Measurement of the rate of substrate cycling between acetate and acetyl-CoA in sheep muscle *in vivo*

Effects of infusion of acetate

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1. The rate of substrate cycling between acetate and acetyl-CoA in sheep hindlimb muscle was measured from the decrease of [^{14}C]acetate specific radioactivity across the tissue *in vivo*. 2. The rate of cycling was 0.005–0.015 $\mu\text{mol}/\text{min}$ per g and, in two of the three sheep investigated, was approximately doubled as a result of infusing 2 mol acetate/day intravenously. 3. The results indicate that this cycle contributes only approx. 0.5% of the total heat produced by the animal and would probably make only a relatively small contribution to any heat increment of feeding associated with diets producing large amounts of acetate.

INTRODUCTION

Much of the acetate in ruminant blood is derived from rumen fermentations, but a significant amount (approx. 30% in normal fed animals) is derived from tissue metabolism (Annison & White, 1962; Annison & Armstrong, 1970; Bergman & Wolff, 1971). This 'endogenous' acetate production, which occurs in many tissues of both ruminants and non-ruminants (Knowles *et al.*, 1974; Buckley & Williamson, 1977; Pethick *et al.*, 1981), has been suggested to represent a 'safety valve' for preventing an excessive accumulation of acetyl-CoA (Snoswell & Koundakjian, 1972). However, this hypothesis was not supported by the results of Pethick *et al.* (1981).

An alternative explanation is that endogenous acetate formation represents the reverse reaction of a substrate cycle (Newsholme & Crabtree, 1976) between acetate and acetyl-CoA. This cycle, catalysed by the simultaneous activities of acetyl-CoA synthetase (EC 6.2.1.1) and acetyl-CoA hydrolase (EC 3.1.2.1), has been shown to operate in rat hepatocytes, where its rate was increased by an increased concentration of acetate (Jessop *et al.*, 1986). Since the cycle results in the hydrolysis of ATP (AMP formation is equivalent to two molecules of ATP hydrolysed), its physiological role may be to dissipate excess fuel and hence produce heat and/or restrict weight gain (Jessop *et al.*, 1986).

If this cycle in ruminants responds similarly to changes in acetate concentration, it may help to explain why the efficiency of utilization of metabolizable energy is lowered by feeding diets that give rise to large quantities of acetate, for example forage diets (Blaxter, 1962). MacRae & Loble (1982) have suggested that this lowered efficiency may result from a restricted rate of fat synthesis from acetate, and hence increased rate of acetate oxidation. However, an increased rate of acetate oxidation could also result from an increased rate of acetate cycling, since this would increase the utilization of ATP.

Pethick *et al.* (1981) observed a decrease in specific radioactivity of acetate across sheep hindlimb, which

most probably reflects acetate cycling. Therefore it was decided to use a similar preparation both to measure the rate of cycling, for estimating its contribution to total heat production, and to investigate whether increasing the concentration of blood acetate increases the rate of cycling sufficiently to account for a significant part of the HIF associated with acetate.

EXPERIMENTAL

Materials

Isotopes were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.; enzymes, cofactors and buffers were obtained from Boehringer Corporation Ltd., Lewes, East Sussex, U.K.; butyrate, isopentanoate and all inorganic reagents were obtained from BDH Ltd., Poole, Dorset, U.K., and were of the highest purity available; heparin was obtained from C.P. Pharmaceuticals, Wrexham, Clywd, Wales, U.K.; silastic tubing (int. diam. 0.8 mm, outer diam. 1.6 mm) was obtained from McCarthy's Surgical, Glasgow, Scotland, U.K.; polyvinyl tubing (NT2, int. diam. 1 mm, outer diam. 1.6 mm) was obtained from Portex, Hythe, Kent, U.K.

Animals

Greyface wethers (3–4 years old) were housed in metabolism crates and fed continuously with 1 kg of grass pellets (88% dry matter; 20 MJ gross energy and 28 g of N/kg of dry matter)/24 h. Water was freely available. Approx. 4 weeks after implanting a silastic catheter into the dorsal aorta via the mesenteric artery (see Pethick *et al.*, 1981) a polyvinyl catheter was inserted approx. 25 cm into a jugular vein (for substrate infusion) and a silastic catheter inserted approx. 20 cm into a tarsal vein (to sample a region of hindlimb that is predominantly muscle (Domanski *et al.*, 1974)). The tarsal catheter was continuously infused with NaCl (9 g/l) containing heparin (10^5 units/l) at 6 ml/h. The jugular catheter was continuously infused with NaCl (9 g/l) at 20 ml/h.

After 2 days the jugular infusion was changed to 4 M-NaCl and at approx. 08:30 h on the following day

this was supplemented with [$1\text{-}^{14}\text{C}$]acetate (approx. 20 nmol; $2\text{ }\mu\text{Ci/ml}$). After 2 h, 15 ml samples of blood were withdrawn simultaneously from the arterial and tarsal-venous catheters and mixed with 100 units of heparin. Further blood samples (approx. 1 ml) were withdrawn into small plastic syringes containing approx. 100 units of heparin for the measurement of pH and $p\text{O}_2$. Blood sampling was repeated at 30 min intervals for a further 2 h, after which the jugular infusion was continued with 4 M-NaCl alone.

After repeating these control infusions for several more days, the jugular infusion was changed to 4 M-sodium acetate, to supply acetate at approx. 2 mol/day. The radioactive infusions were carried out as described above, and, after several more days, the experiment was completed by returning the jugular infusion to the control solution (4 M-NaCl) and repeating the control radioactive infusions.

Metabolite determination

Approx. 0.5 g of blood was mixed with 0.5 ml of 6% (w/v) HClO_4 , and centrifuged at 2000 g for 15 min. The pH of the supernatant was adjusted to approx. 7 with 2 M-KOH and the tubes placed on ice for 30 min, after which a sample of supernatant was withdrawn and stored at $-15\text{ }^\circ\text{C}$ until required for assay.

Ketone bodies (acetoacetate and 3-hydroxybutyrate) and glucose were measured using the methods of Williamson *et al.* (1962) and Kunst *et al.* (1984) respectively. Lactate was measured by using the method of Noll (1984); to minimize changes in pH due to dissociation of the NH_4^+ added with the enzymes, most of the $(\text{NH}_4)_2\text{SO}_4$ was removed from the commercial enzymes by centrifugation at 2000 g for 15 min, removing the supernatant and redissolving the enzymes in distilled water. These enzyme solutions were used only on the day of preparation.

Acetate concentration and specific radioactivity

Approx. 8 ml of plasma was mixed with 2 ml of 1.99 mM-isopentanoic acid and 1 g of citric acid. The mixture was steam-distilled (Markham still) into 50 μl of 1 M-NaOH and the alkaline solution was evaporated at $40\text{ }^\circ\text{C}$ on a rotary evaporator. The residue was dissolved in 2 ml of 2.66 mM-butyric acid and 1.2–1.4 g was added to a scintillation vial containing 10 ml of Lumax/xylene (1:1, v/v) and 4 ml of 1 M-acetic acid. The ^{14}C radioactivity was measured by using a Packard Tri-Carb liquid-scintillation counter and assumed to represent acetate. Further samples of extract (0.2 μl) were applied to a g.l.c. column to determine the concentration of acetate (Pethick *et al.*, 1981).

Plasma acetate concentration was calculated from the extract acetate concentration and the recovery of the internal standard, isopentanoic acid; this was expressed as a blood concentration by using the packed cell volume (measured values were 23–30%). Recoveries of acetate were 70–95%.

The following preliminary experiments were carried out to determine whether the presence of radioactive volatile compounds other than acetate could interfere with the determination of the specific radioactivity of acetate. Firstly, the acetate in the butyric acid extract from arterial plasma was purified by preparative g.l.c. (Hancock, 1981, p. 68). With three samples from a sheep (no. 6584) infused with 4 M-acetate the specific radio-

activities of purified acetate were 505, 517 and 498 d.p.m./ μmol ; the corresponding specific radioactivities calculated from the radioactivity of the unpurified extract were 525, 530 and 505 d.p.m./ μmol respectively. Since the specific radioactivities could not be determined more accurately than $\pm 5\%$ by either method, the similarity of these values suggests that there is no significant interference from other volatile compounds present in the general circulation. Secondly, since there may be labelling of both CO_2 and 3-hydroxybutyrate across the muscle, possible interference by these compounds in venous plasma was investigated by adding ^{14}C -labelled bicarbonate or 3-hydroxybutyrate to plasma, followed by distillation and evaporation. Less than 0.1% of added bicarbonate and less than 2% of added 3-hydroxybutyrate was recovered in the final extract; therefore any interference by these compounds would be insignificant. (Lactate is unlikely to become significantly labelled from [$1\text{-}^{14}\text{C}$]acetate in muscle and, in any case, enzymic analysis of the butyric acid extract of three samples of venous plasma showed that its molar concentration was less than 6% that of acetate.) Consequently, the method used to determine the specific radioactivity of acetate is considered to be sufficiently accurate for the present investigations.

Blood oxygen and pH

The pH and $p\text{O}_2$ of the blood samples were measured using a Radiometer Blood Micro System (BMS3 MK2) attached to a Radiometer Acid-Base Analyser (PHM71 MK2). $p\text{O}_2$ was adjusted to that at pH 7.4 by using the equation:

$$p\text{O}_2(7.4) = p\text{O}_2(\text{measured}) \cdot 10^s$$

where $s = -0.48(7.4 - \text{pH})$, and the percentage saturation of haemoglobin calculated from a dissociation curve for sheep blood at pH 7.4 (Bartels & Harms, 1959). The concentration of O_2 was obtained as the sum of that dissolved in the plasma and that bound to the haemoglobin, assuming that 1 g of haemoglobin binds 1.34 ml of O_2 when fully saturated (see Adams & Hahn, 1982). The haemoglobin concentration was measured as described by Adams & Hahn (1982).

Blood flow

Since it is not possible to isolate the arterial blood supply to the hindlimb muscle area, blood flow through the muscle cannot be measured by the dilution of dyes or other added substances. However, Pethick *et al.* (1981) showed that, provided the animal is standing quietly, blood flow through the muscle can be calculated from the following equation (which incorporates the correction recently reported by Lindsay *et al.*, 1986):

$$\text{Blood flow [l/(min/kg)]} = (0.16 - 0.019) [\text{arteriovenous difference for } \text{O}_2 (\mu\text{mol/ml})]$$

This equation was therefore used to measure blood flow, with lactate output being used to check that the animals were resting.

Estimation of tricarboxylic-acid-cycle flux

Assuming that the principal fuels supplying acetyl-CoA to the tricarboxylic acid ('Krebs') cycle (K) are acetate, glucose and long-chain fatty acids and that the net flux of acetate into the muscle (U) reflects oxidation, ($K - U$) is the flux through the cycle due to the oxidation

of fuels other than acetate. If these fuels are completely oxidized so that the ratio acetyl-CoA formation/ O_2 consumption is equal to 2 for acetate and approx. 3 for glucose or long-chain fatty acid oxidation:

$$O = 2U + 3(K - U),$$

whence

$$K = (O + U)/3$$

where O is the O_2 consumption.

These assumptions are reasonable for muscle, because alternative pathways of acetyl-CoA utilization (e.g. fat synthesis, ketone-body formation) are of minor importance (Jarrett *et al.*, 1976). However, significant amounts of intramuscular fat could give rise to errors, because U will then include acetate conversion into fat and hence K is overestimated.

Calculation of the rate of acetate cycling

This model assumes that (i) the venous concentration and specific radioactivity of acetate reflect those of the cellular pool and (ii) there is a single pool of acetyl-CoA. In muscle this pool is likely to be intramitochondrial, since this is the predominant location of acetyl-CoA hydrolase (Knowles *et al.*, 1974). If all pathways and tracers are in a steady state, the net labellings of acetate and acetyl-CoA are both zero. Therefore, for acetate:

$$B \cdot c_a \cdot s_a + C \cdot s_{\text{acetyl-CoA}} = s_v \cdot (B \cdot c_v + C + U) \quad (1)$$

where B is the blood flow, c_a and s_a are the arterial acetate concentrations and specific radioactivities and c_v and s_v are the venous concentrations and specific radioactivities.

For acetyl-CoA:

$$(C + U) \cdot s_v = (K + C) \cdot s_{\text{acetyl-CoA}} \quad (2)$$

so that:

$$s_{\text{acetyl-CoA}} = (C + U) \cdot s_v / (K + C)$$

Therefore eqn. (1) becomes:

$$B \cdot c_a \cdot s_a + C(C + U)s_v / (K + C) = s_v(B \cdot c_v + C + U)$$

which can be rearranged to:

$$C = \frac{BKc_a \cdot r - UK - BKc_v}{(Bc_v + K - Bc_a \cdot r)}$$

where $r = s_a/s_v$.

Since $U = B(c_a - c_v)$, this equation can be further simplified to:

$$C = \frac{BKc_a(r - 1)}{K + B(c_v - c_a \cdot r)} \quad (3)$$

K , i.e. the tricarboxylic-acid-cycle flux, is calculated as $(U + O)/3$, where O is the O_2 uptake (see above), and eqn. (3) is then used to calculate the rate of acetate cycling. A more sophisticated model, which allowed for a continuous variation of acetate concentration across the muscle bed instead of assuming it to be equal to the venous concentration, gave rates of cycling only approx. 10% lower than those produced by the simple model. The latter was therefore considered to be sufficiently accurate for measuring the rate of the cycle *in vivo*.

Although acetate cycling will decrease the specific radioactivity of acetate across the muscle, an observed decrease in specific radioactivity across the hindlimb does not necessarily indicate cycling, for two reasons: (i) acetate production (C) may take place in a different tissue from uptake (U); (ii) even if C and U occur in the same tissue or cell, they may not share a common pool of acetyl-CoA. The first possibility is unlikely, because the enzymes for acetyl-CoA formation from, and hydrolysis to, acetate occur in the same tissue (Knowles *et al.*, 1974). Moreover, since both these enzymes are mitochondrial in muscle (Knowles *et al.*, 1974) the second possibility would require at least two non-communicating mitochondrial pools of acetyl-CoA. This is theoretically possible, although evidence obtained with rat liver mitochondria indicates only one pool of acetyl-CoA (Lopes-Cardozo *et al.*, 1978).

RESULTS AND DISCUSSION

Conditions of animals during experiments

During infusions the sheep ate normally, but drank large quantities of water, presumably because of the disposal of extra Na^+ ions by the kidneys. Each animal stood quietly during collection of blood samples, and this is reflected in a low net output of lactate (Table 2). Therefore, calculating blood flow from the arteriovenous difference of O_2 is considered to be satisfactory. Indeed the blood flows reported here (Table 2 below) are similar to those reported by Domanski *et al.* (1974) and by Pethick *et al.* (1981), who used a similar preparation. [The muscle blood flows, and hence metabolite uptakes, in these papers were overestimated by a factor of 2 (Lindsay *et al.*, 1986). The comparisons in the present paper refer to the corrected and not the original data.] Furthermore, the uptakes of glucose, O_2 and ketone bodies in the control animals were similar to those measured in normal resting sheep (Domanski *et al.*, 1974), which suggests that the infusion of NaCl did not seriously disturb resting-muscle metabolism.

The arterial specific radioactivity of acetate reached a near-plateau within 2 h after the start of infusions and varied by less than 10% of the mean value during the infusion. In contrast, the arterial acetate concentration varied by as much as 30% of the mean value. These observations are similar to those reported by Pethick *et al.* (1981).

Blood haemoglobin contents and packed cell volume tended to decrease during an experiment from initial values of approx. 12 and 30% to approx. 7 and 22% respectively. This may be a cumulative effect of the withdrawal of blood samples or an expansion of plasma volume by Na^+ infusion.

In all animals the pH of the arterial blood increased by approx. 0.4 pH unit during the infusion of 4 M-acetate.

Table 1. Arterial and venous concentrations of metabolites in hindlimbs of sheep infused with NaCl or sodium acetate

Values are expressed as $\mu\text{mol/ml}$ of blood and represent means $\pm$ s.e.m. Significance testing (t test) refers to the effect of acetate infusion (***) $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

Sheep	Infusion	Acetate		Lactate		3-Hydroxybutyrate + acetoacetate		Glucose		Oxygen	
		Arterial	Venous	Arterial	Venous	Arterial	Venous	Arterial	Venous	Arterial	Venous
1	NaCl ($n = 35$) Acetate ($n = 30$)	0.61 ± 0.02	0.46 ± 0.01	0.91 ± 0.02	0.88 ± 0.07	0.32 ± 0.02	0.25 ± 0.03	2.86 ± 0.01	2.61 ± 0.08	9.94 ± 0.05	7.01 ± 0.11
		$0.92 \pm 0.02^{***}$	$0.57 \pm 0.02^{***}$	$1.15 \pm 0.04^{***}$	$1.18 \pm 0.09^{**}$	0.31 ± 0.03	0.33 ± 0.03	$2.41 \pm 0.02^{***}$	$2.38 \pm 0.04^{**}$	$9.41 \pm 0.06^{***}$	$5.99 \pm 0.24^{***}$
2	NaCl ($n = 16$) Acetate ($n = 15$)	0.56 ± 0.02	0.41 ± 0.01	0.89 ± 0.13	0.78 ± 0.03	0.37 ± 0.04	0.35 ± 0.08	2.92 ± 0.03	2.64 ± 0.09	9.73 ± 0.06	6.93 ± 0.19
		$1.29 \pm 0.08^{***}$	$0.87 \pm 0.08^{***}$	0.97 ± 0.04	$0.99 \pm 0.08^{**}$	0.41 ± 0.07	0.42 ± 0.08	$2.42 \pm 0.02^{***}$	2.45 ± 0.05	9.64 ± 0.03	6.84 ± 0.15
3	NaCl ($n = 40$) Acetate ($n = 37$)	0.44 ± 0.02	0.27 ± 0.02	0.79 ± 0.03	0.86 ± 0.11	0.39 ± 0.01	0.31 ± 0.02	2.71 ± 0.01	2.44 ± 0.04	8.30 ± 0.02	4.86 ± 0.13
		$1.38 \pm 0.04^{***}$	$0.99 \pm 0.04^{***}$	$1.16 \pm 0.05^{***}$	$1.18 \pm 0.06^{**}$	$0.46 \pm 0.03^*$	$0.47 \pm 0.03^{***}$	$2.47 \pm 0.03^{***}$	2.41 ± 0.03	$8.22 \pm 0.02^{**}$	5.02 ± 0.09

Table 2. Net uptakes and calculated rates of tricarboxylic acid cycle and acetate cycling in sheep hindlimb muscle

Values are expressed as means $\pm$ s.e.m. Each rate or ratio was calculated for each individual data point and the results averaged. (Significance testing was as in Table 1). Notes: $^{(1)}B = 0.16-0.019$ (arteriovenous difference for O_2) (see the Experimental section); $^{(2)}\text{uptake} = B \times (\text{arteriovenous difference})$; $^{(3)}r = \text{specific radioactivity of arterial acetate} / \text{specific radioactivity of venous acetate}$; $^{(4)}K = (U + O)/3$ (see the Experimental section); $^{(5)}C = \{B \times (r - 1) [\text{arterial acetate}] / [K + B (\text{venous acetate}) - r \cdot [\text{arterial acetate}]]\}$ (see the Experimental section); $^{(6)}C_0 = B \times [\text{venous acetate}] \times (1 - 1/r)$ (see the Results section). Abbreviation: TCA, tricarboxylic acid.

Sheep	Infusion	Blood flow (<i>B</i>) [l/(kg/ min)] ⁽¹⁾	Net uptake (mmol/min per kg of muscle) ⁽²⁾					<i>r</i> ⁽³⁾	Cycling (mmol/min per kg)		
			Acetate (<i>U</i>)	Lactate	3-Hydroxy- butyrate + acetoacetate	Glucose	<i>O</i> ₂ (<i>O</i>)		TCA cycle (<i>K</i>) ⁽⁴⁾	Acetate cycling	
										(<i>C</i>) ⁽⁵⁾	<i>C</i> ₀ ⁽⁶⁾
1 (6584)	NaCl (<i>n</i> = 35)	0.11 ± 0.002	0.018 ± 0.002	0.003 ± 0.004	0.008 ± 0.003	0.03 ± 0.005	0.32 ± 0.01	1.12 ± 0.01	0.11 ± 0.001	0.016 ± 0.002	0.006 ± 0.001
	Acetate (<i>n</i> = 30)	0.10 ± 0.003***	0.036 ± 0.002***	-0.002 ± 0.008	-0.001 ± 0.002**	0.003 ± 0.005**	0.32 ± 0.02	1.12 ± 0.01	0.12 ± 0.003	0.022 ± 0.002*	0.006 ± 0.002
2 (7842)	NaCl (<i>n</i> = 16)	0.11 ± 0.003	0.014 ± 0.002	0.01 ± 0.01	0.004 ± 0.005	0.03 ± 0.01	0.30 ± 0.03	1.13 ± 0.01	0.11 ± 0.003	0.011 ± 0.002	0.006 ± 0.001
	Acetate (<i>n</i> = 15)	0.11 ± 0.003	0.037 ± 0.001***	-0.001 ± 0.009	0.001 ± 0.003	-0.003 ± 0.01	0.31 ± 0.04	1.09 ± 0.02	0.12 ± 0.001**	0.024 ± 0.003***	0.008 ± 0.002
3 (90034)	NaCl (<i>n</i> = 40)	0.08 ± 0.003	0.013 ± 0.001	-0.005 ± 0.01	0.007 ± 0.002	0.02 ± 0.003	0.27 ± 0.01	1.11 ± 0.01	0.10 ± 0.004	0.006 ± 0.002	0.002 ± 0.002
	Acetate (<i>n</i> = 37)	0.10 ± 0.002***	0.037 ± 0.002***	-0.001 ± 0.005	-0.001 ± 0.002**	0.01 ± 0.02	0.30 ± 0.04	1.03 ± 0.01***	0.12 ± 0.002***	0.007 ± 0.001	0.003 ± 0.001

Unlike the changes in metabolite concentrations (see below), this change took 3–4 days to develop fully.

Effect of acetate infusion on blood concentrations and metabolite uptake in hindlimb muscle

The infusion of approx. 2 mol of acetate/day increased the arterial acetate concentration by as much as 3-fold, but decreased the concentration of glucose (Table 1). It had no significant effect on the concentrations of ketone bodies and no consistent effect on those of lactate. The net rate of acetate utilization (U) was increased, and that of glucose and ketone bodies decreased (Table 2). All these effects occurred within 2 h of starting the infusion and, except for one animal (no. 6584), showed no further significant changes after several days. Therefore all the samples obtained during infusion of acetate or NaCl were pooled to give the values in Tables 1–3. In sheep no. 6584 there was a reproducible, but transient, further increase in blood acetate concentration (approx. 0.7 mol/ml) during the second day of infusion with acetate; therefore data for this animal on this day have been excluded.

Cycling between acetate and acetyl-CoA

The rates of substrate cycling between acetate and acetyl-CoA (C) were calculated from the decrease of acetate specific radioactivity across the limb, using eqn. (3), and the results are presented in Table 2. Values of C calculated from eqn. (3) are quite sensitive to errors in the specific radioactivities of acetate (s_a and s_v), especially when the blood acetate concentration is increased. Since the specific radioactivities could not be determined more accurately than approx. $\pm 5\%$ of the mean values, a large number of measurements was needed to determine whether there was a significant effect of acetate infusion on the rate of the cycle.

The results in Table 2 show that acetate infusion significantly increased the rate of cycling in two of the

three sheep investigated, but the effect was rather small.

Pethick *et al.* (1981) calculated the rate of endogenous acetate production (i.e. cycling) by assuming that the net uptake of acetate radioactivity across the muscle was equal to the specific radioactivity of arterial acetate multiplied by the 'gross uptake' (i.e. the forward rate of the cycle, $U + C$). Consequently (using the symbols defined above):

$$B \cdot c_a s_a - B \cdot c_v s_v = s_a \cdot (U + C_0)$$

so that, by using $U = B(c_a - c_v)$:

$$C_0 = B \cdot c_v \cdot (1 - 1/r) \quad (4)$$

where $r = s_a/s_v$ and C_0 denotes an apparent rate of cycling (since this derivation implicitly assumes that the specific radioactivity of acetyl-CoA is zero).

An estimate of the ratio $s_{\text{acetyl-CoA}}/s_{\text{acetate}}$ can be made from the fluxes in Table 2 (by using eqn. 2), and the results (Table 3) show that this ratio may be as high as 0.5. Consequently, the rate of cycling calculated by ignoring $s_{\text{acetyl-CoA}}$, C_0 , is only 25–50% of that calculated using the model described here (see Table 2).

Fluxes in the whole animal

In Table 3 the fluxes involved in the postulated acetate/acetyl-CoA cycle are calculated in terms of their possible contributions to the whole animal. These values are only approximate because of the assumptions that 25% of the body weight is skeletal muscle (Table 2) and hindlimb muscle metabolism reflects that of the entire muscle mass. Table 3 also shows values for the total flux of acetate in the body ('entry rate') before and after correcting for acetate recycling from acetyl-CoA in muscle (see the Appendix). An interesting observation is that the net entry of acetate from the gut (i.e. the difference between the corrected entry rate and the rate of acetate infusion) was significantly lower than the corrected entry rate when the animals were infused with

Table 3. Acetate fluxes in the whole animal and the heat produced by muscle acetate cycling

Values are means $\pm$ S.E.M. All rates are expressed as mol/day. Each rate, ratio or percentage was calculated for each individual data point and the results averaged, except for the rates of heat production, which were calculated directly from C_m . Notes: ⁽¹⁾calculated from rates in Table 2, assuming muscle wt. = 25% body wt. (Pethick *et al.*, 1981); body wts. were 50, 45 and 42 kg for sheep 1, 2 and 3 respectively; ⁽²⁾ $E = (I^*/s_{\text{acetate}}) - C_m(1 - s_{\text{acetyl-CoA}}/s_{\text{acetate}})$ (see the Appendix); ⁽³⁾ $s_{\text{acetyl-CoA}}/s_{\text{acetate}} = (C + U)/(C + K)$ (see the Experimental section); ⁽⁴⁾heat production = $C_m \times 2 \times 80$, since 2 mol of ATP are hydrolysed by the cycle and 1 mol of ATP = approx. 80 kJ of heat (see Newsholme & Crabtree, 1976). See Table 1 for details of significance testing.

Sheep	Infusion	Acetate uptake by muscle ⁽¹⁾ (U_m)	Acetate cycling by muscle ⁽¹⁾ (C_m)	Apparent entry rate (I^*/s_{acetate})	Corrected entry rate ⁽²⁾ (E)	U_m as a % of E	$s_{\text{acetyl-CoA}}/s_{\text{acetate}}$ ⁽³⁾	Heat production due to muscle acetate cycling ⁽⁴⁾ (kJ/day)
1 (6584)	NaCl ($n = 35$)	0.33 ± 0.03	0.27 ± 0.03	3.0 ± 0.07	2.81 ± 0.08	12 ± 1	0.28 ± 0.009	43 ± 5
	Acetate ($n = 30$)	$0.64 \pm 0.03^{***}$	$0.40 \pm 0.05^*$	$4.2 \pm 0.15^{***}$	$4.00 \pm 0.14^{***}$	$16 \pm 1^{***}$	$0.42 \pm 0.01^{***}$	$64 \pm 7^*$
2 (7842)	NaCl ($n = 16$)	0.23 ± 0.03	0.17 ± 0.02	2.33 ± 0.03	2.20 ± 0.06	11 ± 1	0.21 ± 0.006	26 ± 3
	Acetate ($n = 15$)	$0.59 \pm 0.09^{***}$	$0.40 \pm 0.09^{***}$	$4.07 \pm 0.05^{***}$	$3.87 \pm 0.12^{***}$	$15 \pm 2^{**}$	$0.51 \pm 0.01^{***}$	$65 \pm 14^{***}$
3 (90034)	NaCl ($n = 40$)	0.19 ± 0.02	0.09 ± 0.03	2.27 ± 0.06	2.19 ± 0.07	9 ± 1	0.15 ± 0.008	14 ± 4
	Acetate ($n = 37$)	$0.56 \pm 0.02^{***}$	0.10 ± 0.02	$4.2 \pm 0.02^{***}$	$4.14 \pm 0.03^{***}$	14 ± 1	$0.35 \pm 0.02^{***}$	12 ± 2

Table 4. Residual whole-animal acetate flux in sheep infused with acetate

Values are expressed as means $\pm$ S.E.M.. Each value was calculated for each individual data point and the results averaged. *E* is the 'corrected' entry rate (Table 3). The significance testing refers to the difference between *E*—infusion rate and *E* for infusion with NaCl (** $P < 0.001$; ** $P < 0.01$).

Sheep	Rate (mol of acetate/day)		
	Acetate infusion rate	<i>E</i> —infusion rate	<i>E</i> for infusion with NaCl
1 (<i>n</i> = 30)	2.23 $\pm$ 0.04	1.77 $\pm$ 0.12***	2.81 $\pm$ 0.08 (<i>n</i> = 35)
2 (<i>n</i> = 15)	2.23 $\pm$ 0.01	1.64 $\pm$ 0.13***	2.20 $\pm$ 0.06 (<i>n</i> = 16)
3 (<i>n</i> = 37)	2.23 $\pm$ 0.01	1.91 $\pm$ 0.05**	2.19 $\pm$ 0.07 (<i>n</i> = 40)

NaCl (Table 4). This may indicate the operation of a feedback mechanism which reduces acetate entry from the rumen (or its production by fermentation in the rumen) in response to an elevated blood acetate concentration.

Table 3 also shows that only 9–16% of the entry rate of acetate was accounted for by muscle, and this percentage was only slightly increased by acetate infusion. Although these contributions are only approximate, and are somewhat lower than the values of 20–25% reported by Pethick *et al.* (1981) (see the note above concerning overestimation), they suggest that skeletal muscle may not be the major site of acetate utilization in sheep.

The heat produced as a result of cycling between muscle acetate and acetyl-CoA was 14–43 kJ/day in the controls and increased to approx. 65 kJ/day in the two sheep responding to the acetate infusions (Table 3). Since this is only approx. 0.5% of the total heat production (approx. 7 MJ/day), this cycle in muscle is unlikely to contribute significantly to the total heat production of a normal resting animal.

Acetate cycling and HIF

A small but significant increase in cycling after infusing acetate was observed in two of the three sheep investigated (Tables 2 and 3). However, this would represent an extra heat production of only approx. 25 kJ/day (Table 3), which is only 1.5% of the total energy supplied by the infused 2 mol of acetate (approx. 1750 kJ). This suggests that the cycle in muscle is unlikely to contribute significantly to any HIF as a direct result of changes in blood acetate concentrations.

Although dietary manipulations could change the rate of cycling indirectly (e.g. by hormonal effects which are

bypassed by infusing acetate into the blood), this small direct effect of acetate on a potentially important heat-producing cycle in muscle suggests that other mechanisms must be involved in varying the efficiency of utilization of different diets. These mechanisms may be postabsorptive (see MacRae & Lobley, 1982) and/or preabsorptive (Ørskov *et al.*, 1979).

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APPENDIX

Calculation and correction for cycling of whole-body entry rate of acetate

In this model the fluxes and the pools of acetate and acetyl-CoA refer to the whole animal. I^* is the rate of infusion of [^{14}C]acetate and is equal to the infusion rate (ml/day) multiplied by the infusate radioactivity (d.p.m./ml). E_t (mol/day) is the 'entry rate' of acetate, i.e. its net rate of entry into the blood from the gut. C_t (mol/day) is the total rate of acetate cycling, i.e. the sum of cycling in all tissues in which the cycle operates.

Assuming isotopic steady state at acetate:

$$I^* + C_t s_{\text{acetyl-CoA}} = s_{\text{acetate}}(C_t + E_t)$$

so that:

$$\begin{aligned} I^*/s_{\text{acetate}} &= C_t + E_t - C_t (s_{\text{acetyl-CoA}}/s_{\text{acetate}}) \\ &= E_t + C_t(1 - s_{\text{acetyl-CoA}}/s_{\text{acetate}}) \end{aligned}$$

where s_{acetate} is the specific radioactivity of arterial acetate.

Consequently the 'apparent entry rate', I^*/s_{acetate} , is only equal to the actual entry rate, E_t , if there is no recycling.

The effect of muscle cycling on the entry rate can be allowed for by assuming that the rate of cycling measured in the hindlimb is the same for the entire muscle mass. In this case the ratio of the radioactivities of acetyl-CoA and acetate can be calculated from eqn. (2) of the main paper:

$$s_{\text{acetyl-CoA}}/s_{\text{acetate}} = (C + U)/(C + K)$$

and a corrected entry rate, E , then calculated from the equation:

$$E = I^*/s_{\text{acetate}} - C_m(1 - s_{\text{acetyl-CoA}}/s_{\text{acetate}})$$

in which C_m (mol/day) is the whole-body rate of cycling due to muscle (Table 3 of the main paper). Although E is a better approximation to the entry rate, E_t , than I^*/s_{acetate} , it still contains the effects of cycling in other tissues (e.g. liver; Pethick *et al.*, 1981) and may therefore be an overestimate.

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