

Interactions between propionate and amino acid metabolism in isolated sheep hepatocytes

BY C. DEMIGNÉ, C. YACOUR, C. MORAND AND C. RÉMÉSY

INRA Laboratoire des Maladies Métaboliques, Centre de Recherches, Theix-F-63122, Ceyrat, France

(Received 9 February 1990 – Accepted 13 July 1990)

The purpose of the present study was to evaluate the contribution of various substrates to glucose synthesis in isolated sheep hepatocytes, and more specifically to quantify the contribution of propionate to gluconeogenesis. Liver cells from fed sheep have a very high capacity for propionate utilization and conversion into glucose. The glucogenicity of lactate or amino acids was very low in hepatocytes from fed sheep, but was significantly increased in hepatocytes from starved animals. Amino acids such as alanine or glutamine were characterized by a substantial utilization towards ureogenesis, whereas their conversion to glucose was very low. Propionate utilization and conversion into glucose was inhibited by butyrate, ammonia and especially ethanol (by up to 80%). Ethanol promoted a striking accumulation of intracellular malate in hepatocytes incubated with propionate (reaching 14.9 $\mu\text{mol/g}$ cell) and led to a depletion of phosphoenolpyruvate; ethanol inhibition could be counteracted by pyruvate. Propionate and butyrate enhanced ureogenesis from ammonia in ruminant liver cells but their effects were not additive. Propionate also elicited a marked increase in cellular concentrations of phosphoserine and serine, particularly in the presence of ammonia; such effects could influence phospholipid metabolism in the liver. These findings emphasize the contribution of propionate, compared with the other glucogenic substrates, to glucose synthesis in ruminants and point to the possibilities of modulation of the glucogenicity of propionate by various substrates which may be present in portal blood.

Gluconeogenesis: Hepatocytes: Propionate: Amino acids: Sheep

Ruminants generally absorb only small amounts of glucose from the alimentary tract and, thus, rely on hepatic gluconeogenesis for much of their glucose supply. The chief precursor of glucose in fed animals is propionate, which arises essentially from microbial fermentation in the rumen. The rate of glucose synthesis is highest in fed animals, decreasing during fasting (Bergman *et al.* 1966). This contrasts with the non-ruminant, where fasting stimulates gluconeogenesis. In vivo, propionate uptake by the liver is generally quantitative but its metabolic fate may be modified by other substrates (e.g. ammonia, short-chain or long-chain fatty acids) which could limit the extent of its conversion to glucose (Weekes *et al.* 1978; Demigné *et al.* 1986; Faulkner & Pollock, 1986; Aiello & Armentano, 1987). In turn, propionate may alter the rate of various metabolic pathways, especially ureogenesis or ketogenesis (Bush & Milligan, 1971; Rattenbury *et al.* 1983; Emmanuel & Kenelly, 1984; Demigné *et al.* 1986).

The pathway for glucose synthesis from propionate is well-established, but little is known of the control mechanisms which regulate this pathway in the ruminant liver. This point is important since the actual contribution of propionate to the overall synthesis of glucose in ruminants is still a matter of controversy: frequently, relatively low values (approximately 40%) have been proposed and, as a result, some investigations did not account for all the glucose produced by the liver (Bergman *et al.* 1966; Judson *et al.* 1968; Wiltout & Satter, 1972; Reynolds & Huntington, 1988). Quantification of the amount of glucose carbon derived from propionate involves various radiolabelled intermediates (particularly in the

tricarboxylic acid cycle) which may be incompletely channelled towards gluconeogenesis. On the other hand, propionate-C atoms can also be replaced by C from other sources. Furthermore, it is conceivable that various metabolic factors may also affect, positively or negatively, propionate glucogenicity.

To address this problem, and to further investigate the effects of propionate on various metabolic pathways in the liver (ureogenesis, ketogenesis), the present study was carried out on isolated hepatocytes from sheep. This model affords estimation of the glucogenicity of the substrates under simplified metabolic conditions and is particularly useful for studies on the impact of substrates on intracellular metabolism.

MATERIAL AND METHODS

Preparation of isolated hepatocytes

Limousine sheep (6 months old) weighing about 50 kg were used; the animals were offered hay *ad lib.* plus 0.5 kg/d of concentrate (g/kg: barley 400, maize 300, soya seed cake 250, and minerals). Male Wistar rats (IFFA-CREDO, L'Arbresle, France), each weighing about 180 g, were used in some experiments for comparative purposes. They were fed *ad lib.* on commercial pellets (AO3, UAR, Villemoisson/Orge, France) containing (g/kg): digestible protein 208, carbohydrate (essentially starch) 504, lipid 50 and cellulose 34.

Sheep hepatocytes were prepared from portions of liver (about 30 g, on the edge of the median lobe) removed immediately after slaughter in a local abattoir. The portal branch was cannulated, and the liver sample was perfused (by gravity using a portable infusion device) with a Ca^{2+} -free Krebs buffer containing 0.2 mM-EGTA at 38°, and transferred to the laboratory perfusion system (Masterflex pump, Cole Palmer, Chicago, IL, USA) within about 3 min. All the subsequent steps of hepatocyte isolation from rat and sheep liver were similar, essentially as described by Krebs *et al.* (1974), except for (1) perfusion flow (about 3 ml/g liver) and amounts of collagenase (*EC* 3.4.24.7) used (about 0.5 units/g liver) that were in proportion to the weight of the liver sample, and (2) a two-step collagenase perfusion was used for sheep hepatocytes (step 1, 10 min; step 2, about 20 min). The cells were first separated from undigested debris by filtration through a 0.2 mm nylon mesh and were collected by centrifugation at 100 g for 90 s.

Cell viability estimated by cell membrane refractoriness in phase-contrast microscopy or Trypan Blue exclusion, was over 95% for both rat and sheep hepatocytes. Incubations were performed under agitation at 37° in a 27 mM-bicarbonate Krebs-Henseleit buffer, pH 7.4 (Krebs *et al.* 1974) containing 20 g albumin (Boehringer, fraction V)/l and cells were continuously gassed with carbogen (oxygen-carbon dioxide; 95:5, v/v).

Metabolic studies

Hepatocyte suspensions (3 ml; 20–25 mg fresh weight/ml) were taken in 10-ml glass Erlenmeyer flasks containing the substrates under study, and incubated at 37° with gassing by carbogen. At the required intervals, portions (0.5 ml) were withdrawn and immediately centrifuged (8000 g, 10 s) and the supernatant fraction subsequently analysed. For measurements of intracellular metabolites, two procedures were used on separate incubations: (1) 2 ml cell suspension were centrifuged (8000 g, 10 s) and, after careful removal of the supernatant fraction, the tubes were immediately plunged in liquid nitrogen until further processing (for ATP, glucose-6-phosphate, phosphoenolpyruvate (PEP), malate and oxaloacetate), or (2) 500 μl cell suspension were layered over 1500 μl ice-cold Krebs buffer, centrifuged and treated as described later (p. 303), for determination of cellular amino acid.

Laboratory procedures

Glucose, L-lactate, pyruvate, malate, oxaloacetate, glucose-6-phosphate, PEP, ATP and ammonia were determined spectrophotometrically on neutralized (potassium carbonate) supernatant fractions of perchloric extracts of blood or plasma (1 vol:2 vol 0.6 M-perchloric acid) or of cell pellets (120 μ l 0.4 M-HClO₄ for 40 mg pellet) using standard enzymic methods (Siess *et al.* 1977). Urea was determined by a colorimetric method (Marsh *et al.* 1965) and propionate by gas-liquid chromatography after ethanolic extraction of the samples (Rémésy & Demigné, 1974). Protein concentrations were measured using the bicinchoninic acid (BCA) protein reagent (Smith *et al.* 1985).

Glucose formation was measured using either unlabelled substrates (taking into account glucose release consecutive to glycogenolysis) or using labelled precursors. Labelled substrates are used (1) when there is an endogenous production of the precursor (L-lactate), (2) for substrates with a low glucogenicity which is difficult to detect by the chemical measurement of glucose (alanine, glutamine), or (3) for propionate in the presence of effectors which could distinctly affect its rate of utilization or conversion to glucose. The conversion of ¹⁴C-labelled glucogenic substrates into glucose was estimated after deproteinization of a 500 μ l supernatant fraction by 165 μ l 1.2 M-HClO₄, then neutralization by 165 μ l 0.75 M-K₂CO₃. The supernatant fraction was then applied to microcolumns of Dowex AG1-X8 (100–200 mesh, acetate form) for incubations with [2-¹⁴C]propionate or [U-¹⁴C]lactate, or of Dowex AG50W-X2 (100–200 mesh, H⁺ form) for incubations with [U-¹⁴C]alanine or [U-¹⁴C]glutamine. The columns were eluted four times each with 600 μ l deionized water, and the neutral fraction counted in InstaGel (Packard, Rungis, France).

Amino acids were determined after deproteinization of the samples by sulphosalicylic acid (50 g/l) containing α -amino adipic acid as an internal standard. The separation and the quantification of amino acids were performed by high performance liquid chromatography using lithium buffers on a Chromakon 500 (Kontron, Zürich, Switzerland) fitted with a 4.6 \times 250 mm MCI Gel column (Mitsubishi, Tokyo, Japan) using post-column derivatization by ninhydrin.

Glutaminase (EC 3.5.1.2) activity was measured on intact mitochondria isolated from samples of liver, as previously described (Rémésy *et al.* 1988). Glutamine hydrolysis was determined according to Zaleski *et al.* (1986) in a medium containing various concentrations of L-glutamine from 0 to 20 mM.

Materials

[2-¹⁴C]propionate was purchased from Amersham International plc (Amersham, Bucks, UK) and [U-¹⁴C]lactate, [U-¹⁴C]alanine and [U-¹⁴C]glutamine were from the CEA (Gif/Yvette, France). Collagenase (H grade), bovine serum albumin (fraction V), enzymes and coenzymes were obtained from Boehringer (Meylan, France) and Dowex resins from Biorad (Richmond, CA, USA). The BCA protein reagent test was from Pierce (Interchim, Montluçon, France). Other reagents were commercially available products of the best analytical grade.

Statistics

The means for given variables were tested using a one-way analysis of variance. If a statistically significant result was obtained ($P < 0.05$) *a priori* contrasts of the groups were tested with least significance differences (LSD). For experiments with only two conditions, the Student's *t* test was used (Snedecor & Cochran, 1967). In all statistical tests we have chose to reject the null hypothesis at the 5% level ($P < 0.05$).

Table 1. *Gluconeogenesis from various substrates* in sheep and rat hepatocytes*†
(Mean values with their standard errors for no. of animals shown in parentheses; each assay was carried out in triplicate)

	Glucose released ($\mu\text{mol}/\text{min}$ per g cell)					
	Sheep				Rat	
	Fed (5)‡		72 h-starved (3)		24 h-starved (5)	
	Mean	SEM	Mean	SEM	Mean	SEM
Propionate	0.73	0.06	0.48	0.03	0.30	0.03
Pyruvate	0.25	0.03	0.30	0.02	0.60	0.06
Lactate	0.03	0.01	0.18	0.02	0.70	0.06
Glycerol	0.15	0.03	0.17	0.02	0.65	0.05
Dihydroxyacetone	0.39	0.04		nd	1.01	0.09
Fructose	0.60	0.05		nd	1.44	0.12
Sorbitol	0.25	0.03		nd		nd
Alanine	0.05	0.01	0.07	0.02	0.40	0.03
Glutamine	0.04	0.01	0.13	0.02	0.14	0.02

nd, Not determined.

* Initial concentration 2.5 mM.

† For details of procedures, see p. 303.

‡ In these assays glycogenolysis was high, thus, glucose flux from propionate, lactate, alanine and glutamine were assessed in parallel using ^{14}C -labelled substrates.

RESULTS

Gluconeogenesis from propionate and other glucogenic substrates

The substrates studied were generally used at the concentration of 2.5 mM, rather than the concentrations of 5–10 mM frequently encountered, in order to limit some artifacts (for instance: ATP or free CoA depletion, maximal rate of metabolism with poor responsiveness to effectors, etc). To obviate the risks of extensive depletion of substrates under some conditions (incubations with 1.25 mM-propionate, for example), sampling was carried out at short intervals (every 10 min) to check the linearity of the metabolic process. Nevertheless, the substrate concentrations were generally higher than the physiological concentrations (except for free fatty acids): portal vein propionate is 0.2–0.5 mM, L-lactate 0.4–1.0 mM, alanine 0.15 mM and glutamine about 0.2 mM (Bergman *et al.* 1966; Baird *et al.* 1980; Heitmann & Bergman, 1980; Reynolds & Huntington, 1988).

Glucose production by isolated sheep hepatocytes has been compared in fed or 72 h-starved sheep, and in 24 h-starved rats (Table 1). In sheep, propionate was the most effective glucogenic substrate (glucose flux, up to 1 $\mu\text{mol}/\text{min}$ per g wet weight cell) but this flux was lower during starvation (–34%). There was practically no gluconeogenesis from lactate in hepatocytes from fed sheep, but this process was detectable in hepatocytes from starved animals. Nevertheless, gluconeogenesis from lactate never represented more than one-third of gluconeogenesis from propionate and, in all conditions, lactate glucogenicity was much lower in sheep than in rat liver cells. In contrast, there was a noticeable conversion of pyruvate into glucose in hepatocytes from fed sheep (also lower than with rat hepatocytes). The glucogenicity of glycerol was low in ruminant hepatocytes, and dihydroxyacetone was more efficiently utilized than glycerol in both rat and sheep hepatocytes. Although they are not physiological substrates, fructose and sorbitol were efficiently converted to glucose by ruminant liver cells. Gluconeogenesis from alanine and

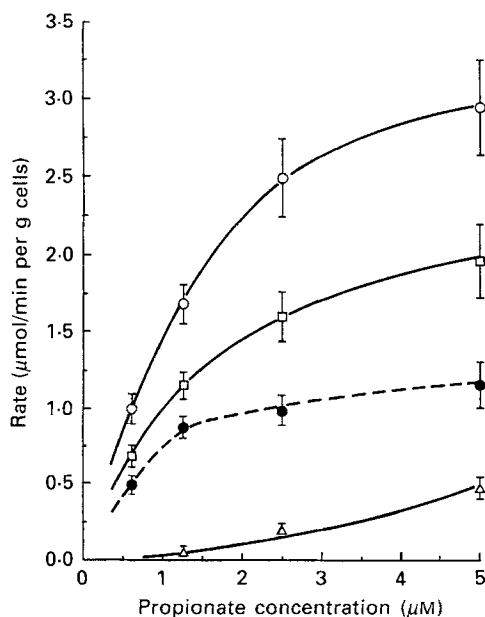


Fig. 1. Relationships between propionate concentration and the rate of propionate utilization (\circ — \circ) and of glucose (\square — \square) and L-lactate production (\triangle — \triangle) by sheep hepatocytes. For comparison, the rate of propionate utilization in rat hepatocytes is presented (\bullet — \bullet). The rate of glucose production is expressed as equivalent to three-carbon units produced/min per g cells. Values are means with their standard errors represented by vertical bars for four animals.

glutamine was very low in sheep hepatocytes whereas, for the same concentrations, alanine was effectively glucogenic in rat liver cells. There was a relatively more efficient conversion of glutamine into glucose (close to that of lactate or glycerol) in hepatocytes from starved sheep.

Fig. 1 shows the relationship between extracellular propionate concentrations and its rate of utilization and conversion to glucose. Hepatocytes from fed sheep had a very high capacity for propionate utilization (up to $3 \mu\text{mol}/\text{min}$ per g cell at 5 mM) and for subsequent glucose synthesis. However, the percentage incorporation into glucose of propionate utilized was lower with increasing concentrations (80% at 0.62 mM v. 66% at 5 mM) and a significant release of lactate could be observed for propionate concentrations higher than 2 mM .

Factors affecting gluconeogenesis from propionate

Ammonia and butyrate are continuously absorbed from the rumen and, under certain circumstances, detectable amounts of ethanol may also be present in the portal vein. As shown in Table 2, ethanol turned out to be a potent inhibitor of propionate utilization and, to a higher degree, of gluconeogenesis (inhibition up to 75–80%). The inhibitory effects were detectable at low concentrations of ethanol, in the range of 1 mM . Furthermore, the percentage of inhibition by ethanol was as high at 2.5 mM as that at 1.25 mM propionate (values not shown). Low concentrations of ammonia (1 mM) did not affect propionate utilization, but ammonia was inhibitory at higher concentrations, conversion to glucose being particularly affected (–43% at 4 mM -ammonia). As previously reported (Demigné *et al.* 1986), butyrate is an effective inhibitor of propionate utilization and conversion to

Table 2. *Effects of ethanol, ammonia and butyrate on the utilization of propionate and on the conversion of 1.25 mM-2[¹⁴C]propionate into glucose by sheep hepatocytes*†

(Mean values with their standard errors for three animals (Expt 1) or two animals (Expt 2) in which each assay was carried out in triplicate)

		Propionate utilization‡			[¹⁴ C]glucose synthesized		
		Flux (μ mol/min per g cells)		Inhibition (%)	Flux (μ mol/min per g cells)		Inhibition (%)
		Mean	SEM		Mean	SEM	
Expt 1	None	1.55	0.12	—	0.61	0.05	—
Ethanol (mM):	1.25	0.98*	0.08	37	0.27*	0.03	56
	2.50	0.83*	0.08	46	0.21*	0.03	66
	5.00	0.62*	0.05	60	0.15*	0.03	75
Ammonia (mM):	1.25	1.42	0.11	8	0.60	0.06	≈ 0
	2.50	1.39	0.12	10	0.45*	0.04	26
	5.00	1.09*	0.09	30	0.35*	0.04	43
Butyrate (mM):	1.25	0.96*	0.08	48	0.29*	0.04	52
	2.50	0.70*	0.07	55	0.25*	0.04	61
SED (residual df)		0.17 (18)			0.08 (18)		
Expt 2	None	1.45	0.13	—	0.64	0.06	—
Ethanol (mM):	2.50	0.86*	0.07	50	0.18*	0.03	72
Ammonia (mM):	2.50	1.55	0.11	20	0.58	0.06	< 10
Pyruvate (mM):	2.50	1.35	0.11	30	0.58	0.05	< 10
Ethanol 2.50 mM + pyruvate 2.50 mM		1.43†	0.15	0	0.66†	0.04	0
Ethanol 2.50 mM + ammonia 2.50 mM		1.25†	0.09	14	0.30*†	0.04	50
SED (residual df)		0.18 (6)			0.12 (6)		

* Mean values were significantly different from those for hepatocytes incubated with propionate alone ($P < 0.05$).† Mean values were significantly different from those for hepatocytes incubated with ethanol ($P < 0.05$).

‡ For details of procedures, see p. 303.

§ Propionate utilization was measured by gas-liquid chromatography.

glucose. In Expt 2, the effects of substrates which could thwart the effect of ethanol via changes in cytosolic or mitochondrial redox state (namely pyruvate and ammonia) were investigated. In this experiment, neither 2.5 mM-ammonia nor 2.5 mM-pyruvate inhibited propionate metabolism. Pyruvate completely abolished the strong inhibitory effect of ethanol (72% inhibition with ethanol alone) on gluconeogenesis from propionate. Ammonia restored a rate of propionate utilization that was not significantly different from the control value; however, glucose production was still markedly depressed (–50%).

Table 3 presents the interactions between pyruvate, lactate and propionate metabolism. Lactate itself did not affect propionate metabolism (results not shown). The interferences between the pyruvate-yielding substrates (lactate, alanine, pyruvate) and propionate were investigated using pyruvate itself. As reported previously, pyruvate did not affect propionate metabolism, whereas pyruvate utilization was strongly reduced by propionate. Furthermore, under such conditions, most of the pyruvate used could be accounted for by lactate released in the incubation medium. Pyruvate uptake by ruminant hepatocytes was enhanced by even-chain fatty acids, resulting chiefly in lactate release (butyrate) or in glucose release (oleate). In contrast to propionate, pyruvate utilization was dramatically

Table 3. *Pyruvate metabolism and interactions with propionate, ethanol and fatty acids in isolated sheep hepatocytes*†

(Mean values with their standard errors for two animals in which each assay was carried out in triplicate)

	Flux ($\mu\text{mol}/\text{min}$ per g cells)							
	Propionate utilization		Pyruvate utilization		Glucose production		Lactate production	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Pyruvate 2 mM								
None	—	—	0.68	0.04	0.20	0.02	0.25	0.03
Propionate 2 mM	1.98	0.17	0.27*	0.03	0.63*	0.06	0.40	0.04
Butyrate 2 mM	—	—	1.31*	0.15	0.25	0.03	0.62	0.05
Oleate 1 mM	—	—	1.12*	0.10	0.41*	0.04	0.33	0.03
Ethanol 2 mM	—	—	2.15*	0.30	0.22	0.03	1.14	0.15
Propionate 2 mM								
None	—	0.24	—	—	0.65	0.05	0.18	0.02
Ethanol 2 mM	1.02†	0.13	—	—	0.24†	0.03	0.52†	0.05
Ethanol 2 mM + pyruvate 2 mM	1.83	0.16	0.80	0.06	0.59*	0.05	0.90*†	0.10
SED (residual df)	0.24 (4)		0.23 (6)		0.07 (8)		0.09 (8)	

* Mean values were significantly different from those for hepatocytes with pyruvate alone ($P < 0.05$).† Mean values were significantly different from those for hepatocytes with propionate alone ($P < 0.05$).

‡ For details of procedures, see p. 303.

enhanced by ethanol, together with lactate release, whilst glucose production was unchanged.

Table 4 presents the concentrations of intracellular metabolites involved in propionate metabolism. There was a trend to decreased intracellular ATP in the presence of propionate: 1.84 (SEM 0.23) v. 2.54 (SEM 0.33) $\mu\text{mol}/\text{g}$ cell in controls ($P = 0.09$). ATP was significantly depleted ($P < 0.05$) in the presence of propionate plus ammonia. Propionate metabolism brought about a considerable increase in the concentrations of malate and oxaloacetate in ruminant hepatocytes. The probable increase in the cellular redox status by ethanol amplified this phenomenon, leading to a large accumulation of intracellular malate (up to about 14.9 $\mu\text{mol}/\text{g}$ cell) and to a concomitant drop of oxaloacetate. In contrast, ammonia rather counteracted malate accumulation. Aspartate present in high concentrations (6–7 $\mu\text{mol}/\text{g}$ cell) under basal conditions was poorly responsive to substrates, except to the addition of propionate plus ammonia. Glutamate was also present in high concentrations (3–5 $\mu\text{mol}/\text{g}$ cell); it was not affected by propionate alone but markedly increased (up to 10–15 $\mu\text{mol}/\text{g}$ cell) on the addition of ethanol or ammonia to propionate. The changes in PEP concentrations were approximately parallel to those of gluconeogenesis from propionate under most conditions, except in the presence of ethanol which elicited a drastic depletion of PEP whereas gluconeogenesis was not completely inhibited. Changes in glucose-6-phosphate concentrations essentially mirrored those of gluconeogenesis from propionate.

Interactions among gluconeogenesis, ureogenesis and amino acid metabolism

In ruminant hepatocytes, ureogenesis from ammonia levelled off at a relatively low ammonia concentration (about 2 mM); furthermore, ureogenesis was not responsive to the addition of 0.25 mM-ornithine in the medium (results not shown). Table 5 shows that

Table 4. *Effect of propionate, ammonia and ethanol on the intracellular concentrations of ATP and metabolites involved in gluconeogenesis†*

(Mean value with their standard errors for three fed sheep in which each assay was carried out in triplicate)

Metabolites ($\mu\text{mol/g}$ cells)	Control		Propionate 2.5 mM		Ethanol 2.5 mM		Propionate 2.5 mM + ethanol 2.5 mM		Ammonia 2.5 mM		Propionate 2.5 mM + ammonia 2.5 mM		SED (residual df)
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	
ATP	2.54	0.33	1.84	0.23	2.48	0.40	2.04	0.28	2.13	0.32	1.44*	0.20	0.48 (12)
Malate	0.10	0.01	4.31*	0.55	0.38*	0.04	14.90**†	1.67	0.09	0.02	2.87*	0.41	—
Oxaloacetate		0.01 <	0.24	0.04	0.01 <		0.05**†	0.02					—
Aspartate	6.28	0.41	6.46	0.50	6.48	0.45	7.05	0.54	7.06	0.55	12.10**†	1.38	0.82 (12)
Glutamate	3.42	0.29	3.52	0.31	5.05*	0.49	10.51**†	0.96	3.48	0.29	15.22**†	1.66	0.95 (12)
Phosphoenolpyruvate	0.07	0.02	0.21*	0.03	0.04	0.01	0.05†	0.01	0.06	0.01	0.13†	0.03	0.04 (12)
Glucose-6-phosphate	0.09	0.02	0.19*	0.03	0.07	0.02	0.13†	0.02	0.09	0.02	0.17*	0.02	0.04 (12)

* Mean values were significantly different from those for hepatocytes incubated without added substrate (control conditions) ($P < 0.05$).

† Mean values were significantly different from those for hepatocytes incubated with propionate ($P < 0.05$).

‡ For details of procedures, see p. 303.

Table 5. *Ureogenesis from ammonia in the presence of various substrates in hepatocytes from fed and starved sheep†*

(Mean values with their standard errors for three animals in which each assay was carried out in triplicate)

Addition	Fed		72 h-starved	
	Mean	SEM	Mean	SEM
Ammonia 1.5 mM				
None	0.41	0.03	0.25	0.03
Lactate 2.5 mM	0.45	0.03	0.56*	0.05
Pyruvate 2.5 mM	0.50	0.05	nd	
Propionate 2.5 mM	0.65*	0.06	0.49*	0.04
Butyrate 2.5 mM	0.77*	0.06	0.42*	0.03
Propionate 2.5 mM + butyrate 2.5 mM	0.80*	0.07	0.45*	0.04
Oleate 1 mM	0.64*	0.05	0.42*	0.04
Octanoate 2 mM	0.71*	0.06	nd	
SED (residual df):	0.09 (12)		0.05 (12)	

nd, not determined.

* Mean values were significantly different from those for hepatocytes with 1.5 mM-ammonia (NH₄Cl) alone ($P < 0.05$).

† For details of procedures, see p. 303.

propionate activated ureogenesis in vitro, as did butyrate, octanoate and oleate. Lactate and pyruvate were ineffective. In hepatocytes from starved sheep, basal ureogenesis was lower but, besides propionate and even-chain fatty acids, lactate was also stimulatory.

Ammonia in the incubation medium led to an increase in the intracellular concentrations of some amino acids, especially glutamate and alanine (Table 6). Propionate elicited a rise in the concentrations of serine and, to a lesser extent, of phosphoserine. In the presence of propionate and ammonia, there was an accumulation of glutamate and aspartate and a dramatic increase in phosphoserine and serine (together with an increase in glycine and alanine concentrations). On the addition of butyrate, there was an increase in glutamate and glycine, which was accentuated by the addition of ammonia. Modifications in phosphoserine and serine concentrations were less apparent than in the presence of propionate. Lactate did not affect intracellular amino acids but, in the presence of ammonia, lactate led to a noticeable rise of glutamate and alanine (values not shown).

When sheep hepatocytes were incubated in the presence of amino acids at near-physiological concentrations (Table 7), there was a noticeable release of urea (0.26 $\mu\text{mol}/\text{min}$ per cell) which appeared to be dissociated from gluconeogenesis (0.05 $\mu\text{mol}/\text{min}$ per cell). The most readily utilized amino acids were glutamine, alanine, serine and, to a lesser extent, aromatic amino acids (not shown), glycine and threonine. It must be noted that glutamine was significantly utilized by ruminant hepatocytes at low extracellular concentrations (namely 0.5 mM), in contrast to rat hepatocytes. In parallel, a net production of glutamate could be observed. Compared with values obtained with rat hepatocytes (Fafournoux *et al.* 1983; Rémésy *et al.* 1988), glutamine accumulation into ruminant liver cells was quite limited, together with relatively high basal concentrations of ornithine (about 0.30 $\mu\text{mol}/\text{g}$). Most other glucogenic amino acids such as alanine, serine and glycine were concentrated into sheep liver cells (about 10-fold for alanine or glycine). In the presence of propionate in the medium, the intracellular concentrations of phosphoserine and serine were dramatically increased, up to 2.09 and 3.30 $\mu\text{mol}/\text{g}$ cell respectively. Serine uptake by hepatocytes was depressed by 70% accordingly. Fur-

Table 6. *Effects of volatile fatty acids, ammonia, lactate, alanine and glutamine on the intracellular concentrations ($\mu\text{mol/g cells}$) of amino acids*[†]

(Mean values with their standard errors for three fed sheep in which each assay was carried out in triplicate)

	Phosphoserine		Aspartate		Serine		Glutamate		Glutamine		Glycine		Alanine	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Control	0.24	0.03	6.32	0.41	0.15	0.02	3.41	0.22	nd	nd	2.39	0.30	0.80	0.10
Alanine 1.25 mM	0.87*	0.10	6.20	0.49	0.70*	0.09	5.15*	0.62	nd	nd	2.31	0.28	10.76*	1.34
Glutamine 1.25 mM	0.30	0.04	6.57	0.39	0.39	0.05	12.53*	1.95	5.07	0.77	2.22	0.36	1.15	0.12
Ammonia (Amm) 1.5 mM	0.30	0.04	6.74	0.42	0.23	0.03	4.22	0.31	nd	nd	3.08	0.25	1.28	0.13
Propionate (Pro) 2.5 mM	0.42	0.05	6.90	0.44	1.11*	0.13	3.58	0.30	nd	nd	2.68	0.22	0.92	0.12
Butyrate (But) 2.5 mM	0.30	0.03	6.67	0.45	0.25	0.03	4.56*	0.37	nd	nd	3.98*	0.40	0.74	0.10
Pro 2.5 mM + Amm 1.5 mM	2.05*†	0.36	13.10*†	0.84	1.74*†	0.19	12.23*†	1.56	0.37	0.05	3.08	0.31	1.50*†	0.15
But 2.5 mM + Amm 1.5 mM	0.42	0.04	6.80	0.44	0.38	0.04	12.88*	1.66	nd	nd	4.55*	0.32	1.03	0.14
Pro 2.5 mM + But 2.5 mM + Amm 1.5 mM	1.46*†	0.19	12.45*†	1.33	1.50*	0.17	21.47*†	2.12	0.18	0.03	3.02	0.39	1.26	0.14
SED (residual df)	0.21 (18)		0.82 (18)		0.14 (18)		1.59 (18)				0.40 (18)		0.58 (18)	

nd, not detectable (< 0.1 mM).

* Mean values were significantly different from those for hepatocytes in control conditions ($P < 0.05$).

† Mean values were significantly different from those for hepatocytes incubated with 2.5 mM propionate ($P < 0.05$).

‡ For details of procedures, see p. 303.

Table 7. Gluconeogenesis, ureogenesis and amino acid utilization in sheep hepatocytes: effect of propionate†
(Mean values with their standard errors for three animals in which each assay was carried out in triplicate)

	Control						+ Propionate 2.5 mM					
	Extracellular concentration (mM)		Net flux (nmol/min per g cells)		Intracellular concentration (μmol/g cells)		Net flux (nmol/min per g cells)		Intracellular concentration (μmol/g cells)		SEM	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Urea	—											
Glucose	—		+260	30	—	—	+250	40	—	—	—	—
Phosphoserine	0		+50	10	0.23	0.03	+740*	80	2.09*	0.33	8.07	0.81
Taurine	0		—	—	7.80	0.69	—	—	8.40*	0.77	—	—
Aspartate	0		—	—	5.52	0.70	—	—	—	—	—	—
Threonine	0.25		-12	3	0.71	0.05	-10	3	0.92	0.06	—	—
Serine	0.25		-64	5	1.34	0.10	-19*	3	3.28*	0.45	—	—
Glutamate	0		+77	6	4.90	0.57	+90	8	4.52	0.50	—	—
Glutamine	0.50		-171	10	1.90	0.23	-165	12	2.47	0.32	—	—
Glycine	0.50		-35	4	5.10	0.66	-30	3	6.95*	0.69	—	—
Alanine	0.50		-175	13	4.50	0.48	-170	11	6.51*	0.56	—	—

* Mean values were significantly different from those for hepatocytes incubated in control conditions (in the presence of the amino acid mixture) ($P < 0.05$).

† For details of procedures, see p. 303.

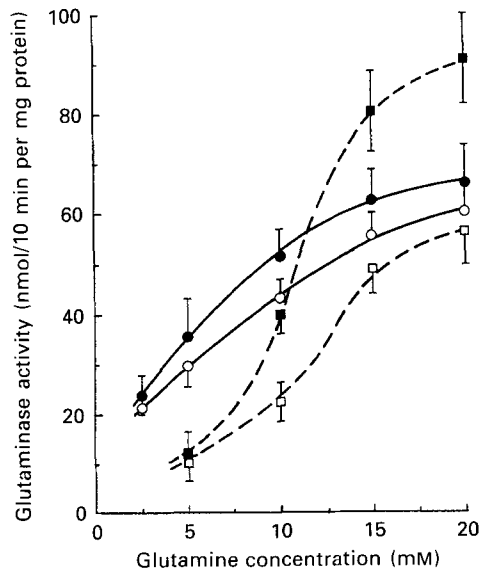


Fig. 2. Glutaminase (*EC* 3.5.1.2) activity in isolated mitochondria from sheep (—) and rat (---) liver. Values are means with their standard errors represented by vertical bars for three animals in which the assays were carried out in triplicate. Glutamine hydrolysis was measured with (●, ■) or without (○, □) the addition of 0.5 mM-ammonium chloride at pH 7.4 (corresponding to a concentration of the unionized ammonia form of 15 μ M).

thermore, propionate enhanced the intracellular concentrations of glycine and alanine, but without altering their net uptake.

The relationship between glutaminase activity and glutamine concentrations in the medium is presented in Fig. 2, for mitochondria isolated from sheep and rat liver. The greatest activity was observed in mitochondria from rats, but the activity at low concentrations of glutamine (2.5–5.0 mM) was higher in sheep mitochondria. Ammonia (equivalent to 15 μ M) strikingly enhanced glutaminase activity in rat, but not in sheep, mitochondria. The reduction of the incubation medium pH to 7.0 depressed glutaminase activity in rat mitochondria as well as, although to a lesser extent, in sheep mitochondria (results not presented).

DISCUSSION

The flux of propionate determined with comparable concentrations of propionate (about 0.5 mM) appears very similar *in vivo* and *in vitro*, in the range of 0.4–0.8 μ mol/min per g liver tissue or g liver cells (Bergmann *et al.* 1966; Judson *et al.* 1968; Baird *et al.* 1980; Gill *et al.* 1985; Demigné *et al.* 1988; Veenhuizen *et al.* 1988). Nevertheless, it must be kept in mind that a variety of factors, including hormones, are present *in vivo* and may modulate the utilization of the various substrates. Compared with ruminants, rat hepatocytes have a lower capacity of propionate uptake and metabolism but they exhibit higher capacities of butyrate utilization (Demigné *et al.* 1986). It appears that gluconeogenesis from propionate is markedly depressed in hepatocytes from starved ruminants; this may reflect the decrease in propionyl-CoA carboxylase (*EC* 4.1.1.41) activity reported by Baird & Young (1975); in addition, propionyl-CoA synthetase (*EC* 6.2.1.17) could be responsive to changes in the availability of propionate (Wahle *et al.* 1981).

Hepatocytes isolated from fed sheep failed to utilize lactate (present results; Harmon

et al. 1984); a peculiarity also reported *in vivo* (Huntington *et al.* 1981; Naylor *et al.* 1984). The fact that pyruvate is more efficiently utilized than lactate suggests that the L-lactate dehydrogenase (*EC* 1.1.1.27) step could be rate-limiting for gluconeogenesis from lactate. In sheep, L-lactate dehydrogenase activity is relatively low (160 nmol/min per mg 15000 g supernatant protein against about 1000 nmol/min per mg in the rat; Smith & Osborne-White, 1971); furthermore, the redox status in the cytosol could be unfavourable to lactate oxidation (Herriman & Heitzman, 1978). Another controlling step for gluconeogenesis from pyruvate-yielding substrates is pyruvate carboxylase (*EC* 6.4.1.1), which has a low activity in growing ruminants (Smith & Walsh, 1982). Pyruvate carboxylase has been reported to be induced during pregnancy or lactation (Smith & Walsh, 1982). It is noteworthy that oleate was very effective in channelling pyruvate towards glucose synthesis, apparently at the expense of lactate formation. A part of the PEP produced could be removed from the glucogenic pathway by pyruvate kinase (*EC* 2.7.1.40), since its activity is relatively high in sheep liver (Vernon *et al.* 1987), but the actual degree of activation, by dephosphorylation during hepatocyte isolation (Riou *et al.* 1985), is still poorly known in the ruminant. The rate of gluconeogenesis from glycerol, dihydroxyacetone and fructose was relatively low in sheep liver cells: since the overall activity of gluconeogenesis from C₃ precursors is practically as high in sheep as in the rat, this could be ascribed to a lower activation of the previously mentioned substrates by sheep hepatocytes, possibly at the level of the specific kinases. In contrast to glucose, ruminant liver cells metabolized some fructose or sorbitol, which could be relevant for some clinical purposes (as antiketogenic agents).

Propionate metabolism is not dependent on pyruvate carboxylase but is dependent on mitochondrial PEP carboxykinase (*EC* 4.1.1.49), which is very active in ruminants (Ballard *et al.* 1969; Smith & Osborne-White, 1971). Our results suggest that propionate might increase the NADH:NAD ratio in hepatocytes, which results in an accumulation of malate in ruminant hepatocytes. It must be noted that 2.5 mM-propionate brought about a dramatic rise in both malate and oxaloacetate concentrations which suggests that the overall process of PEP synthesis may become rate-limiting. In the presence of high concentrations of propionate, the production of lactate could be mediated either by malic enzyme (of low activity in ruminants, Vernon *et al.* 1987) or by pyruvate kinase.

In vitro, a large part of the propionate is converted to glucose (65–80%, depending on propionate concentration). Similarly, Aiello & Armentano (1987) found that the glucose:CO₂ ratio obtained from 2-[¹⁴C]propionate was generally higher than 10. This efficient conversion of propionate to glucose may reflect the fact that this requires only 4 ATP, against 6 ATP for lactate and 10 ATP for alanine. *In vivo*, a lower conversion of propionate to glucose might reflect reducing redox conditions prevailing in sheep liver mitochondria.

It appears that propionate utilization by sheep hepatocytes is inhibited by several metabolites: ethanol, butyrate and ammonia. Ethanol is the most potent inhibitor, effective at concentrations as low as 1 mM. This observation has some agronomic relevance since ruminants may consume on occasions silages containing up to 40 g ethanol/kg dry matter. A large part escapes bacterial metabolism and is absorbed; information about ethanol concentration in the portal vein is still lacking but it may be assumed that concentrations in the range of 1–2 mM could be attained with such silages. The inhibitory effect of ethanol could be strengthened by the fact that propionate itself leads to malate accumulation. That ethanol inhibition is mediated by a rise in the cellular NADH:NAD ratio is supported by the restoration of propionate utilization on the addition of pyruvate. The fact that gluconeogenesis is affected more than propionate utilization itself raises the question of the metabolic fate of propionate-C escaping gluconeogenesis. A large part is likely to be

transferred to organic acids, including lactate. This is reminiscent of the effect of ethanol on pyruvate metabolism.

An inhibitory effect of butyrate on gluconeogenesis from propionate has been previously reported (Demigné *et al.* 1986; Aiello & Armentano, 1987) and contrasts with the activating effects of butyrate on pyruvate utilization. Butyrate is unlikely to act via an enhanced supply of energy since octanoate or oleate had no effect on propionate metabolism (results not shown). Butyrate may compete with propionate for activation to acyl-CoA in the mitochondria (Ricks & Cook, 1981), but propionate metabolism could also be inhibited by butyrate at a site independent of the activation reaction (Aiello & Armentano, 1987). Some other short-chain fatty acids (isobutyrate, valerate, 2-methylbutyrate) may, on the other hand, channel propionate-C towards gluconeogenesis rather than to oxidation (Aiello *et al.* 1989). Furthermore, propionate is a potent inhibitor of pyruvate utilization (present results; Smith, 1971), possibly by inhibition of pyruvate carboxylase by the various CoA-esters appearing during propionate metabolism (propionyl CoA, methylmalonyl-CoA, succinyl CoA).

Inhibitory effects of ammonia on propionate metabolism require relatively high concentrations (> 2 mM) which bring about a marked depletion of cellular ATP together with an accumulation of aspartate and glutamate, probably at the expense of oxaloacetate and 2-oxoglutarate. In contrast, amino acids did not inhibit gluconeogenesis from propionate. Stimulation of ureogenesis by propionate, butyrate or fatty acids may arise from energy provision or from alterations of some intermediates of the urea cycle. Propionate stimulated ureogenesis from ammonia (but not from amino acids) whilst cellular ATP was depleted. Furthermore, propionate, butyrate and fatty acids (values not presented) considerably enhanced the intracellular concentrations of glutamate (and aspartate with propionate). These results contrast with the inhibitory effects of propionate on ureogenesis reported in the rat (Cathelineau *et al.* 1979) and in sheep (Rattenbury *et al.* 1983).

The concomitant availability of large amounts of amino groups and of organic acids may lead to the synthesis of various amino acids, as shown in the rat (Brosnan & Williamson, 1974). However, there are particular effects of propionate in ruminants, especially the concomitant rise in the cellular concentrations of phosphoserine and serine. In the rat, the biosynthesis of serine is generally coupled to a high glycolytic activity (along with induction of certain key-enzymes such as 3-phosphoglycerate dehydrogenase (*EC* 1.1.1.95) and phosphoserine aminotransferase (*EC* 2.6.1.52); (Snell, 1980). In contrast, serine synthesis in ruminant hepatocytes seems to accompany a high rate of gluconeogenesis (high concentrations of PEP); however, a possible effect of propionate on serine catabolism could not be ruled out. It is noteworthy that the synthesis of phosphoserine and serine also depends on the availability of the NH₂ group, whether provided by ammonia or amino acids (alanine for instance). The physiological significance of this process is still to be ascertained, however it is established that serine is involved in the synthesis of various classes of phospholipids and recent works shows that the biosynthesis of long-chain bases (such as sphingosine) is stimulated by a high availability of serine (Messmer *et al.* 1989). This feature could provide an explanation of the antisteatotic effects of propionate in ruminants.

Sheep hepatocytes can catabolize certain amino acids with, however, some peculiarities compared with the rat. In hepatocytes from fed ruminants, there is a similar utilization of alanine and glutamine, whereas in rat hepatocytes alanine is more efficiently utilized than glutamine (Lund & Watford, 1976; Hayes & McGivan, 1982; Rémésy *et al.* 1988). The rate of alanine or glutamine utilization plateaued (about 0.3 $\mu\text{mol}/\text{min}$ per g cell) at extracellular concentrations of about 1 mM. It is noteworthy that glutamine (which yields, like

propionate, an intermediate of the Krebs cycle) is not efficiently glucogenic: for a rate of glutamine utilization of $0.35 \mu\text{mol}/\text{min}$ per g cell at 2.5 mM , only $0.04 \mu\text{mol}/\text{min}$ per g cell glucose was produced in vitro. This process appears more efficient during starvation. This low conversion of glutamine into glucose could reflect the fact that gluconeogenesis, when coupled to ureogenesis, is an energy-consuming process. However, addition of fatty acids failed to stimulate gluconeogenesis from glutamine or alanine (results not shown). Another explanation is that glutamine may give rise to other amino acids, especially glutamate; glutamate accumulates in liver cells incubated in the presence of glutamine, and a part may be released in the external medium. Both glutamine catabolism and glutaminase activity are relatively high in ruminant liver cells under basal conditions, namely in the absence of effectors of glutamine catabolism. Furthermore, it appears that ammonia has little effect on glutamine metabolism in ruminant hepatocytes whereas it is a potent activator of glutaminolysis in rat liver cells (Lund & Watford, 1976).

Ruminant hepatocytes are also characterized by high intracellular concentrations of glutamate, aspartate and glycine. When hepatocytes were incubated in the presence of glutamine, a considerable accumulation of glutamate was observed whereas that of glutamine was quite limited, in contrast with the rat (Rémésy *et al.* 1988). Under such conditions (or when intracellular glutamate is elevated in the presence of ammonia + propionate + butyrate), there is a net release of glutamate by ruminant liver cells, a phenomenon also observed in vivo (Heitmann & Bergman, 1980). As suggested previously, glutamate release by liver cells may lessen the contribution of certain amino acids to gluconeogenesis. Glycine accumulation is also a characteristic feature of ruminant hepatocytes. Such an accumulation has been observed in blood plasma and in the liver of ketotic ewes (Demigné *et al.* 1988).

In conclusion, the present work emphasizes the high capacity of sheep hepatocytes to metabolize propionate (chiefly towards glucose), compared with liver cells of simple-stomached species. However, it appears that propionate metabolism is subject to inhibition by various metabolites present in portal blood (butyrate, ammonia, occasionally ethanol) and that there may be complex interactions between these effectors. Changes in the redox status of the cytosolic or mitochondrial compartments are likely to modulate the conversion of propionate to glucose, which seems rather depressed under conditions of elevated NADH:NAD ratios. In turn, it appears that propionate is a potent effector of a variety of metabolic processes including not only gluconeogenesis, ureogenesis and ketogenesis but also the metabolism of certain amino acids such as phosphoserine and serine.

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