary evaporator to 3–4 ml. and mixed with 15 g. of anhydrous $CaSO_4$. The product was mixed with 2 ml. of N-H_2SO_4 and extracted with ether for 6 hr. in a Soxhlet

Table 1. *Metabolism of* D(−)-β-*hydroxybutyrate by sheep tissue* in vitro

Tissue (250 mg.) was incubated in Warburg flasks (in duplicate) in Krebs–Ringer phosphate (3 ml.) for 2 hr. at 39° with β-hydroxy[1-^{14}C]butyrate (10 μmoles; 100 μmc). Oxygen uptakes were measured with and without added substrate. Numbers in parentheses indicate the numbers of separate experiments.

Tissue	Ketone body disappearance (μmoles/g./hr.)	$^{14}CO_2$ production (μmc/g./hr.)	O_2 consumption (μl./g./hr.) With added substrate	O_2 consumption (μl./g./hr.) Without added substrate
Liver	−5·2* (2)	3·7 (3)	387 (2)	257 (3)
Heart	1·4 (2)	28·3 (5)	1716 (3)	464 (4)
Kidney	9·4 (2)	117·3 (5)	1972 (3)	1408 (2)
Rumen epithelium	0·8 (2)	5·8 (2)	366 (1)	308 (1)
Rumen-wall muscle	0·2 (2)	3·7 (2)	—	—
Striated muscle	0·2 (1)	8·0 (2)	279 (2)	219 (2)
Spleen	3·4 (1)	12·6 (1)	546 (1)	532 (1)
Lung	−0·6* (1)	3·6 (1)	—	—
Brain cerebrum	—	1·4 (1)	540 (1)	444 (1)
Brain cerebellum	—	3·4 (1)	434 (1)	—

* Negative values indicate ketone body production.

apparatus. β-Hydroxybutyrate was separated from butyrate and other acids by silicic acid chromatography with the system $CHCl_3$–'*tert.*-pentanol' described by Leng & Annison (1963). The β-hydroxybutyrate fraction was chromatographed again, and the slight amounts of material emerging from the column before or after the β-hydroxybutyrate band were discarded. The eluate was neutralized with CO_2-free 0·1 N-NaOH and the β-hydroxybutyrate obtained showed no evidence of inhomogeneity when chromatographed again. The product (sodium salt) was freeze-dried in a concentrated aqueous solution to yield a white powder which was dried *in vacuo* over P_2O_5. The radiochemical yield of β-hydroxy[^{14}C]butyrate was about 25%, and the specific activity of the product was 80–85% of that of the starting material (sodium [^{14}C]butyrate) in both systems. About 30% of the starting material was recovered from the liver-slice system, and about 25% from rumen epithelium. Partial degradation of the β-hydroxybutyrate by procedures described by Leng & Annison (1963) showed that in both systems [1-^{14}C]butyrate gave rise to β-hydroxybutyrate with 80–84% of total radioactivity in C-1 and 16–20% in C-3; the product from [2-^{14}C]butyrate showed less than 0·1% of radioactivity in C-1, and it was assumed that the distribution of radioactivity between C-2 and C-4 was about 82 and 18%. For convenience the two products have been termed β-hydroxy-[1-^{14}C]butyrate and β-hydroxy[2-^{14}C]butyrate.

Measurements of specific rotation and analyses for sodium and carbon content were made on unlabelled samples of sodium β-hydroxybutyrate prepared by procedures identical with those described for the preparation of labelled material (Found: for liver preparation, Na, 17·3; C, 37·3; for rumen-epithelium preparation, Na, 17·1; C, 37·5. Calc. for $C_4H_7NaO_3$: Na, 18·3; C, 38·1%). The $[\alpha]_D$ values were −13·3° (liver preparation) and −13·8° (rumen-epithelium preparation); the expected value was −14·6° [Ottaway (1962), who quoted previous authors].

The somewhat low specific rotations of both preparations of β-hydroxybutyrate relative to the values reported previously must be attributed to contamination with other materials, or to the presence of small amounts of the laevorotatory form of β-hydroxybutyrate. Lactate, the most likely contaminant in the preparations in view of the relative R_F values of lactate and β-hydroxybutyrate in the chromatographic system used to isolate β-hydroxybutyrate, could not be detected in the preparations when examined by the enzymic procedure of Barker & Britton (1957).

Measurement of β-*hydroxybutyrate entry rate.* The general procedure described by Annison & White (1961) for the measurement of glucose entry rate was used. β-Hydroxy-[1-^{14}C]butyrate or β-hydroxy[2-^{14}C]butyrate was infused intravenously at a rate of 1 μmole (0·1 μc)/min. without a priming dose.

Chemical methods. Blood ketone body concentrations and specific radioactivities (Leng & Annison, 1963), blood glucose specific radioactivity (Annison & White, 1961), blood lactate specific radioactivity (Annison, Lindsay & White, 1963), blood acetate specific radioactivity (Annison & White, 1962) and blood CO_2 specific radioactivity (Annison & Lindsay, 1961) were determined as described previously.

RESULTS

Metabolism of D(−)-β-*hydroxybutyrate in isolated tissues.* Tissue preparations of rumen epithelium, liver, kidney, heart, spleen, muscle, brain and lung were incubated in Krebs–Ringer phosphate with β-hydroxy[1-^{14}C]butyrate, and oxygen consumption, conversion of substrate into carbon dioxide and total ketone body disappearance were measured (Table 1).

All tissues converted β-hydroxybutyrate into carbon dioxide, but kidney, heart and spleen were the most active, and brain, liver and rumen epithelium the least active of the tissues. Similar results were observed with the bicarbonate medium. The effects of added β-hydroxybutyrate on the endogenous metabolism of heart, kidney and striated muscle were examined with β-hydroxy[1-^{14}C]butyrate and β-hydroxy[2-^{14}C]-

butyrate. The ratios of the specific radioactivities of liberated carbon dioxide and added substrates provided estimates of the contribution of endogenous respiration to total oxidative metabolism. Comparison of these values with those based on oxygen consumption indicated that endogenous respiration was largely unaffected by the presence of β-hydroxybutyrate (Table 2).

The conversion of β-hydroxybutyrate into acetoacetate and the non-enzymic decarboxylation of this material during or after incubation would give misleading results when $^{14}CO_2$ production from β-hydroxy[1-^{14}C]butyrate was used as an index of tissue oxidation. Acetone, however, could not be detected in the flask contents. The oxidation of β-hydroxybutyrate in animal tissues occurs after reduction to acetoacetate and cleavage into two C_2 fragments which enter the tricarboxylic acid cycle as acetyl-CoA (see Campbell & Best, 1956). The higher specific radioactivities of carbon dioxide produced by β-hydroxy[1-^{14}C]butyrate with that by β-hydroxy[2-^{14}C]butyrate (Table 2) were in agreement with observations on $^{14}CO_2$ production from [1-^{14}C]- and [2-^{14}C]-acetate in other systems (Weinman, Strisower & Chaikoff, 1957), and were attributed to influx into the tricarboxylic acid cycle of other cycle intermediates (Weinman *et al.* 1957).

Liver slices continued to produce ketone bodies in the presence of added β-hydroxybutyrate (Table 1).

The apparent endogenous production of ketone bodies by lung was unexpected, and to the authors' knowledge has not been previously reported.

No detectable incorporation of ^{14}C into lactate or glucose occurred when liver slices were incubated with β-hydroxy[1-^{14}C]butyrate.

Metabolic interrelations of β-hydroxybutyrate, acetate, propionate and butyrate. Tissue preparations of liver, heart and kidney were incubated in bicarbonate medium with the combinations of 1-^{14}C-labelled substrates shown in Table 3. The low rate of oxidation of β-hydroxybutyrate by liver was lowered by acetate, propionate and butyrate. The effects of acetate and butyrate were probably due

Table 2. *Effects of β-hydroxybutyrate on endogenous respiration of heart and kidney slices and striated muscle*

Tissue (1 g.) was incubated with β-hydroxy[1-^{14}C]butyrate or β-hydroxy[2-^{14}C]butyrate (40 μmoles; 200 μmc; specific activity 104 μc/g. of C) in Krebs–Ringer phosphate (12 ml.). *A*, Apparent contribution (%) of endogenous substrates to total oxidative metabolism calculated from oxygen consumption in the presence and absence of substrate. *B*, Actual contribution (%) of endogenous respiration to total oxidative metabolism calculated from specific activities of substrates and liberated $^{14}CO_2$.

Tissue	Substrate	O_2 uptake (μl./g./hr.)	Specific radioactivity of CO_2 (μc/g. of C)	*A*	*B*
Heart	None	494 (2)	—	—	—
	β-Hydroxy[1-^{14}C]butyrate	827 (4)	42·6	60	59
	β-Hydroxy[2-^{14}C]butyrate		35·4		67
Kidney	None	1276 (2)	—	—	—
	β-Hydroxy[1-^{14}C]butyrate	2347 (4)	49·9	54	52
	β-Hydroxy[2-^{14}C]butyrate		32·8		68
Striated muscle	None	210 (2)	—	—	—
	β-Hydroxy[1-^{14}C]butyrate	252 (4)	18·3	83	82
	β-Hydroxy[2-^{14}C]butyrate		12·3		88

Table 3. *Metabolic interrelations of β-hydroxybutyrate and acetate, propionate and butyrate*

Tissue (250 mg.) was incubated in Krebs–Ringer bicarbonate (3 ml.) for 2 hr. at 39° with β-hydroxybutyrate (10 μmoles; 100 μmc), acetate (20 μmoles; 100 μmc), propionate (20 μmoles; 50 μmc) and butyrate (10 μmoles; 100 μmc), as indicated. The average results of two experiments are shown.

Substrate	$^{14}CO_2$ production (μmc/g./hr.) Liver	Heart	Kidney
β-Hydroxy[1-^{14}C]butyrate	6·1	11·9	93·6
β-Hydroxy[1-^{14}C]butyrate + acetate	4·6	8·2	65·9
β-Hydroxy[1-^{14}C]butyrate + propionate	3·4	9·8	73·4
β-Hydroxy[1-^{14}C]butyrate + butyrate	1·3	11·0	60·2
[1-^{14}C]Acetate	26·6	3·4	144·4
[1-^{14}C]Acetate + β-hydroxybutyrate	26·4	2·6	68·6
[1-^{14}C]Propionate	45·2	1·6	88·0
[1-^{14}C]Propionate + β-hydroxybutyrate	46·0	1·6	85·6
[1-^{14}C]Butyrate	35·2	5·0	128·2
[1-^{14}C]Butyrate + β-hydroxybutyrate	34·0	5·0	84·4

Table 4. *Interrelations of β-hydroxybutyrate and glucose when incubated with heart, kidney, brain and striated muscle*

Tissue (250 mg.) was incubated in Krebs–Ringer phosphate (3 ml.) with β-hydroxy[1-^{14}C]butyrate (10 μmoles; 100 μmc) and uniformly ^{14}C-labelled glucose (10 μmoles; 100 μmc), as indicated. The average results of two experiments are shown.

Substrate	$^{14}CO_2$ production (μmc/g./hr.) Heart	Kidney	Brain	Striated muscle
β-Hydroxy[1-^{14}C]butyrate	25·6	97·7	1·6	7·4
β-Hydroxy[1-^{14}C]butyrate + glucose	26·2	95·6	2·2	6·0
[^{14}C]Glucose	7·1	15·8	15·3	1·2
[^{14}C]Glucose + β-hydroxybutyrate	3·6	10·1	12·7	0·8

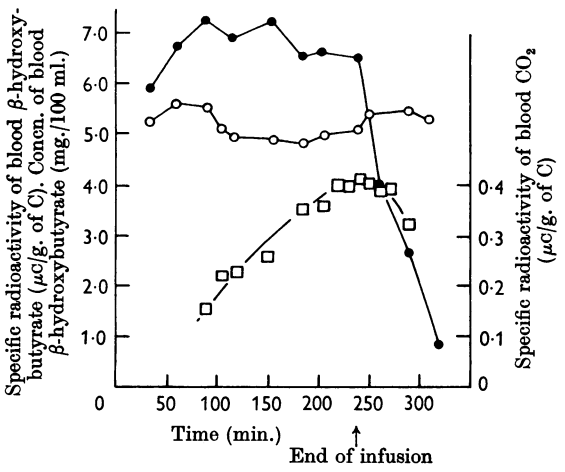

Fig. 1. The concentration and specific radioactivity of blood β-hydroxybutyrate, and the specific radioactivity of blood carbon dioxide, during the infusion of sodium D(−)-β-hydroxy[2-^{14}C]butyrate. The infusion rate was 1 μmole (0·1 μc)/min. ●, Specific radioactivity of blood β-hydroxybutyrate; ○, blood β-hydroxybutyrate concentration; □, specific radioactivity of blood CO_2.

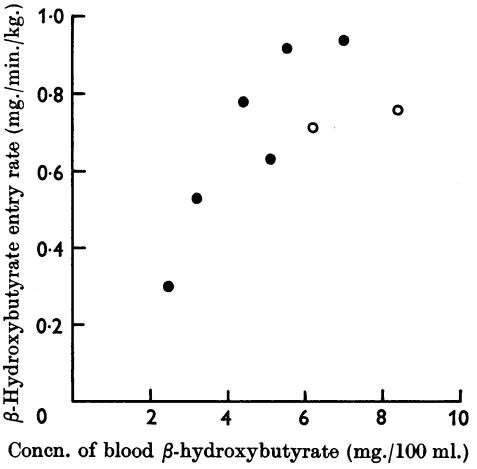

Fig. 2. Relationship between β-hydroxybutyrate entry rate and blood β-hydroxybutyrate concentration. ●, Sheep starved for 1 day; ○, sheep starved for 5 days.

to increased ketone body production from these substrates, which would dilute added labelled β-hydroxybutyrate. β-Hydroxybutyrate was without effect on the oxidation of short-chain fatty acids. Heart tissue oxidized β-hydroxybutyrate more readily than the short-chain acids, and no mutual interactions were observed. The rate of oxidation of all substrates by kidney slices was relatively high. Acetate and butyrate competed with β-hydroxybutyrate as substrates for oxidation, but the oxidation of propionate was unchanged in the presence of β-hydroxybutyrate, although propionate decreased β-hydroxybutyrate oxidation (Table 3).

Metabolic interrelations of β-hydroxybutyrate and glucose. Glucose had little effect on the oxidation of β-hydroxybutyrate by tissue preparations of heart, kidney, brain and striated muscle, but glucose oxidation by these tissues was decreased by β-hydroxybutyrate (Table 4).

Measurement of β-hydroxybutyrate entry rates. The infusion of β-hydroxy[^{14}C]butyrate gave rise to constant specific activities of blood β-hydroxybutyrate within 50–70 min. Blood concentrations of β-hydroxybutyrate were less subject to fluctuations in response to excitement than those of plasma free fatty acid (C. E. West & E. F. Annison, unpublished work) or glucose (Annison & White, 1961), and concentrations of β-hydroxybutyrate during most infusion experiments were relatively constant (Fig. 1). The mean value for the entry rate of β-hydroxybutyrate in six starved (24 hr.) sheep was 0·68 mg./min./kg., and in two sheep starved for 5 days was 0·74 mg./min./kg. The dependence of entry rate on blood concentrations is shown in Fig. 2.

Recycling of β-hydroxybutyrate. The extent of recycling through acetyl-CoA was studied by measuring the relocation of ^{14}C from C-1 to C-3 during the infusion of β-hydroxy[1-^{14}C]butyrate.

Table 5. *Transfer of* ^{14}C *to blood acetate, glucose and lactate after the infusion of β-hydroxy*[1-^{14}C]*butyrate*

β-Hydroxy[1-^{14}C]butyrate was infused at a rate of 1 μmole (0·1 μc)/min. for 2 hr. The results of two experiments are shown.

Substrate	Specific activity (μc/g. of C) Sheep 1	Sheep 2
β-Hydroxybutyrate	22·6	12·8
Acetate	0·14	0·10
Glucose	0·06	0·10
Lactate	0·05	0·09

At the end of a 2 hr. infusion the labelling pattern of blood β-hydroxybutyrate relative to that of the material infused indicated some degree of recycling. The proportion of radioactivity located at C-1 declined from 82 % in the original material to 78 % in blood β-hydroxybutyrate.

Conversion of blood β-hydroxybutyrate into carbon dioxide. Rough estimates of the contribution of β-hydroxybutyrate to total carbon dioxide production were obtained by comparing the specific radioactivities of blood carbon dioxide and β-hydroxybutyrate in the terminal stages (180–240 min.) of β-hydroxy[2-^{14}C]butyrate infusions. The slow increase in specific radioactivity of blood carbon dioxide at the end of the infusion (Fig. 1) indicated that the calculated values for the contribution of β-hydroxybutyrate to overall oxidation must be regarded as minimal values. Values of 5 and 7 % were obtained in two experiments on starved (24 hr.) sheep.

Incorporation of ^{14}C *from β-hydroxybutyrate into glucose, lactate and acetate.* Glucose, lactate and acetate were isolated from blood after the infusion for 120 min. of β-hydroxy[1-^{14}C]butyrate, and their specific radioactivities compared with that of blood β-hydroxybutyrate. The results of two experiments are shown in Table 5.

DISCUSSION

D(−)-β-Hydroxybutyrate, which accounts for about 85 % of the ketone bodies of sheep blood (Leng & Annison, 1963), was isolated from the urine of a diabetic by Magnus-Levy (1901). Lehninger & Greville (1953) prepared the D(−)- and L(+)-forms of β-hydroxybutyric acid by resolving a racemic mixture, and Ottaway (1962) described the preparation of D(−)-β-hydroxybutyric acid from poly-β-hydroxybutyric acid obtained from *Bacillus megaterium* and *B. cereus*. The ready conversion of sodium butyrate into sodium β-hydroxybutyrate by sheep-liver slices (Leng & Annison, 1963) and by rumen epithelium (Pennington, 1952) led us to investigate the use of these systems to prepare ^{14}C-labelled D(−)-β-hydroxybutyrate from ^{14}C-labelled sodium butyrate.

The relatively high entry rate and degree of oxidation of β-hydroxybutyrate in the intact sheep and the considerable capacity of isolated tissues to utilize this substrate provided unequivocal evidence of the metabolic importance of β-hydroxybutyrate. Entry rates in sheep starved for 1 day and for 5 days were not markedly different, suggesting that even in the well-nourished animal ketone body utilization made a significant contribution to caloric requirements. Butyrate is an important source of ketone bodies in fed sheep, and evidence has been obtained that a substantial fraction of butyrate is converted into β-hydroxybutyrate as a C_4 unit (Annison, Leng, Lindsay & White, 1963). In starved sheep, however, in which ruminal butyrate production is low, ketone bodies presumably arise largely by condensation of acetyl-CoA (Krebs, 1961) produced during fatty acid degradation. Considerable transfer of ^{14}C to β-hydroxybutyrate has been observed during the infusion of uniformly ^{14}C-labelled palmitate into starved (24 hr.) sheep (C. E. West & E. F. Annison, unpublished work).

The observation that D(−)-β-hydroxybutyrate was readily oxidized by heart, kidney and muscle was in agreement with previous work on the utilization of ketone bodies by extrahepatic tissues (see Campbell & Best, 1956). The major sites of ketone body production in ruminants, the liver and rumen epithelium, showed little capacity to oxidize β-hydroxybutyrate. The oxidation of D(−)-β-hydroxybutyrate by isolated tissues was only slightly decreased in the presence of short-chain fatty acids, and was unaffected by glucose. Glucose oxidation, however, was substantially decreased in the presence of D(−)-β-hydroxybutyrate. These observations on substrate interrelations in isolated tissues are in agreement with results obtained previously with eviscerated animals. Drury & Wick (1955) found that β-hydroxybutyrate decreased the rate of oxidation of glucose in eviscerate rabbits, and many workers have failed to demonstrate any effect of glucose (or insulin) on the utilization of ketones (see Campbell & Best, 1956). The rate of oxidation of D(−)-β-hydroxybutyrate by striated muscle, although low, was significant since this tissue constitutes a large fraction of total body mass. Blixenkrone-Møller (1938) showed that perfused muscles used ketone bodies more extensively when contracting than when resting, the rate being proportional to oxygen uptake. The oxygen uptake of muscle tissue in the present studies was low. The oxidation of β-hydroxybutyrate by brain tissue was in agreement with the oxidation of this substrate by brain mitochondria reported by McCann (1957).

The metabolism of endogenous substrates in isolated tissues was found to be largely unchanged in the presence of β-hydroxybutyrate, in contrast with the results in Kulka, Krebs & Eggleston (1961), who reported that acetoacetate depressed the endogenous metabolism of sheep-heart homogenates.

SUMMARY

1. Sodium D(−)-β-hydroxy[^{14}C]butyrate was prepared by incubating sodium [^{14}C]butyrate with sheep-liver slices or sheep-rumen epithelium in a bicarbonate medium. The products from [1-^{14}C]- and [2-^{14}C]-butyrate were labelled mainly (80–84%) in C-1 and C-2 respectively.

2. Tissue preparations of rumen epithelium, liver, kidney, heart, spleen, muscle, brain and lung all showed some capacity to oxidize β-hydroxybutyrate. Kidney, heart and spleen were the most active, and brain, liver and rumen epithelium the least active of the tissues.

3. The endogenous respiration of isolated tissues was unaffected by added β-hydroxybutyrate.

4. Heart tissue oxidized β-hydroxybutyrate more readily than the short-chain acids, and no mutual interactions were observed. Acetate and butyrate competed with β-hydroxybutyrate as substrates for oxidation by kidney slices, but the oxidation of propionate was unchanged in the presence of β-hydroxybutyrate.

5. Glucose had little effect on the oxidation of β-hydroxybutyrate by heart, kidney, brain and striated muscle, but glucose oxidation by these tissues was decreased by β-hydroxybutyrate.

6. Entry rates of β-hydroxybutyrate were measured by isotope dilution with continuous-infusion techniques. The mean value for the entry rate of β-hydroxybutyrate in six starved (24 hr.) sheep was 0·68 (range 0·30–0·94) mg./min./kg. body wt., and in two sheep starved for 5 days values of 0·53 and 0·92 mg./min./kg. were obtained.

7. Comparison of the specific radioactivities of blood β-hydroxybutyrate and blood carbon dioxide observed during the terminal stages of β-hydroxy-[2-^{14}C]butyrate infusions provided a rough estimate of the contribution of β-hydroxybutyrate to total oxidative metabolism. Values of 5 and 7% were obtained in two experiments.

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