Metabolism of Acetate, Propionate and Butyrate by Sheep-Liver Slices

By R. A. LENG and E. F. ANNISON
School of Rural Science, University of New England, Armidale, N.S.W., Australia

(*Received* 16 *July* 1962)

Short-chain fatty acids constitute a major energy source in ruminants (Annison & Lewis, 1959). These products of ruminal fermentation are absorbed directly into portal blood and pass to the liver before reaching the general circulation. Examination of arterial and portal blood in sheep has indicated that propionate and butyrate are almost completely removed by the liver under normal feeding conditions, but substantial quantities of acetate appear in peripheral blood (Annison, Hill & Lewis, 1957). A substantial glucose turnover in sheep has been indicated (Annison & White, 1961; Kronfeld & Simesen, 1961), focusing attention on the nature of glucose precursors in this species since very little glucose is absorbed from the alimentary tract. Propionate is well recognized as a source of glucose, but the possibility of butyrate and, to a less extent, of acetate being a precursor of glucose has been the subject of much speculation. In the present investigation no evidence was obtained of net synthesis of carbohydrate from butyrate or acetate in liver slices. The possible synthesis of glycogen from glucose or propionate in liver slices was studied in media containing several combinations of inorganic ions. Glycogen loss occurred in all media examined, although in a medium with high potassium content reported to favour glycogen synthesis in rat-liver slices (Hastings, Teng, Nesbett & Sinex, 1952) glycogen loss was minimal.

The metabolic interrelations of acetate, propionate and butyrate in liver slices were also examined with ^{14}C-labelled substrates. Incorporation of substrate into glucose, oxidation to [^{14}C]-carbon dioxide and production of lactate and ketone bodies were examined. Considerable ketone-body production from butyrate was observed, and the labelling pattern in β-hydroxybutyrate derived from [1-^{14}C]butyrate was similar to that reported by Hird & Symons (1959) with rumen or omasum epithelium and the same substrate.

MATERIALS AND METHODS

Experimental animals. Merino wethers, age 2–3 years, were housed indoors and fed once daily on a standard ration of 800 g. of lucerne chaff. Starved animals were allowed access to water.

Preparation of liver slices. Animals were killed by exsanguination, and samples of liver tissue immediately removed with a stainless-steel cork borer (1·3 cm. diam.). Tissue samples were placed in incubating medium at 4° and slices (0·02–0·04 mm. thick) were cut by hand at this temperature.

Incubation procedures. Double-side-bulb Warburg flasks were used as incubation vessels. Tissue slices (250 mg.), which were randomized before incubation, were blotted dry on filter paper, weighed and incubated in 3·0 ml. of medium. Substrates were added in 0·9% NaCl (0·1 ml.), and 0·2 ml. of 6 N-H_2SO_4 was placed in one side arm. The gas phase was 100% O_2 when phosphate medium was used, and $O_2 + CO_2$ (95:5) with media based on bicarbonate. Incubations (in duplicate) were carried out for 2 hr. at 39° with a shaking rate of 100 strokes/min. Krebs–Ringer phosphate and Krebs–Ringer bicarbonate media were prepared as described by Umbreit, Burris & Stauffer (1957). Results were expressed as μmc (or μmoles or mg.)/hr./g. of tissue (μmc/hr./g.).

Purification of substrates. After distillation AnalaR-grade acetic acid, propionic acid and *n*-butyric acid (British Drug Houses Ltd.) were found to be homogeneous when examined by gas–liquid partition chromatography (James & Martin, 1952). Aqueous solutions of these substrates were neutralized (pH 7·0) with NaOH and stored at −10°.

Radioactive substrates. The sodium salts of [1-^{14}C]acetic acid, [2-^{14}C]acetic acid, [1-^{14}C]propionic acid, [2-^{14}C]-propionic acid and [1-^{14}C]butyric acid, and $NaH^{14}CO_3$, were obtained from The Radiochemical Centre, Amersham, Bucks. Sodium [2-^{14}C]butyrate and sodium [3-^{14}C]butyrate were purchased from California Foundation for Biochemical Research, Calif., U.S.A. The radioactivity of labelled substrates was checked by wet oxidation (Van Slyke & Folch, 1940) to CO_2, which was assayed as $BaCO_3$ by the procedure described by Annison & White (1961).

Leuconostoc mesenteroides. A culture of this organism (strain 39) was obtained from the Department of Bacteriology, Indiana University, Bloomington, Ind., U.S.A.

Ion-exchange resins and silicic acid. These materials were obtained from Mallinckrodt Chemical Works, St Louis, Mo., U.S.A.

Chemical methods

After acidification of the medium at the end of the incubation period and absorption of CO_2 the flask contents were diluted with 3 ml. of water, and the tissue was removed by centrifuging. Analyses were made on the supernatant.

Glucose. This was estimated by the glucose-oxidase method of Huggett & Nixon (1957).

Glycogen. Glycogen was isolated from liver tissue by the method of Good, Kramer & Somogyi (1933) and hydrolysed with N-HCl at 100° for 3 hr. Neutralized hydrolysates were assayed for glucose.

Lactic acid. A method based on lactate dehydrogenase (Barker & Britton, 1957) was used. The reduction of NAD^+

to NADH at 340 mμ in the presence of semicarbazide (0·1 M) in a glycine buffer (0·6 M, pH 10·1) provided a measure of lactate content.

Ketone bodies. Total ketone bodies (acetone, acetoacetate and β-hydroxybutyrate) were estimated by the method of Bakker & White (1957). Recoveries of acetone from β-hydroxybutyrate were in the range 91–95%. The concentration of salicylaldehyde used for colour development was decreased from 20 to 10%, resulting in reduced colour production in blank estimations. When β-hydroxybutyrate values were required the filtrates (5 ml.) were made acid with 5 N-H_2SO_4 (1 ml.) and heated in a boiling-water bath to remove acetoacetate and acetone before assay.

Analysis of volatile fatty acids. These were estimated by titration with alkali after steam-distillation (Annison, 1954), and mixtures of fatty acids were analysed by gas–liquid partition chromatography (James & Martin, 1952) as described by Annison (1954).

Assay of labelled carbon dioxide. When Krebs–Ringer phosphate was used as incubating medium, CO_2 was collected in 0·2 ml. of 5% (w/v) CO_2-free NaOH in the centre well of the incubation flasks. Carbon dioxide produced during incubations in bicarbonate media was collected at the end of the incubation by injecting 0·4 ml. of 5% (w/v) CO_2-free NaOH through a rubber stopper into one side arm, and tipping 0·2 ml. of 6 N-H_2SO_4 from the second side arm. The flasks were left 6 hr. to allow absorption of CO_2. Solutions of NaOH containing absorbed CO_2 were transferred quantitatively to tubes containing 0·4 ml. of 5% (w/v) NH_4Cl and 1 ml. of 0·5% Na_2CO_3 as carrier where necessary, and precipitated as $BaCO_3$ with 0·4 ml. of 20% (w/v) $BaCl_2,2H_2O$. The $BaCO_3$ was assayed for radioactivity as described by Annison & White (1961).

Assay of labelled glucose. Supernatant fluid (3 ml.) was placed in a centrifuge tube with 0·5 ml. of 3·2% (w/v) glucose, deproteinized with 72% (w/v) $HClO_4$ (0·05 ml.) and the precipitate removed by centrifuging. The supernatant was transferred to another 15 ml. centrifuge tube containing phenylhydrazine hydrochloride (0·5 g.) and 2 ml. of sodium acetate–acetic acid buffer (containing 25 mg. of sodium acetate/ml. and 25 mg. of acetic acid/ml.) was added to adjust the solution to pH 4·7. After thorough mixing the solution was heated in a boiling-water bath until a heavy precipitate of glucosazone appeared (usually after about 30 min.). The glucosazone was collected by centrifuging, washed twice with water (10 ml.), dissolved in pyridine (0·5 ml.) and precipitated with water (10 ml.). This process was repeated four times, the final precipitate being filtered and plated out as described for $BaCO_3$ (Annison & White, 1961). The plated osazone was dried under suction in air by using an infrared lamp before removal of the planchet from the filter stem. Oven-drying of the osazone was found to cause curling and breaking of the precipitate. The osazone was assayed for radioactivity as described for $BaCO_3$ and corrected for self-absorption by the method of Hendler (1959).

The possible contamination of glucosazone with labelled substrates or labelled metabolic products was checked in control experiments. Glucose was isolated as glucosazone from an aqueous solution containing labelled acetate, propionate, butyrate, pyruvate and fumarate (10 μmoles, 5 μmc of each substrate/ml.) and unlabelled glucose (30 μmoles/ml.). The glucosazone was without detectable radioactivity.

Isolation and step-wise degradation of glucose. Carrier glucose (96 mg.) was added to flask contents after incubation and before deproteinization with $Ba(OH)_2$–$ZnSO_4$. The precipitate was removed by filtration, and the filtrate and washings were concentrated to 2–3 ml. *in vacuo* at 50° in a rotary evaporator. Purification of the glucose was effected with ion-exchange resins, the material being passed successively through 10 g. columns of Amberlite IR-120 (H^+ form) and Amberlite IR-4 B (OH^- form). The final effluent was concentrated to 2–3 ml. as above, made up to 10 ml. and assayed for glucose content. The glucose was fermented to lactic acid, ethanol and CO_2 with *Leuconostoc mesenteroides* by the general procedure described by Bernstein & Wood (1957).

(i) C-1. The $^{14}CO_2$ produced during fermentation was collected in CO_2-free NaOH and assayed for specific activity as $BaCO_3$.

(ii) C-2 *and* C-3. The ethanol was oxidized to acetic acid (Bernstein & Wood, 1957), which was isolated and purified by steam-distillation at pH 4·0. Acetic acid was decarboxylated by the Schmidt reaction, by the following modification of the method of Phares (1951), suggested by Dr D. B. Lindsay. Sodium acetate (0·2 m-mole) from the oxidation of ethanol was obtained dry in a 1 oz. screw-cap bottle containing a small tube fitted with a filter-paper wick. Sodium azide (20 mg.) and 100% H_2SO_4 (0·2 ml.) were added to the chilled bottle (0°), which was immediately tightly closed and evacuated by suction applied through a hypodermic needle (24-gauge) inserted through the cap and rubber liner. The bottle was then placed in a water bath at 45° and the temperature raised to 70° during a period of 10 min. After 45 min. at 70° the bottle was cooled and 1 ml. of CO_2-free 2 N-NaOH was injected into the small tube through the bottle cap. The bottle was allowed to stand overnight to allow absorption of CO_2. The NaOH solution, which in addition to absorbed CO_2 also contained SO_2, was placed in a CO_2-transfer apparatus (Kornberg, Davies & Wood, 1952) and acidified with 2 N-lactic acid containing H_2O_2 (5 vol. %), which oxidized sulphite to sulphate. The liberated CO_2 was absorbed in CO_2-free 0·5 N-NaOH and assayed for specific activity as $BaCO_3$. Methylamine produced in the reaction was steam-distilled (pH 10·0, Markham still) into 2 ml. of 2 N-H_2SO_4, the distillate concentrated *in vacuo* and oxidized (Van Slyke & Folch, 1940) to CO_2, which was assayed for specific activity as before.

(iii) C-4, C-5 *and* C-6. Lactic acid was isolated by silica-gel chromatography as described below, and degraded to CO_2 (C-4) and acetic acid by the procedures described by Bernstein & Wood (1957). Liberated CO_2 was assayed for specific activity as $BaCO_3$ and the distribution of radioactivity in acetic acid was determined as described above.

Isolation of lactic acid and β-hydroxybutyric acid. Silica-gel chromatography was used to isolate lactate and β-hydroxybutyrate produced during the incubation of liver slices with labelled butyrate. The acids were extracted from flask contents with ether (after acidification) and separated by chromatography on silica gel with $CHCl_3$–*tert.*-pentanol (Pennington & Sutherland, 1956). Columns (1 cm. diam.) containing 12 g. of silicic acid were used with loadings of 0·2–0·5 m-mole of acid mixtures. Progressively changing solvent mixture was applied to the column by the mixing device of Donaldson, Tulane & Marshall (1952), the mixing vessel containing 320 ml. of $CHCl_3$ (equilibrated with 0·25 N-H_2SO_4) and the upper vessel $CHCl_3$–*tert.*-

Table 1. *Effect of concentrations of* Na^+ *and* K^+ *ions on the metabolism of* [1-^{14}C]*propionate*

Liver slices from a fed sheep were incubated with [1-^{14}C]propionate (100 μmc, 40 μmoles).

Composition of medium (mM)		Glycogen content (g./100 g.)		Uptake of propionate (μmoles/hr./g.)	Production of $^{14}CO_2$ (μmc/hr./g.)	Incorporation of radioactivity into glucose (μmc/hr./g.)
K^+ ions	Na^+ ions	Before incubation	After incubation			
0	163·3	0·77	0·52	36·0	38·8	6·1
38	126·3	0·72	0·51	33·4	26·4	4·5
76	89·3	0·68	0·58	30·4	23·8	2·4
113	51·3	0·78	0·60	21·2	23·4	2·8
150	13·3	0·75	0·61	12·8	11·2	1·6

Table 2. *Comparison of propionate metabolism in media with high and low contents of* K^+ *ion*

Krebs–Ringer bicarbonate (low K^+ ion) and the medium of high K^+ ion content of Hastings *et al.* (1952) were used. Liver slices from fed and starved (6 days) sheep were incubated with [1-^{14}C]propionate (40 μmoles, 100 μmc).

Content of K^+ ion	Production of $^{14}CO_2$ (μmc/g./hr.)		Incorporation of radioactivity into glucose (μmc/g./hr.)	
	Fed sheep	Starved sheep	Fed sheep	Starved sheep
Low	32·0	1·2	6·7	0
High	16·2	1·7	1·8	0

pentanol (4:1, v/v). Fractions (5 ml.) were titrated with 0·01 N-NaOH in methanol under CO_2-free conditions with cresol red (0·1 % in methanol) as indicator. Separations of acids similar to those reported by Pennington & Sutherland (1956) were obtained except that β-hydroxybutyric acid preceded and slightly overlapped lactic acid.

Stepwise degradation of β*-hydroxybutyrate.* The acid isolated by silica-gel chromatography was chromatographed a second time in the system described above, isolated and the specific activity determined by wet oxidation (Van Slyke & Folch, 1940). Decarboxylation of β-hydroxybutyrate to acetone and CO_2, followed by conversion of acetone into iodoform and acetic acid, gave the relative distribution of radioactivity in C-1 and C-3, and a mean value for C-2 and C-4.

(i) C-1. Sodium β-hydroxybutyrate was decarboxylated by a modification of the procedure of Bakker & White (1957). An aqueous solution (3 ml.) containing 10–12 mg. of sodium β-hydroxybutyrate was added to a 1 oz. screw-cap bottle containing 5 ml. of 0·2 % $K_2Cr_2O_4$ in N-H_2SO_4. A small tube was placed upright in the bottle, which was fitted with a cap containing a silicone-rubber liner. The bottle was heated in an autoclave (15 lb./in.^{2}) for 30 min., cooled and 1 ml. of CO_2-free N-NaOH injected into the small tube through the bottle cap. After standing overnight to allow absorption of CO_2, the contents of the tube were removed and the absorbed CO_2 was assayed for specific activity as $BaCO_3$.

(ii) C-2, C-3 *and* C-4. The acetone produced in 91–95 % yield (Bakker & White, 1957) by the decarboxylation reaction was isolated from the bottle contents by distillation in the apparatus described by Reid (1961). Acetone was distilled into 2 ml. of 2,4-dinitrophenylhydrazine reagent prepared by dissolving 1 g. of the solid in 7·5 ml. of conc. H_2SO_4, 75 ml. of 95 % (v/v) ethanol and then water being added to a volume of 100 ml. The hydrazone, which appeared in the receiving vessel as a red precipitate, was filtered off, recrystallized from ethanol–water, dried and assayed for radioactivity by oxidation to CO_2 (Van Slyke & Folch, 1940).

(iii) C-2 *and* C-4. The acetone produced by decarboxylation of β-hydroxybutyrate was distilled into 1 ml. of 5 % (w/v) NaOH and a solution of iodine (1 %, w/v) in aqueous KI (10 %, w/v) was added dropwise to the distillate (2 ml.). The precipitate of iodoform was filtered off, recrystallized from ethanol–water, dried and assayed for radioactivity by oxidation to CO_2 (Van Slyke & Folch, 1940).

RESULTS

Effects of medium on glycogenolysis and propionate metabolism. Sodium [1-^{14}C]propionate was incubated with liver slices from fed sheep in media containing varying ratios of Na^+ ion: K^+ ion (Table 1). Glycogenolysis was extensive during the preparation of tissue in all media. In the typical experiment shown in Table 1 the liver-glycogen content immediately after removal of tissue at death was 1·8 g./100 g., which declined to about 0·7 g./100 g. during preparation of tissue. The rate of glycogen loss was slowed but not halted during incubations, particularly in media with a high content of K^+ ion (Table 1). Changes in glycogen content during incubation were unaffected by the presence of substrate. Propionate metabolism, measured by propionate uptake, oxidation to CO_2 and incorporation into glucose, was favoured by increased concentration of Na^+ ion (Table 1). In further experiments on the influence of the ionic composition of media on propionate metabolism, the medium reported by Hastings *et al.* (1952) to favour glycogen synthesis in rat-liver slices was compared with Krebs–Ringer bicarbonate medium with tissue from fed and starved (6 days) sheep (Table 2). No incorporation of propionate into glycogen was observed with either medium, but propionate oxidation and incorporation into glucose were greatest in Krebs–Ringer bicarbonate, and this medium of low K^+ ion

Table 3. *Metabolism of acetate, propionate and butyrate by sheep-liver slices*

Slices were incubated in Krebs–Ringer bicarbonate with acetate (40 μmoles, 200 μmc), propionate (40 μmoles, 100 μmc) and butyrate (20 μmoles, 200 μmc). The results of four experiments on fed sheep are compared with those from one experiment with tissue from a starved (6 days) sheep.

Substrate	Production of $^{14}CO_2$ (μmc/hr./g.)		Incorporation into glucose (μmc/hr./g.)	
	Fed sheep	Starved sheep	Fed sheep	Starved sheep
[1-^{14}C]Acetate	20·8±4·2	3·6	1·7± 0·4	0·2
[2-^{14}C]Acetate	8·4±1·4	1·6	5·8± 1·1	0
[1-^{14}C]Propionate	43·4±6·8	7·8	8·1± 2·5	0·6
[2-^{14}C]Propionate	6·2±1·0	2·8	28·3±10·7	3·0
[1-^{14}C]Butyrate	33·8±4·8	14·4	1·6± 0·5	0·2
[2-^{14}C]Butyrate	24·8±3·2	9·6	7·6± 4·2	0·4
[3-^{14}C]Butyrate	31·2±6·6	4·8	1·5± 0·7	0·2

content was used in subsequent experiments. Propionate metabolism was considerably depressed in tissue from starved animals (Table 2).

Concentrations of glycogen in the livers of fed and starved sheep were 4·6 ± 0·3 (17) and 1·2 ± 0·2 (4) g./100 g. of tissue respectively.

Oxidation of fatty acids and their incorporation into glucose. Differentially ^{14}C-labelled acetate, propionate and butyrate were incubated in Krebs–Ringer bicarbonate with liver slices from fed and starved sheep, and $^{14}CO_2$ production and incorporation of radioactivity into glucose measured (Table 3). Propionate and butyrate were more readily oxidized than acetate, and propionate was incorporated into glucose to a greater extent than acetate or butyrate. As expected, 1-^{14}C-labelled substrates produced more $^{14}CO_2$ than substrates labelled in other positions, and [2-^{14}C]propionate gave rise to more highly labelled glucose than did [1-^{14}C]propionate.

The mean ratio of $^{14}CO_2$ produced from [1-^{14}C]- and [2-^{14}C]-acetate was 2·1, range 1·6–2·5 (6), and the ratio for [1-^{14}C]- and [2-^{14}C]-butyrate was 1·7, 1·4–2·0 (4). These CO_2 ratios indicate substantial influx of unlabelled tricarboxylic acid-cycle intermediates (Weinman, Strisower & Chaikoff, 1957).

The effect of propionate concentration on propionate metabolism in bicarbonate medium is shown in Fig. 1. Oxidation of propionate and incorporation into glucose reached a maximum at a propionate concentration of about 6 mM.

Propionate metabolism was considerably depressed in phosphate medium (Table 4). Although slight oxidative activity was detected, some depression of oxidation of endogenous substrates was revealed by measurements of oxygen uptakes (Fig. 2). Metabolism of acetate and butyrate in phosphate medium was similar to that in bicarbonate. Comparison of oxygen uptakes in the presence and absence of added substrates suggested that endogenous respiration accounted for 66 and

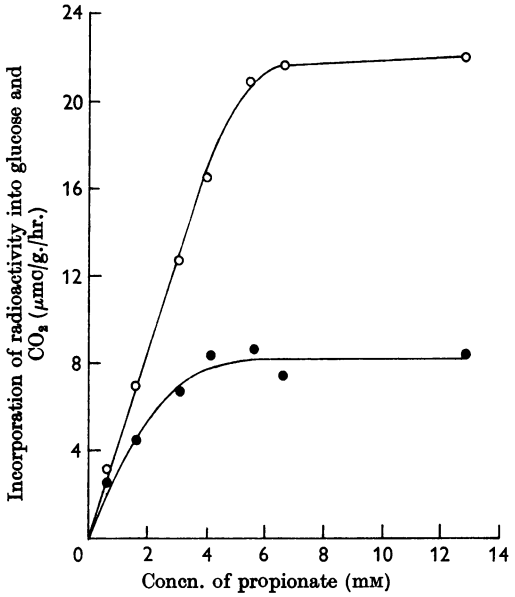

Fig. 1. Effect of concentration of propionate on [2-^{14}C]-propionate metabolism by sheep-liver slices from a fed sheep. Tissues (250 mg.) were incubated in Krebs–Ringer bicarbonate (3 ml.) at 39° for 2 hr. Gas phase, $O_2 + CO_2$ (95:5). ○, Incorporation into glucose; ●, oxidation to $^{14}CO_2$.

51 % of total oxidative metabolism in the presence of acetate and butyrate respectively (Fig. 2). The possible suppression or enhancement of endogenous metabolism in the presence of added substrates was examined by incubating liver slices in phosphate medium with uniformly labelled acetate and with butyrate labelled equally at C-1 and C-2. The ratios of the specific activities of liberated CO_2 and added substrates examined in relation to uptake of O_2 indicated that endogenous respiration was unaffected by the presence of acetate or butyrate (Table 5).

Table 4. *Metabolism of acetate, propionate and butyrate by liver slices from fed sheep*

Slices were incubated in Krebs–Ringer phosphate with acetate (40 μmoles, 200 μmc), propionate (40 μmoles, 100 μmc) and butyrate (200 μmoles, 200 μmc). All results are the mean values from four experiments.

Substrate	Production of $^{14}CO_2$ (μmc/hr./g.)	Radioactivity incorporated into glucose (μmc/hr./g.)
[1-^{14}C]Acetate	24·3±3·4	1·6±0·2
[1-^{14}C]Acetate and propionate	0·9±0·3	0
[1-^{14}C]Acetate and butyrate	1·7±0·3	0·1±0·1
[1-^{14}C]Propionate	3·5±0·8	0·3±0·1
[1-^{14}C]Propionate and acetate	5·1±0·9	0·3±0·1
[1-^{14}C]Propionate and butyrate	14·3±4·3	2·9±0·8
[1-^{14}C]Butyrate	31·0±1·1	1·2±0·2
[1-^{14}C]Butyrate and acetate	31·4±1·1	1·9±0·3
[1-^{14}C]Butyrate and propionate	21·6±0·2	1·8±0·1

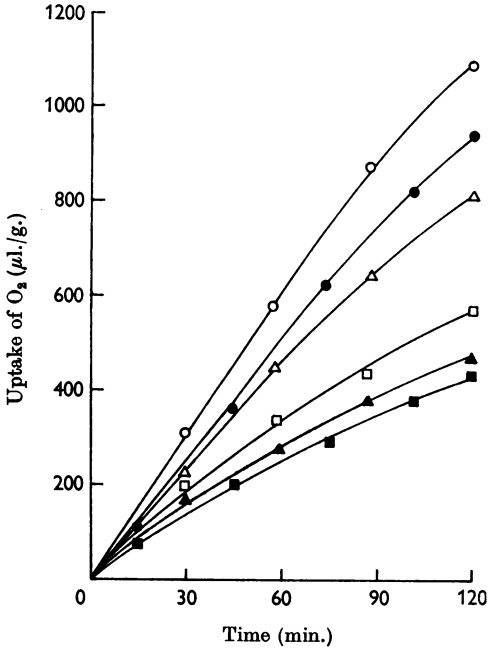

Fig. 2. Uptake of oxygen of liver slices (from a fed sheep) incubated with fatty acids. Tissue (250 mg.) was incubated in Krebs–Ringer phosphate (3 ml.) at 39°. Gas phase, 100% O_2. ○, Butyrate; ●, butyrate + propionate; △, acetate; ▲, acetate + propionate; □, no substrate; ■, propionate.

The stimulation of propionate metabolism by butyrate in phosphate medium (Table 4) was not solely due to increased CO_2 production, since the uptake of O_2 of liver slices was only doubled in the presence of butyrate (Table 5), but propionate incorporation into glucose increased tenfold (Table 4).

Glucose production from acetate, propionate and butyrate. The distribution of radioactivity in glucose isolated after the incubation of liver slices from fed sheep with acetate and propionate labelled at C-1 or C-2 and butyrate labelled at C-1, C-2 or C-3 (Table 6) indicated that propionate can act as a source of glucose, but the incorporation of acetate and butyrate was consistent with entry into glucose as acetyl-CoA through the tricarboxylic acid cycle (Weinman *et al.* 1957). The labelling patterns in glucose obtained from [1-^{14}C]acetate, [1-^{14}C]-butyrate and [3-^{14}C]butyrate were closely similar, and [2-^{14}C]acetate and [2-^{14}C]butyrate were also equivalent in this respect (Table 6).

Table 5. *Effects of acetate and butyrate on endogenous respiration*

Liver slices from fed sheep were incubated with uniformly labelled acetate (40 μmoles, 200 μmc) and [1,2-$^{14}C_2$]-butyrate (20 μmoles, 200 μmc) in Krebs–Ringer phosphate. *A*, Apparent contribution of endogenous substances to total oxidative metabolism calculated from O_2 uptake values 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 CO_2.

Substrate	Uptake of O_2 (μl./hr./g.)	Specific activity of substrates (μc/g. of C)	Specific activity of liberated CO_2 (μc/g. of C)	*A*	*B*
None	422	—	—	—	—
Acetate	636	208	68	66	67
Butyrate	829	208	82	50	61

Metabolic interrelations of acetate, propionate and butyrate. These interrelations were investigated with 1-^{14}C-labelled substrates and production of labelled $^{14}CO_2$ and incorporation of radioactivity into glucose were followed. Results were obtained with liver slices from fed and starved sheep with phosphate and bicarbonate media. Acetate and butyrate stimulated propionate metabolism by tissues from starved sheep in bicarbonate medium

Table 6. *Labelling patterns in glucose produced by incubation of sheep-liver slices with labelled acetate, propionate and butyrate*

Tissue (1 g.) was incubated at 39° for 3 hr. in Krebs–Ringer bicarbonate medium (12 ml.), with each substrate (acetate and propionate, 5 μc, 160 μmoles; butyrate 5 μc, 80 μmoles). The gas phase was $O_2 + CO_2$ (95:5). Mean values from two experiments are shown.

Substrate	Radioactivity recovered in glucose (μmc)	Distribution of radioactivity in glucose (%)					
		C-1	C-2	C-3	C-4	C-5	C-6
[1-^{14}C]Acetate	20	1	3	41	43	7	5
[2-^{14}C]Acetate	77	25	19	4	8	26	18
[1-^{14}C]Butyrate	17	2	4	43	48	2	1
[2-^{14}C]Butyrate	83	28	25	4	6	20	17
[3-^{14}C]Butyrate	13	5	5	39	44	5	2
[1-^{14}C]Propionate	346	< 1	< 1	56	43	< 1	< 1
[2-^{14}C]Propionate	685	34	14	1	3	48	

Table 7. *Interrelations of acetate, propionate and butyrate metabolism*

Liver slices from fed and starved (6 days) sheep were incubated in Krebs–Ringer bicarbonate with acetate (40 μmoles, 200 μmc), propionate (40 μmoles, 100 μmc) and butyrate (20 μmoles, 200 μmc).

Substrate	Production of $^{14}CO_2$ (μmc/hr./g.)		Radioactivity incorporated into glucose (μmc/hr./g.)	
	Fed sheep	Starved sheep	Fed sheep	Starved sheep
[1-^{14}C]Acetate	16·2±1·5 (3)	5·6	1·7±0·3 (3)	0·2
[1-^{14}C]Acetate + propionate	1·5±0·2 (3)	1·2	0·7±0·3 (4)	0·2
[1-^{14}C]Acetate + butyrate	1·6±0·1 (3)	0·5	0·4±0·3 (4)	—
[1-^{14}C]Propionate	41·7±5·3 (3)	10·8	9·2±1·3 (3)	0·6
[1-^{14}C]Propionate + acetate	37·2±2·5 (3)	27·2	9·0±2·4 (3)	1·7
[1-^{14}C]Propionate + butyrate	25·1±4·0 (3)	20·4	6·8±1·0 (3)	1·0
[1-^{14}C]Butyrate	29·0±3·5 (3)	18·8	1·8±0·2 (3)	0·3
[1-^{14}C]Butyrate + acetate	29·0±3·5 (3)	18·8	1·8±0·2 (3)	0·2
[1-^{14}C]Butyrate + propionate	28·3±5·3 (3)	11·4	5·6±0·2 (3)	0·7

(Table 7), but with fed animals no significant ($P > 0.05$) effects were observed. Propionate and butyrate both inhibited acetate oxidation ($P < 0.01$) and reduced acetate incorporation into glucose in experiments with liver slices from fed and starved sheep in both media (Tables 4 and 7).

Acetate and propionate had no marked influence on butyrate oxidation, but butyrate incorporation into glucose was augmented in the presence of unlabelled propionate in bicarbonate medium (Table 7). These results suggested either that CO_2 produced by butyrate oxidation was preferentially fixed during propionate metabolism, or that propionate stimulated butyrate incorporation into glucose through acetyl-CoA. The first hypothesis was checked by using labelled bicarbonate. The depression of incorporation of ^{14}C into glucose by butyrate in the system [^{14}C]bicarbonate–unlabelled propionate was compared with the depression of [^{14}C]propionate incorporation produced by butyrate in the system unlabelled bicarbonate–[^{14}C]propionate (Table 8). Butyrate decreased labelled propionate incorporation into glucose, but the depression was not significantly less ($P > 0.05$) than that of butyrate on the fixation of $^{14}CO_2$ during propionate metabolism.

Table 8. *Carbon dioxide and propionate fixation into glucose*

Liver slices were incubated in Krebs–Ringer bicarbonate solution containing [^{14}C]bicarbonate (250 μmc) and unlabelled acetate (40 μmoles), propionate (40 μmoles) or butyrate (20 μmoles). The effect of butyrate on [1-^{14}C]-propionate (100 μmc) incorporation into glucose was examined in the same experiment.

Flask contents	Radioactivity incorporated into glucose (μmc/hr./g.)
$NaH^{14}CO_3$	0·3±0·1 (6)
$NaH^{14}CO_3$ + acetate	0·9±0·1 (4)
$NaH^{14}CO_3$ + butyrate	0·3±0·1 (4)
$NaH^{14}CO_3$ + propionate	9·2±3·8 (6)
$NaH^{14}CO_3$ + propionate + butyrate	4·8±2·0 (6)
[1-^{14}C]Propionate	11·6±2·2 (6)
[1-^{14}C]Propionate + butyrate	7·6±3·8 (6)

Analysis of short-chain fatty acids recovered by steam-distillation from incubation mixtures indicated the absence of production of short-chain fatty acid by liver tissue.

Table 9. *Ketone-body production by sheep-liver slices*

Slices from fed and starved (6 days) sheep were incubated in Krebs–Ringer bicarbonate with acetate (40 μmoles), propionate (40 μmoles) or butyrate (20 μmoles). β-Hydroxybutyrate concentrations were estimated in experiments with tissue from fed sheep.

Substrate	Production of ketone body (μmoles/hr./g.) Fed sheep	Starved sheep	β-Hydroxybutyrate (% of total ketone bodies)
None	4·0±0·4 (7)	3·0	85
Acetate	6·6±0·5 (6)	5·0	85
Propionate	4·3±0·8 (7)	2·8	83
Butyrate	12·4±1·2 (7)	8·2	88
Acetate + propionate	3·9±1·2 (4)	3·0	—
Butyrate + propionate	6·7±2·0 (4)	6·4	—

Table 10. *Lactate production by liver slices*

Slices from fed and starved (6 days) sheep were incubated in Krebs–Ringer bicarbonate, with acetate (40 μmoles), propionate (40 μmoles) or butyrate (20 μmoles).

Substrate	Lactic acid produced (μmoles/hr./g.) Fed sheep	Starved sheep
None	6·2±2·1 (4)	0·8
Acetate	3·9±0·5 (4)	1·3
Propionate	10·2±1·4 (4)	4·4
Butyrate	3·4±0·5 (4)	1·2

Production of ketone body. This was investigated in bicarbonate medium (Table 9). Increased ketone-body production was observed in the presence of acetate and butyrate. Propionate had no effect on endogenous ketone-body production, but ketone-body production from butyrate was decreased, and from acetate abolished, in the presence of propionate (Table 9). The results obtained in two identical experiments with phosphate medium were within the range of values obtained in bicarbonate medium (Table 9).

The distribution of radioactivity in β-hydroxybutyrate produced by the incubation of liver slices with [1-^{14}C]butyrate was investigated. [1-^{14}C]-Butyrate (80 μmoles, 800 μmc) was incubated with liver slices (1 g.) in 12 ml. of Krebs–Ringer bicarbonate for 3 hr. and the resulting β-hydroxybutyrate isolated by column chromatography. The lactate band was also collected. Stepwise degradation of β-hydroxybutyrate indicated substantial relocation of activity from C-1 of the butyrate to C-3 in the hydroxy acid; 82 % of the activity was recovered in C-1 and 17 % in C-3 with negligible activity (< 1 %) in C-2 and C-4. Comparison of the specific activities of added butyrate (10 μmc/μmole) with β-hydroxybutyrate (8·5 μmc/μmole) indicated some suppression of endogenous ketone-body production, since the results in Table 9 suggested a reduction of specific activity of about 25 % if ketone-body production proceeded unchanged in the presence of butyrate.

The proportion of β-hydroxybutyrate in ketone bodies produced by liver slices in the presence and absence of substrate exceeded 80 % in all cases (Table 9).

Production of lactate. Liver slices from fed and starved sheep were incubated in bicarbonate medium with acetate, propionate and butyrate. Acetate and butyrate had no significant effect on lactate production (Table 10) but some accumulation of lactate occurred in the presence of propionate. Starvation markedly reduced lactate production in the presence and absence of substrate (Table 10).

The lactate produced during the incubation of [1-^{14}C]butyrate (see above) was examined for radioactivity. The specific activity of the lactate (0·9 μmc/μmole) was low relative to that of β-hydroxybutyrate (8·5 μmc/μmole) produced during the same experiment. McCarthy, Shaw & Lakshmanan (1958) reported the production of relatively highly labelled lactate from labelled butyrate by a perfused goat-liver preparation.

DISCUSSION

The failure to achieve glycogen synthesis in sheep-liver slices in media known to favour glycogen deposition in rat-liver slices (Hastings *et al.* 1952) suggested that tissue damage associated with preparation of slices was not reversed in the high-potassium 'intracellular' medium. Reasonably constant oxygen uptakes (Fig. 2), however, suggested that the preparation retained sufficient metabolic activities to justify the present studies *in vitro*.

The relative abundance of short-chain fatty acids in ruminants and the limited amounts of glucose absorbed from the alimentary tract (Annison & Lewis, 1959) have led to much speculation on the possible existence in ruminant tissue of pathways leading to the net conversion of fatty acids into

glucose. Butyrate has been the subject of controversy since Potter (1952) found that sodium butyrate injected intravenously relieved insulin convulsions. Several investigators have observed raised blood-sugar concentrations after butyrate administration (Jarrett, Potter & Filsell, 1952; Johnson, 1955; Clark & Malan, 1956; Kronfeld, Campbell, Hooper & Galligan, 1956), and Shaw (1959) has reported the net conversion of large amounts of butyrate into carbohydrate by the perfused goat liver. Kleiber *et al.* (1954) observed appreciable labelling of milk lactose after the intravenous injection of labelled butyrate in cows, but Black, Kleiber & Brown (1961) have convincingly demonstrated the absence of metabolic pathways that would account for the 'glucogenic' behaviour of butyrate in lactating cows. Lauryssens, Verbeke, Peeters & Reinards (1960) compared the incorporation into cow's-milk constituents of [1-^{14}C]butyrate and [3-^{14}C]butyrate and concluded that butyrate metabolism proceeded after cleavage into C_2 fragments. In the present studies close similarities were observed in the labelling patterns in glucose derived from [1-^{14}C]acetate and [1-^{14}C]- or [2-^{14}C]-butyrate. Similarly [2-^{14}C]acetate and [2-^{14}C]butyrate each gave rise to glucose with almost identical labelling patterns (Table 6). The distribution of radioactivity was consistent with incorporation into glucose through the tricarboxylic acid cycle (Weinman *et al.* 1957). The close similarities in glucose labelling obtained with [1-^{14}C]-acetate and [1-^{14}C]- and [3-^{14}C]-butyrate showed the absence of major pathways leading to butyrate incorporation into glucose as a C_3 unit. The liver is a major site of butyrate utilization in ruminants, and these results strongly suggest the absence of net synthesis of carbohydrate from butyrate or acetate. The stimulation by propionate of butyrate incorporation into glucose observed in the present studies *in vitro* (Table 6) was consistent with observations of appreciable labelling *in vivo* of glycogen (Shaw, 1959) and lactose (Kleiber *et al.* 1954) after administration of labelled butyrate. Large quantities of propionate are absorbed by ruminants, and both propionate and butyrate are extensively metabolized by the liver (Annison *et al.* 1957). No evidence was obtained of the transfer of butyrate carbon atoms to lactate, in contrast with the findings of McCarthy *et al.* (1958) with a perfused goat-liver preparation.

Pritchard & Tove (1960*a*) examined short-chain fatty acid metabolism by sheep-liver slices from starved (6–8 days) sheep. Starvation reduces the overall metabolic activity of liver slices and propionate metabolism in particular is severely diminished (Table 7). The suppression of acetate oxidation by propionate and butyrate in both bicarbonate and phosphate media (Tables 4 and 6) confirmed the observations of Pritchard & Tove (1960*a*) and was in agreement with similar results obtained with rumen epithelium (Pennington, 1957; Pennington & Appleton, 1958) and rat liver (Masoro, Felts, Panagos & Rapport, 1957). Since acetate absorbed from the rumen is always accompanied by propionate and butyrate, acetate oxidation by rumen wall and liver *in vivo* is probably low, and oxidation in peripheral tissues must account for a substantial proportion of the total acetate oxidation observed in the whole animal (Annison & Lindsay, 1961). The stimulation of propionate metabolism by acetate and butyrate reported by Pritchard & Tove (1960*b*) was observed only with tissue from starved sheep (Table 6). Butyrate oxidation was unaffected by propionate, the stimulation of labelled butyrate incorporation into glucose by propionate (Table 6) presumably reflecting increased tricarboxylic acid-cycle activity. The possibility that carbon dioxide resulting from butyrate oxidation was preferentially fixed during propionate metabolism was not supported by the results of experiments in which the fixation of labelled bicarbonate into glucose during propionate metabolism was examined in the presence and absence of unlabelled butyrate (Table 7).

In contrast with the findings of Pritchard & Tove (1960*a*) considerable ketone-body production was observed in liver slices from fed and starved sheep in the presence and absence of added substrate. Pennington (1952) reported ketone-body production by sheep liver *in vitro*. The distribution of radioactivity in β-hydroxybutyric acid produced from [1-^{14}C]butyrate was closely similar to that reported by Hird & Symons (1959) with sheep-omasum epithelium. This relocation of radioactivity implies that there is continued breakdown and resynthesis of acetoacetyl-coenzyme A through the intermediate acetyl-coenzyme A. The greater part of the ketone bodies produced from butyrate was present in the reduced form (Table 9). Hird & Symons (1959) showed that, in the presence of glucose, ketone bodies were produced by omasum epithelium mainly in the reduced form. Glucose and glycogen were invariably present during the incubation of sheep-liver slices in the present experiments since even in starvation (6 days) liver glycogen was not fully depleted.

SUMMARY

1. The metabolism of acetate, propionate and butyrate by sheep-liver slices was studied with ^{14}C-labelled substrates. Production of labelled carbon dioxide and incorporation of radioactivity into glucose were followed.

2. Propionate and butyrate were more readily oxidized than acetate, and propionate was in-

corporated into glucose to a greater extent than were the other substrates. Oxidation of propionate and incorporation into glucose reached a maximum at a propionate concentration of about 6 mM.

3. The distribution of radioactivity in glucose isolated after the incubation of liver slices from fed sheep with acetate and propionate labelled at C-1 or C-2 and butyrate labelled at C-1, C-2 or C-3 indicated that propionate could act as a source of glucose, but the incorporation of acetate and butyrate was consistent with entry into glucose as acetyl-coenzyme A through the tricarboxylic acid cycle.

4. The interrelations of acetate, propionate and butyrate were studied. Acetate and butyrate stimulated propionate metabolism by tissues from starved sheep, and propionate and butyrate both inhibited acetate oxidation. Acetate and propionate were without effect on butyrate oxidation, but butyrate incorporation into glucose was augmented in the presence of unlabelled propionate.

5. Increased ketone-body production by liver slices was observed in the presence of acetate and butyrate. The proportion of β-hydroxybutyrate in ketone bodies exceeded 80 % in all cases.

6. Attempts to promote glycogen synthesis in liver slices in media containing varying ratios of Na^+ ion:K^+ ion concentrations were unsuccessful.

These studies were made possible by the generous support of the Australian Wool Research Committee and the University of New England Research Fund.

REFERENCES

Annison, E. F. (1954). *Biochem. J.* **58**, 570.

Annison, E. F., Hill, K. J. & Lewis, D. (1957). *Biochem. J.* **66**, 592.

Annison, E. F. & Lewis, D. (1959). *Metabolism in the Rumen.* London: Methuen and Co. Ltd.

Annison, E. F. & Lindsay, D. B. (1961). *Biochem. J.* **78**, 777.

Annison, E. F. & White, R. R. (1961). *Biochem. J.* **80**, 162.

Bakker, N. & White, R. R. (1957). *N.Z. J. Sci. Tech.* B, **38**, 1001.

Barker, J. & Britton, H. G. (1957). *J. Physiol.* **138**, 3P.

Bernstein, I. A. & Wood, H. G. (1957). In *Methods in Enzymology*, vol. 4, p. 561. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Black, A. L., Kleiber, M. & Brown, A. M. (1961). *J. biol. Chem.* **236**, 2399.

Clark, R. & Malan, J. R. (1956). *Onderstepoort J. vet. Res.* **27**, 101.

Donaldson, K. O., Tulane, V. J. & Marshall, L. M. (1952). *Analyt. Chem.* **24**, 185.

Good, C. A., Kramer, H. & Somogyi, M. (1933). *J. biol. Chem.* **100**, 185.

Hastings, A. B., Teng, C. T., Nesbett, F. B. & Sinex, F. M. (1952). *J. biol. Chem.* **194**, 69.

Hendler, R. W. (1959). *Science*, **130**, 772.

Hird, F. J. R. & Symons, R. H. (1959). *Biochim. biophys. Acta*, **35**, 422.

Huggett, A. St G. & Nixon, D. A. (1957). *Biochem. J.* **66**, 12P.

James, A. T. & Martin, A. J. P. (1952). *Biochem. J.* **50**, 679.

Jarrett, I. G., Potter, B. J. & Filsell, O. H. (1952). *Aust. J. exp. Biol. med. Sci.* **30**, 197.

Johnson, R. B. (1955). *Cornell Vet.* **45**, 273.

Kleiber, M., Black, A. L., Brown, M. A., Luick, J., Baxter, C. F. & Tolbert, B. M. (1954). *J. biol. Chem.* **210**, 239.

Kornberg, H. L., Davies, R. E. & Wood, D. R. (1952). *Biochem. J.* **51**, 351.

Kronfeld, D. S., Campbell, L. A., Hooper, I. L. & Galligan, S. J. (1956). *Amer. J. vet. Res.* **20**, 430.

Kronfeld, D. S. & Simesen, M. F. (1961). *Amer. J. Physiol.* **201**, 639.

Lauryssens, M., Verbeke, R., Peeters, G. & Reinards, M. T. (1960). *J. Dairy Res.* **27**, 151.

McCarthy, R. D., Shaw, J. C. & Lakshmanan, S. (1958). *Proc. Soc. exp. Biol., N.Y.*, **99**, 556.

Masoro, E. F., Felts, J. M., Panagos, S. S. & Rapport, D. (1957). *Arch. Biochem. Biophys.* **68**, 270.

Pennington, R. J. (1952). *Biochem. J.* **51**, 251.

Pennington, R. J. (1957). *Biochem. J.* **65**, 534.

Pennington, R. J. & Appleton, J. M. (1958). *Biochem. J.* **69**, 119.

Pennington, R. J. & Sutherland, T. M. (1956). *Biochem. J.* **63**, 618.

Phares, E. F. (1951). *Arch. Biochem. Biophys.* **33**, 173.

Potter, B. J. (1952). *Nature, Lond.*, **170**, 541.

Pritchard, G. I. & Tove, S. B. (1960*a*). *Biochim. biophys. Acta*, **41**, 130.

Pritchard, G. I. & Tove, S. B. (1960*b*). *Biochim. biophys. Acta*, **41**, 134.

Reid, R. L. (1961). *Analyst*, **85**, 265.

Shaw, J. C. (1959). *Oklahoma Conference on Radioisotopes in Agriculture*, p. 213. U.S. Atomic Energy Commission.

Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1957). *Manometric Techniques and Tissue Metabolism*, 3rd ed., p. 149. Minneapolis: Burgess Publishing Co.

Van Slyke, D. D. & Folch, J. (1940). *J. biol. Chem.* **136**, 509.

Weinman, E. O., Strisower, E. H. & Chaikoff, I. L. (1957). *Physiol. Rev.* **37**, 252.