Regulation of glycolysis and fatty acid synthesis from glucose in sheep adipose tissue

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1. The following were measured in adipose-tissue pieces, obtained from 7–9 month-old sheep, before or after the tissue pieces had been maintained in tissue culture for 24 h: the rates of synthesis from glucose of fatty acids, acylglycerol glycerol, pyruvate and lactate; the rate of glucose oxidation to CO_2 ; the rate of glucose oxidation via the pentose phosphate pathway; the activities of hexokinase, glucose 6-phosphate dehydrogenase, phosphofructokinase, pyruvate kinase, pyruvate dehydrogenase and ATP citrate lyase; the intra- and extra-cellular water content; the concentration of various metabolites and ATP, ADP and AMP. 2. The proportion of glucose carbon converted into the various products in sheep adipose tissue differs markedly from that observed in rat adipose tissue. 3. There was a general increase in the rate of glucose utilization by the adipose-tissue pieces after maintenance in tissue culture; largest changes were seen in the rates of glycolysis and fatty acid synthesis from glucose. These increases are paralleled by an increase in pyruvate kinase activity. There was no change in the activities of the other enzymes as measured, although the net flux through all the enzymes increased. 4. Incubation of fresh adipose-tissue pieces for 2–6 h led to an increase in the affinity of pyruvate kinase for phosphoenolpyruvate. 5. The rate of pyruvate production by glycolysis was greater than the activity of pyruvate dehydrogenase of the tissue. 6. The results suggest that both pyruvate kinase and pyruvate dehydrogenase have important roles in restricting the utilization of glucose carbon for fatty acid synthesis in sheep adipose tissue.

The rate of fatty acid synthesis from glucose in ruminant adipose tissue is very low, but the mechanisms that restrict this pathway are unclear (see Vernon, 1980). The low activities of ATP citrate lyase (Hanson & Ballard, 1967) and pyruvate dehydrogenase (Robertson *et al.*, 1980) are thought to be partially responsible, but the high rates of fatty acid synthesis from lactate *in vivo* (Prior, 1978) and *in vitro* (Whitehurst *et al.*, 1978; Prior & Jacobson, 1979a,b; Smith & Prior, 1981) suggest other regulatory steps, probably during glycolysis, which restrict the utilization of glucose carbon for fatty acid synthesis. In addition, the rate of fatty acid synthesis from glucose in adipose tissue from foetal lambs is higher than in adult sheep, but the difference in pyruvate dehydrogenase activity at the two ages was insufficient to account for this, and there was no difference in the activity of ATP citrate lyase per cell (Robertson *et al.*, 1982), again suggesting regulation at other steps.

The rate of fatty acid synthesis from glucose in ruminant adipose tissue can be increased several

fold by infusion of glucose *in vivo* (Lindsay, 1970; Ballard *et al.*, 1972; Bartley, 1980) or by maintaining tissue pieces in culture (Vernon, 1979). In the present study this tissue-culture system has been used to try to elucidate further mechanisms that restrict the utilization of glucose carbon for fatty acid synthesis, particularly the regulatory steps during glycolysis.

Experimental

Animals

Sheep were either Cheviot or Finn $\times$ Dorset Horn cross-breeds. They were 7–9 months old and were fed on hay *ad libitum* plus a cereal mix (300–400 g/day, depending on age) for at least 1 month before sampling. A catheter was placed in the jugular vein and on the next day the sheep were anaesthetized and left for about 5 min before sampling (Vernon *et al.*, 1981a). Samples of perirenal adipose tissue were removed and placed immediately in liquid N_2 or sterile 0.15 M-NaCl (at

approx. 37°C). Sheep were then killed with a Captive-Bolt Humane Killer before they recovered from anaesthesia.

Tissue culture

Pieces of adipose tissue weighing about 5 mg were cut with scissors and collected in M199 medium {Medium 199 containing Earle's salts, L-glutamine and 25 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.3, from Gibco-Biocult, Paisley, Scotland, U.K., plus 60 µg of penicillin/ml, 100 µg of streptomycin sulphate/ml, 10 µg of neomycin/ml, 5 µg of amphotericin B/ml and 100 ng of insulin/ml}. The insulin used in the preliminary experiment (Table 1) was kindly donated by The Boots Co., Nottingham, U.K., and that used in other experiments was from Sigma (London) Chemical Co. Samples of tissue pieces (total weight approx. 250 mg) were transferred to sterile 250 ml Erlenmeyer flasks containing 25 ml of M199 medium. The flasks were kept in an incubator at 37°C without shaking for 24 or 48 h. This procedure was also used for the 2 h pre-incubation of the tissue.

Measurement of metabolic activities

Pieces of adipose tissue were transferred to 25 ml Erlenmeyer flasks containing 2.5 ml of M199 medium (about 10 mg of tissue/ml). Then 0.25 µCi of [U-¹⁴C]glucose, L-[U-¹⁴C]lactate, [1-¹⁴C]glucose, [2-¹⁴C]glucose, [6-¹⁴C]glucose or DL-[1-¹⁴C]lactate was added. The concentration of glucose in the medium in all cases was 5.5 mM. When [¹⁴C]lactate was used, 4 mM-L-lactate was also included in the flasks. The tissue slices were incubated for 2 h at 37°C and the incorporation of ¹⁴C into fatty acids, acylglycerol glycerol and CO₂ was determined as described previously (Vernon, 1976). Rates of incorporation of ¹⁴C were essentially linear over the 2 h period of measurement and all determinations were carried out in triplicate.

Measurement of enzyme activities

Pyruvate dehydrogenase was assayed as described previously (Robertson *et al.*, 1980), except that pyruvate and Mg²⁺ concentrations of the assay medium were increased to 3 mM and 2 mM respectively and 6 mM-pyruvate was added during the activation step. These modifications to the assay medium resulted in a 70% increase in the activity of the enzyme in the active form but had no effect on the total enzyme activity. The *p*-(*p*-aminophenylazo)benzene sulphonic acid used in the pyruvate dehydrogenase assay was kindly donated by Professor R. M. Denton. Hexokinase, glucose 6-phosphate dehydrogenase and ATP citrate lyase were assayed as described previously (Vernon, 1976; Vernon *et al.*, 1981b).

Phosphofructokinase activity was measured, with both 0.1 mM- and 3 mM-fructose 6-phosphate as substrate, by the method of Zammit & Newsholme (1976), except that antimycin A (12.5 g/ml final concentration) was used instead of cyanide to inhibit non-specific oxidation of NADH. For pyruvate kinase determination the tissue was extracted by using a buffer containing 20 mM-imidazole acetate, 5 mM-EDTA and 10 mM-mercaptoethanol (pH 6.8) and activity was measured by using either 0.05 mM-phosphoenolpyruvate or 3 mM-phosphoenolpyruvate plus 3 mM-fructose 1,6-bisphosphate as described by Pogson (1968).

Assay of metabolites

Samples of tissue slices were quickly blotted and frozen immediately in liquid N₂. The frozen tissue was ground into a powder and a 25% (w/v) homogenate was prepared in 1 M-HClO₄ (Lamprecht & Trautschold, 1974). The homogenate was centrifuged (2000 *g* for 5 min at 4°C). The fat formed a solid layer on top; the infranatant was removed, neutralized using KHCO₃ and re-centrifuged (as above). Portions of the supernatant were then assayed for: glucose and glucose 6-phosphate (Slein, 1963); phosphoenolpyruvate and 2-phosphoglycerate (Czok & Lamprecht, 1974); L-lactate (Hohorst, 1963); citrate (Dagley, 1974); ATP (Lamprecht & Trautschold, 1974); ADP and AMP (Jaworek *et al.*, 1974). Samples of medium after the 2 h incubations with [U-¹⁴C]glucose were deproteinized by using HClO₄, treated as above and assayed for glucose, lactate and pyruvate.

Measurement of tissue water

Pieces of adipose tissue weighing about 5 mg were incubated at 37°C for 15 min in M199 medium containing 0.5 µCi of [U-¹⁴C]sorbitol and 5 µCi of ³H₂O. Preliminary experiments showed that 15 min was sufficient to allow equilibration of ³H and ¹⁴C between the tissue and medium. After incubation, tissue was removed from the flasks, blotted as for measurement of metabolites, weighed and homogenized in 0.15 M-NaCl. The homogenate was centrifuged (as above) to separate the fat layer. The ³H and ¹⁴C content of the medium and the homogenate infranatant were determined and total tissue water, extracellular water (sorbitol space) and hence intracellular water were calculated (Saggerson & Greenbaum, 1970).

Determination of adipocyte volume and number of cells per g of tissue

Perirenal adipocytes from ruminants are very fragile so they were prepared as described previously (Vernon *et al.*, 1981b), that is by collagenase digestion for 2½ h without shaking. Cells were filtered through a nylon mesh (about 1 mm diameter) with

warm M199 medium. Cells were allowed to float (they were not centrifuged) and most of the infranatant was removed. About 3 ml of M199 medium was added and the cells were distributed by gentle swirling; samples (5 μ l) were transferred to a warm haemocytometer. The mean cell volume and adipocytes per g of tissue were determined (Vernon, 1977). Fat cells were maintained at about 37°C throughout these procedures; agitation was kept to a minimum and polypropylene ware was used (Ether-ton & Chung, 1981). Digestion of the tissue was essentially complete after 2½ h and cell rupture was minimal when using adipose tissue from these young sheep.

Calculation of metabolic fluxes

The net total rate of glucose utilization was calculated by summation of the rates of glucose carbon converted into fatty acid, acylglycerol glycerol, pyruvate, lactate and CO₂. It was assumed that very little glucose was converted into, or formed from, glycogen and that all lactate and pyruvate produced were formed from glucose. The rate of glucose oxidation by the pentose phosphate pathway was calculated from the rate of formation of ¹⁴CO₂ from [1-¹⁴C]glucose, [6-¹⁴C]glucose and the total rate of glucose utilization as described by Katz & Wood (1963). Recycling of fructose 6-phosphate through the pentose phosphate pathway is thought to occur (see below), so when calculating the net flux through phosphofructokinase it was assumed that one molecule of triose phosphate was formed by the pentose phosphate pathway for every 3 mol of CO₂ produced by this pathway.

Statistical analysis

Results are expressed as means $\pm$ S.E.M. Statistical analysis was performed by using Student's *t*-test for paired observations (Table 1) or by analysis of variance when results from both breeds of sheep were pooled (Tables 2, 3, 4 and 5).

Results

Results for metabolic rates and enzyme activities are expressed per 10⁶ cells in Tables 1–4; expression of the results per g of tissue led to the same conclusions (results not shown).

In a preliminary experiment using adipose tissue from Cheviot sheep, the rate of fatty acid synthesis from glucose was, on average, 9.7-fold higher after maintenance of the tissue in tissue culture for 24 h, but there was no further increase in the rate over a further 24 h (Table 1). In contrast, the rate of acylglycerol glycerol formation from glucose of the tissue appeared to be 1.7-fold higher after 24 h in tissue culture and a further 2-fold higher by 48 h but this was not statistically significant (Table 1). The rate of fatty acid synthesis from L-lactate in fresh tissue was markedly greater than that from glucose (Table 1) but it increased by only a factor of 2.4 after maintenance in tissue culture for 24 h, with no further increase by 48 h. The rate of acylglycerol glycerol formation from L-lactate and ¹⁴CO₂ production from L-[1-¹⁴C]lactate did not change over the period of culture. The actual rate of ¹⁴CO₂ production from [1-¹⁴C]lactate could not be assessed precisely due to the presence of D-[1-¹⁴C]lactate so values are not quoted in Table 1. There was no

Table 1. Rates of fatty acid and acylglycerol glycerol synthesis from glucose and L-lactate and activities of pyruvate dehydrogenase and ATP citrate lyase of pieces of perirenal adipose tissue, from 8–9 month-old Cheviot sheep, before or after being maintained in tissue culture for 24 or 48 h

The mean cell volume was 457.0 ± 145.6 pl and there were $(2.02 \pm 0.54) \times 10^6$ cells per g of tissue. Results are means $\pm$ S.E.M. for four observations. Values significantly different from that obtained before tissue culture:

P* < 0.05; *P* < 0.02; ****P* < 0.01.

	Period of culture (h) ...	0	24	48
Glucose conversion into fatty acids (μ g-atoms of carbon/ 2 h per 10 ⁶ cells)		0.02 $\pm$ 0.01	0.17 $\pm$ 0.04*	0.12 $\pm$ 0.02***
L-Lactate conversion into fatty acids (μ g-atoms of carbon/ 2 h per 10 ⁶ cells)		0.13 $\pm$ 0.05	0.31 $\pm$ 0.10*	0.20 $\pm$ 0.07
Glucose conversion into acylglycerol glycerol (μ g-atoms of carbon/ 2 h per 10 ⁶ cells)		0.35 $\pm$ 0.09	0.61 $\pm$ 0.18	1.20 $\pm$ 0.38
L-Lactate conversion into acylglycerol glycerol (μ g-atoms of carbon/ 2 h per 10 ⁶ cells)		0.08 $\pm$ 0.01	0.08 $\pm$ 0.02	0.09 $\pm$ 0.03
Pyruvate dehydrogenase (nmol/min per 10 ⁶ cells)				
Active (initial)		5.2 $\pm$ 1.3	4.6 $\pm$ 0.9	3.9 $\pm$ 0.9
Total		10.5 $\pm$ 3.2	7.7 $\pm$ 1.7	8.9 $\pm$ 2.9
ATP citrate lyase (nmol/min per 10 ⁶ cells)		5.0 $\pm$ 1.3	5.3 $\pm$ 1.0	11.1 $\pm$ 2.4**

Table 2. *Metabolic activities of pieces of sheep perirenal adipose tissue before (day 0) or after (day 1) maintenance in tissue culture for 24 h*

Sheep were either 8–9 month-old Finn × Dorset Horn sheep killed in May or 7–8 month-old Cheviot sheep killed in November. The adipocyte mean cell volumes were 770 ± 248 and 174 ± 25 pl and the numbers of cells per g were $(1.25 \pm 0.3) \times 10^6$ and $(4.32 \pm 0.74) \times 10^6$ respectively. Results are means $\pm$ s.e.m. for four observations. Results for both breeds were pooled and analysed by analysis of variance of the paired observations. Pooled values on day 1 significantly different from those obtained before tissue culture: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

		Metabolic activity (μ g-atoms of glucose carbon to products/ 2 h per 10^6 cells)				
Breed	...	Finn $\times$ Dorset Horn		Cheviot		
Time	...	Day 0	Day 1	Day 0	Day 1	<i>P</i>
CO ₂		2.70 $\pm$ 0.48	4.26 $\pm$ 0.90	0.90 $\pm$ 0.36	1.26 $\pm$ 0.42	*
Fatty acids		0.78 $\pm$ 0.36	2.22 $\pm$ 0.72	0.13 $\pm$ 0.08	0.37 $\pm$ 0.14	**
Acylglycerol glycerol		0.90 $\pm$ 0.30	1.56 $\pm$ 0.24	0.30 $\pm$ 0.06	0.36 $\pm$ 0.06	**
Pyruvate		0.78 $\pm$ 0.18	3.0 $\pm$ 0.54	0.48 $\pm$ 0.12	0.96 $\pm$ 0.18	**
Lactate		1.86 $\pm$ 0.36	6.24 $\pm$ 0.78	1.38 $\pm$ 0.24	4.20 $\pm$ 0.60	***
All products		7.02 $\pm$ 0.48	16.32 $\pm$ 1.08	3.18 $\pm$ 0.72	7.14 $\pm$ 1.26	***
CO ₂ , via pentose phosphate pathway		2.40 $\pm$ 1.08	3.42 $\pm$ 0.78	0.72 $\pm$ 0.24	0.96 $\pm$ 0.30	*

change in either pyruvate dehydrogenase (active or total) or ATP citrate lyase activity of the tissue after maintenance in tissue culture for 24 h. There was, however, a significant increase in ATP citrate lyase activity after 48 h of tissue culture (Table 1).

A further experiment was performed using Finn × Dorset Horn sheep initially and then Cheviot sheep. The former sheep were born in July and were sampled the following May, whereas the Cheviot sheep were sampled in the autumn. Adipose tissue pieces from the Cheviot sheep (fresh or after tissue culture) were pre-incubated for 2 h in M199 medium before measurement of metabolic rates, enzyme activities and metabolite concentrations. Pieces of tissue used for measurement of metabolic rates were transferred to fresh M199 medium for incubation. As the rates of glucose utilization and enzyme activities per cell of the two groups of sheep differed, pooled results were analysed by analysis of variance.

The rate of synthesis from glucose of all the various products increased significantly after maintenance of the pieces of adipose tissue in tissue culture for 24 h (Table 2). The rate of incorporation of glucose carbon into fatty acids, CO₂, acylglycerol glycerol, pyruvate and lactate was, on average, 2.8-, 1.5-, 1.5-, 2.9- and 3.2-fold higher respectively, total glucose utilization was 2.3-fold higher and the rate of oxidation via the pentose phosphate pathway was 1.4-fold higher. Glucose oxidation via the pentose phosphate pathway accounted for $81 \pm 5\%$ of CO₂ produced from glucose in adipose tissue pieces from both breeds before or after maintenance in tissue culture.

There was no change in the activities of hexokinase, glucose 6-phosphate dehydrogenase, phos-

phofructokinase, pyruvate dehydrogenase and ATP citrate lyase of the tissue slices after maintenance in tissue culture for 24 h. However, the ratio of activity of phosphofructokinase in the presence of 0.1 mM-fructose 6-phosphate/in the presence of 3 mM-fructose 6-phosphate increased significantly by 20% (Table 3). Pyruvate kinase activity measured in the presence of 0.05 mM- or 3 mM-phosphoenolpyruvate plus 3 mM-fructose 1,6-bisphosphate was 2.3- and 2.5-fold higher respectively after 24 h of tissue culture, but the ratio of activity with the two different phosphoenolpyruvate concentrations did not change (Table 3).

The net flux through all the enzyme reactions increased significantly, but not to the same extent (Table 4); the average net flux through hexokinase was 2.3-fold higher, glucose 6-phosphate dehydrogenase 1.4-fold higher, phosphofructokinase 3.3-fold higher, pyruvate kinase 2.6-fold higher, pyruvate dehydrogenase 2.5-fold higher and ATP citrate lyase 2.5-fold higher. The rate of metabolism of glucose 6-phosphate via the pentose phosphate pathway exceeded its rate of production via hexokinase (Table 4), indicating recycling of fructose 6-phosphate. This is also suggested by the ratio of the rate of ¹⁴CO₂ production from [6-¹⁴C]glucose to that from [2-¹⁴C]glucose (0.28 ± 0.06 and 0.25 ± 0.03 for adipose tissue slices, from Finn × Dorset Horn sheep, before and after maintenance in tissue culture respectively). Of the (net) triose phosphate produced by fresh adipose tissue pieces, 46% and 30% was via the pentose phosphate cycle for tissue from Finn × Dorset Horn and Cheviot sheep respectively; these proportions fell to 26% and 14% respectively after maintenance of the tissue pieces in tissue culture.

Table 3. *Enzyme activities of pieces of sheep perirenal adipose tissue before (day 0) or after (day 1) being maintained in tissue culture for 24 h*

Details of sheep, mean cell volumes and cells/g of tissue are given in Table 2. Results are means $\pm$ S.E.M. for four observations in each case. $\text{PFK}_{0.1}/\text{PFK}_3$ and $\text{PK}_{0.05}/\text{PK}_3$ are the ratios of the activities of phosphofructokinase measured using 0.1 mM- and 3 mM-fructose 6-phosphate and pyruvate kinase measured using 0.05 mM-phosphoenolpyruvate and 3 mM-phosphoenolpyruvate + 3 mM-fructose 1,6-bisphosphate respectively. Statistical analysis was performed as described in Table 2. Pooled values on day 1 significantly different from those obtained on day 0: * $P < 0.05$; ** $P < 0.01$. ns, non-significant.

		Enzyme activity (nmol/min per 10 ⁶ cells)				
Breed	...	Finn × Dorset Horn		Cheviot		
Time	...	Day 0	Day 1	Day 0	Day 1	<i>P</i>
Hexokinase		64.6 ± 22.0	56.1 ± 17.0	9.6 ± 2.3	12.2 ± 1.8	ns
Glucose 6-phosphate dehydrogenase		49.9 ± 9.9	49.5 ± 7.7	64.8 ± 18.5	58.4 ± 13.3	ns
Phosphofructokinase						
0.1 mM-Fructose 6-phosphate		92.5 ± 17.6	91.3 ± 10.1	24.3 ± 5.5	35.4 ± 9.5	ns
3 mM-Fructose 6-phosphate		178.4 ± 19.1	140.4 ± 19.7	61.6 ± 17.2	70.2 ± 14.4	ns
PFK _{0.1} /PFK ₃		0.51 ± 0.05	0.66 ± 0.06	0.46 ± 0.08	0.51 ± 0.08	*
Pyruvate kinase						
0.05 mM-Phosphoenolpyruvate		93.0 ± 13.2	231.4 ± 49.4	55.4 ± 6.6	109.2 ± 22.0	**
3 mM-Phosphoenolpyruvate + 3 mM-fructose 1,6-bisphosphate		455.6 ± 14.6	1007.7 ± 94.7	182.8 ± 40.1	504.5 ± 220.4	**
PK _{0.05} /PK ₃		0.20 ± 0.03	0.24 ± 0.07	0.33 ± 0.05	0.28 ± 0.06	ns
Pyruvate dehydrogenase						
Active		—	—	5.4 ± 1.5	4.9 ± 1.1	ns
Total		—	—	8.8 ± 1.7	7.7 ± 1.9	ns
ATP citrate lyase		—	—	4.7 ± 1.2	4.5 ± 1.1	ns

Table 4. *Net substrate fluxes through enzymes in sheep adipose tissue pieces before (day 0) or after (day 1) being maintained in tissue culture for 24 h*

Details of sheep, mean cell volumes and numbers of adipocyte/g of tissue are given in Table 2. Net fluxes are expressed as nmol of substrate converted and as percentages of the maximum fluxes, assuming these to be equal to the enzyme activities reported in Table 3, measured in the presence of saturating amounts of substrate. Results are means $\pm$ S.E.M. of four observations in each case. Statistical analysis was performed by analysis of variance as described in Table 2. Values on day 1 significantly different from those obtained before tissue culture: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. ns, non-significant.

		Net flux (nmol of substrate converted/ min per 10 ⁶ cells)					Net flux (% of maximum flux)					
Breed	...	Finn × Dorset Horn		Cheviot			Finn × Dorset Horn		Cheviot			
Time	...	Day 0	Day 1	Day 0	Day 1	<i>P</i>	Day 0	Day 1	Day 0	Day 1	<i>P</i>	
Hexokinase		9.8 ± 0.6	22.7 ± 1.5	4.4 ± 0.9	9.9 ± 1.8	***	12.8 ± 3.2	32.4 ± 4.5	46.7 ± 4.4	82.0 ± 9.4	**	
Glucose 6-phosphate dehydrogenase		20.3 ± 7.8	28.6 ± 6.7	5.9 ± 2.1	7.8 ± 2.3	*	—	—	8.6 ± 1.6	13.0 ± 1.9	*	
Phosphofructokinase		3.8 ± 2.0	13.3 ± 2.1	2.5 ± 0.4	7.8 ± 1.6	**	1.9 ± 0.8	10.6 ± 3.3	2.9 ± 0.7	14.0 ± 1.9	**	
Pyruvate kinase		10.2 ± 1.3	26.8 ± 6.2	6.0 ± 1.1	16.3 ± 2.7	**	2.2 ± 0.3	2.7 ± 0.6	3.6 ± 1.0	4.1 ± 0.7	ns	
Pyruvate dehydrogenase (active)		3.0 ± 0.8	8.5 ± 2.3	0.9 ± 0.6	2.0 ± 0.8	*	—	—	15.5 ± 8.0	36.9 ± 10.9	*	
ATP citrate lyase		3.1 ± 1.6	6.5 ± 1.7	0.5 ± 0.4	1.5 ± 0.6	*	—	—	8.3 ± 4.9	30.4 ± 8.6	*	

The rate of pyruvate production (the flux through pyruvate kinase, Table 4) was greater than the activity of pyruvate dehydrogenase (Table 3). The

net flux through the enzyme reactions as a percentage of maximum enzyme activity (as measured), with the exception of pyruvate kinase, also in-

Table 5. *Amounts of various metabolites and adenine nucleotides of pieces of sheep perirenal adipose tissue before (day 0) or after (day 1) being maintained in tissue culture for 24 h*

Details of sheep, mean cell volumes and cells/g of tissue are given in Table 2 as are details of the statistical analysis. Amounts of metabolite/g of tissue were measured before and after the tissue pieces were incubated for 2 h; as there was no significant change in the amounts/g, mean values are reported. Results are means $\pm$ S.E.M. of four observations in each case. Pooled values on day 1 significantly different from those obtained on day 0: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. ns, non-significant.

		Concentration (nmol/g of tissue)				
Breed	...	Finn × Dorset Horn		Cheviot		
Time	...	Day 0	Day 1	Day 0	Day 1	P
Glucose		897.6 ± 109.4	458.3 ± 156.5	1200.0 ± 109.3	957.0 ± 29.9	***
Glucose 6-phosphate		7.8 ± 2.4	6.6 ± 0.9	8.3 ± 0.9	9.0 ± 3.1	ns
2-Phosphoglycerate		14.0 ± 7.2	16.8 ± 6.0	6.3 ± 1.9	6.4 ± 2.4	ns
Phosphoenolpyruvate		17.0 ± 1.9	15.3 ± 3.9	14.4 ± 4.3	11.4 ± 3.0	ns
Pyruvate		51.6 ± 28.1	101.5 ± 48.7	38.1 ± 6.2	60.3 ± 8.9	*
Lactate		149.1 ± 27.9	642.4 ± 148.2	340.7 ± 24.7	718.9 ± 70.7	***
Citrate		29.9 ± 6.9	47.4 ± 12.6	19.2 ± 4.6	20.0 ± 9.0	ns
ATP		44.3 ± 12.7	93.2 ± 23.4	65.4 ± 7.9	74.0 ± 6.1	*
ADP		49.0 ± 16.1	41.1 ± 15.3	35.8 ± 2.9	29.0 ± 3.0	ns
AMP		8.1 ± 2.8	8.6 ± 4.4	13.8 ± 2.5	9.2 ± 2.2	ns

creased significantly: the net flux through hexokinase was 2.1-fold higher, glucose 6-phosphate 1.5-fold higher, phosphofructokinase 5.2-fold higher, pyruvate dehydrogenase 2.4-fold higher and ATP citrate lyase 3.7-fold higher (Table 4).

The amounts of the various metabolites per g of adipose-tissue pieces listed in Table 5, before or after maintenance in tissue culture, did not change during incubation for 2 h in M199 medium, so values reported are means of pre- and post-incubation values. The glucose content of the tissue pieces was significantly lower ($P < 0.01$) after 24 h in tissue culture, whereas the amount of pyruvate, lactate and ATP per g of tissue was 1.8-, 3.2- and 1.6-fold higher respectively. The amounts of the other metabolites measured did not change significantly during culture.

The total amount of water per g of adipose tissue ($255.1 \pm 30.3 \mu\text{l/g}$) and the amounts of intracellular ($32.4 \pm 3.9 \mu\text{l/g}$) and extracellular ($222.7 \pm 34.6 \mu\text{l/g}$) water of the tissue slices did not change during incubation of fresh tissue for 2 h and so mean values are reported; these values are for Cheviot sheep only. In addition there was no change in the total amount of water per g or its distribution after maintenance of the tissue slices in culture for 24 h (results not shown).

Values for the intracellular water were used to calculate the mean intracellular concentration of the various metabolites in adipose tissue from Cheviot sheep. The calculation makes the assumption that the concentration of the metabolite in the extracellular water is negligible or the same as

that of the incubation medium; hence values for glucose, pyruvate and L-lactate are not included. Values for tissue pieces before tissue culture were (mm): glucose 6-phosphate, 0.26 ± 0.03 ; 2-phosphoglycerate, 0.22 ± 0.09 ; phosphoenolpyruvate, 0.45 ± 0.12 ; citrate, 0.67 ± 0.23 ; ATP, 2.2 ± 0.5 ; ADP, 1.1 ± 0.15 ; AMP, 0.47 ± 0.14 (means $\pm$ S.E.M. of four observations). These values did not change significantly after maintenance of the tissue pieces in tissue culture (results not shown).

Further studies with adipose tissue from 8–9-month-old Finn $\times$ Dorset Horn sheep showed that, with the exception of pyruvate kinase, the activities of various enzymes listed in Table 3 did not change when tissue pieces (fresh or post-culture) were incubated for 2 or 4 h in M199 medium. No significant change in pyruvate kinase (measured using 3 mM-phosphoenolpyruvate plus 3 mM-fructose 1,6-bisphosphate) was found under these conditions but there was a significant increase in the activity of pyruvate kinase measured using 0.05 mM-phosphoenolpyruvate after fresh tissue was incubated for 2, 4 or 6 h (Fig. 1). There was no change in pyruvate kinase activity measured with either 0.05 mM- or 3 mM-phosphoenolpyruvate in tissue pieces incubated for a further 2 or 4 h in fresh M199 medium after tissue culture for 24 h. In addition, incubation of fresh tissue in M199 medium for 4 or 6 h resulted in a significant increase in the ratio of the activity of pyruvate kinase measured with 0.05 mM-phosphoenolpyruvate: activity measured with 3 mM-phosphoenolpyruvate plus 3 mM-fructose 1,6-bisphosphate, but after 24 h of tissue culture this ratio

Fig. 1. Pyruvate kinase activity measured using 0.05 mM-phosphoenolpyruvate (○) or 3 mM-phosphoenolpyruvate plus 3 mM-fructose 1,6-bisphosphate (□) and the ratio of these two activities ($PK_{0.05}:PK_3$, △) of sheep perirenal adipose tissue pieces before and after incubation in M199 medium for up to 24 h

The sheep were 8–9 month-old Finn × Dorset Horn breed; the mean cell volume was 487.1 ± 11.0 pl and there were $(1.56 \pm 0.13) \times 10^6$ cells per g of tissue. Results are means $\pm$ S.E.M. for four observations. Value significantly different from that at zero time: * $P < 0.05$; ** $P < 0.02$; *** $P < 0.01$.

returned to the value found with fresh tissue at zero time (Fig. 1). In addition, pre-incubation of pieces of fresh adipose tissue for 2 h in M199 medium resulted in a 25% increase in the rates of fatty acid, acylglycerol glycerol and lactate synthesis, although the increases were not significant (with the exception of acylglycerol glycerol; results not shown).

Discussion

The mean cell volume and diameter distribution profile of adipocytes from the Finn × Dorset Horn sheep were the same as those reported for Merino × Dorset Horn sheep for a similar age by using an OsO_4 fixation technique (Hood & Thornton, 1979).

The two breeds of sheep used differed in their metabolic rates and enzyme activities per adipocyte, with the cells from larger, more rapidly developing

Finn × Dorset Horn sheep having greater activities. These differences could be due to season rather than to breed as the Finn × Dorset Horn sheep were killed in May, whereas the Cheviot sheep were killed in the autumn. The proportion of glucose metabolized to the various products, however, was similar for both breeds.

Yang & Baldwin (1973) reported that 1.4, 26, 24.5 and 45% of the glucose carbon used by bovine adipocytes was converted into fatty acids, acylglycerol, CO_2 and lactate plus pyruvate respectively; conversion into other products accounted for less than 4% of the glucose used. The results from the present study with sheep adipose tissue are in general agreement with these findings. About 50% of the glucose uptake *in vivo* by the tail fat-pad of the Syrian fat-tailed sheep could be accounted for by lactate production (Khachadurian *et al.*, 1966). A high rate of lactate production and a low rate of fatty acid synthesis from glucose are characteristic of ruminant adipose tissue. In addition 80% of the glucose oxidation to CO_2 occurred via the pentose phosphate pathway (confirming previous speculation; see Vernon, 1980). The estimated rate of NADPH production from glucose oxidation via the pentose phosphate pathway in the adipose pieces exceeded the requirement of NADPH for fatty acid synthesis from glucose; this apparent discrepancy is accounted for by concurrent fatty acid synthesis from acetate, the major lipogenic precursor in ruminant adipose tissue (see Vernon, 1980), for the M199 medium contained 0.6 mM-acetate. There is also considerable recycling of fructose 6-phosphate through the pentose phosphate cycle as suggested for bovine adipose tissue from the relative rates of $^{14}CO_2$ production from $[6-^{14}C]$ - and $[2-^{14}C]$ -glucose (Baldwin *et al.*, 1973). The pattern of glucose metabolism in ruminant adipose tissue thus differs markedly from that of the rat (Flatt & Ball, 1964, 1966; Saggerson & Greenbaum, 1970; Zaragoza-Hermans, 1973, 1974).

The low activities of pyruvate dehydrogenase and ATP citrate lyase are in agreement with previous reports (see Vernon, 1980; Robertson *et al.*, 1980). The greater proportion of pyruvate dehydrogenase in the active form compared with that of our previous report is due to a modification of the assay conditions (see the Experimental section). The hexokinase activity is also low in adipose tissue from Cheviot sheep as noted previously (Vernon, 1976).

The amount of total water, extracellular water and intracellular water per g of adipose tissue have not previously been reported for a ruminant. The values, however, were similar to those reported for rat adipose tissue (Crawford & Renold, 1965; Denton *et al.*, 1966; Saggerson & Greenbaum, 1970). In addition, the amounts of most metabolites on a per-g-of-tissue basis were similar to those found in

rat (Denton *et al.*, 1966; Saggerson & Greenbaum, 1970; Ballard, 1972) and also in bovine adipose tissue (Baldwin *et al.*, 1969). However the phosphoenolpyruvate concentration of bovine (Baldwin *et al.*, 1969) and ovine adipose tissue appears to be markedly greater than that of rat adipose tissue (Denton *et al.*, 1979). The glucose levels in tissue removed from anaesthetized sheep and frozen immediately in liquid N₂ (321.8 ± 50.9 nmol/g for Cheviot sheep and 188.2 ± 38.2 nmol/g for Finn $\times$ Dorset Horn sheep) are similar to those of rat (Ballard, 1972) and steer (Smith & Prior, 1981); the glucose concentration rose during incubation, probably due to the high concentration of glucose (5.5 mM) in the medium compared with sheep blood (see Leng, 1970). The concentrations of adenine nucleotides and the ratios of their concentrations were similar to those reported for rat adipose tissue (Denton *et al.*, 1966; Ballard, 1972).

In all experiments, maintenance of sheep adipose tissue in tissue culture for 24 h resulted in an increase in the rate of total glucose utilization. The rate of glucose metabolism to the various products did not all increase to the same extent, the largest (proportionate) increase being in the rate of glycolysis and fatty acid synthesis from glucose. The rate of glucose oxidation via the pentose phosphate pathway showed the smallest (proportionate) increase but this is probably determined by changes in the rate of fatty acid synthesis from acetate and this increases by only 50% or so under such conditions (Vernon, 1979).

For adipose tissue from Cheviot sheep, the net rate of glucose utilization rose to 83% of the measured hexokinase activity after 24 h of tissue culture. The low hexokinase activity may prevent much further increase in glucose utilization in adipose tissue from this breed, as observed in the preliminary experiment (Table 1).

The increased flux through phosphofructokinase was not associated with any change in the activity of the enzyme as measured, but there was an increase in the ratio of activity measured at the two fructose 6-phosphate concentrations, suggesting a change in the affinity of the enzyme for this substrate. Phosphofructokinase activity is modulated by a variety of allosteric effectors, including ATP, AMP, citrate, fructose 1,6-bisphosphate and fructose 6-phosphate (see Newsholme & Start, 1973). The mean concentration of AMP and citrate in the cell did not change, whereas that of ATP (an inhibitor) increased, suggesting that it is changes in the concentration of other effectors, such as fructose 2,6-bisphosphate (Van Schaftingen & Hers, 1980; Pilkis *et al.*, 1981), that are responsible.

The activity of pyruvate kinase changed during both short-term and prolonged incubation, and the changes appeared to parallel the change in the rate

of glycolysis. During short-term incubation there appeared to be an increase in the affinity for phosphoenolpyruvate as indicated by the increase in the ratio of pyruvate kinase activity with 0.05 mM-phosphoenolpyruvate/that with 3 mM-phosphoenolpyruvate (Fig. 1) as previously observed in epididymal adipose tissue from rats (Denton *et al.*, 1979). After incubation for 24 h, however, the affinity for phosphoenolpyruvate returned to that observed at zero time, but the activity had increased; whether this is due to an increase in the amount of enzyme or to a further change in its properties is not yet clear. The increase in pyruvate kinase activity during this period paralleled the increase in the rate of glycolysis, suggesting that pyruvate kinase has an important role in controlling the glycolytic flux in sheep adipose tissue.

Most pyruvate produced by the tissue was released as lactate and little was further metabolized through pyruvate dehydrogenase. The activity of this enzyme, as measured, was not sufficient to meet the rate of pyruvate production. However, more pyruvate could have been converted into acetyl-CoA than was observed, suggesting that allosteric effectors are probably modulating the activity of pyruvate dehydrogenase in the tissue, as is also suggested by the observation that the flux through the enzyme increases during tissue culture, whereas there was no apparent change in the activity. The concentration of pyruvate within the cell would also seem to be important. The higher rates of fatty acid synthesis from glucose observed after tissue culture are associated with a higher pyruvate concentration, and incubation of the tissue with 4 mM-L-lactate, which is likely to increase the intracellular pyruvate concentration, resulted in a higher flux of carbon to fatty acids than was observed with glucose. These observations are consistent with our previous suggestion that pyruvate dehydrogenase has an important role in limiting the further metabolism of glucose in sheep adipose tissue (Robertson *et al.*, 1980) as previously shown for rat adipose tissue (see Denton, 1975; Randle, 1978).

Another possibility that has yet to be explored in sheep adipose tissue is that pyruvate translocase of the mitochondria could be rate-limiting. An inhibitor (α -cyanohydroxycinnamate) of the pyruvate translocase markedly inhibited fatty acid synthesis from lactate in bovine adipose tissue (Prior *et al.*, 1981).

The view that a low ATP citrate lyase activity is responsible for the low rate of fatty acid synthesis from glucose in ruminant adipose tissue has been challenged recently for various reasons (Prior & Jacobson, 1979a; Vernon, 1980; Smith & Prior, 1981; Whitehurst *et al.*, 1981). The results of the first experiment (Table 1) suggest that changes in ATP citrate lyase activity follow changes in the rate

of fatty acid synthesis from glucose, as previously shown for the rat (Foster & Srere, 1968).

The mean ATP concentration increased during 24 h in tissue culture, suggesting that the tissue is being maintained satisfactorily during tissue culture.

The low rate of fatty acid synthesis from glucose is probably due to restrictions at several steps in the pathway. The rate appears to be linked to the rate of glycolysis, which in turn seems to be regulated by pyruvate kinase. A major control point at pyruvate kinase rather than at an earlier point in glycolysis perhaps reflects the fact that glucose is the major source of glycerol 3-phosphate for esterification and that glucose oxidation via the pentose phosphate pathway probably supplies the majority of the NADPH required for fatty acid synthesis in ruminant adipose tissue (see Vernon, 1980). In addition, the results indicate an important role for pyruvate dehydrogenase in limiting the further metabolism of pyruvate to acetyl-CoA and ensuring that most of the pyruvate produced is released from the cell as lactate.

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