

The pathway of ketogenesis in rumen epithelium of the sheep

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1. A method for the fractionation of sheep rumen epithelium with limited mitochondrial breakage is described. 2. The distributions of the enzymes of the 3-hydroxy-3-methylglutaryl-CoA pathway of ketogenesis indicate that this process is exclusively mitochondrial. Enzyme activities are sufficient to account for the ketogenic rates found *in vivo*. The failure of (–)-hydroxycitrate to block ketogenic flux supports this view. 3. 3-Hydroxybutyrate dehydrogenase activity is largely associated with particulate material in the mitochondrial fraction. 4. ATP citrate lyase activity was found, with appreciable acetoacetyl-CoA thiolase and 3-hydroxy-3-methylglutaryl-CoA synthase in the cytoplasmic fraction.

Ketogenesis in ruminants occurs in both liver and rumen epithelium (Pennington, 1952; Hird & Weidemann, 1964; Goosen, 1976); the latter tissue may be quantitatively more significant in the fed state (Leng & West, 1969).

In theory, acetoacetate can be formed from acetoacetyl-CoA by direct deacylation or through the longer pathway involving 3-hydroxy-3-methylglutaryl-CoA. Baird *et al.* (1970) measured the activities of enzymes of these two pathways in bovine rumen epithelium and showed clearly that the 3-hydroxy-3-methylglutaryl-CoA pathway was present and sufficiently active to generate ketone bodies at the known rates. However, the intracellular distribution of the ketogenic enzymes was not unambiguously established by these workers. Of the 3-hydroxy-3-methylglutaryl-CoA synthase (EC 4.1.3.5) and 3-hydroxy-3-methylglutaryl-CoA lyase (EC 4.1.3.4) activities, 74% appeared to be cytoplasmic, but there was some evidence of a considerable degree of mitochondrial breakage. Watson & Lindsay (1972) also reported finding these activities, together with 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) activity, in the cytoplasmic fraction from sheep rumen epithelium; again, however, mitochondrial breakage could have been a significant factor.

The possibility of a cytoplasmic pathway for ketogenesis in rat liver (particularly in the fed state) was raised by Brunengraber *et al.* (1978). More systematic studies have, however, cast some doubt on this proposal (Endemann *et al.*, 1982) and support the established view that ketogenesis may be wholly a mitochondrial process. Nevertheless, because ketone body synthesis in the rumen epi-

thelium is more significant in the fed than in the starved animal, and in view of the previous uncertainties, we have critically re-examined the problem of the intracellular location of ketogenic enzymes in the rumen wall.

This paper describes a method for the fractionation of rumen epithelium with limited mitochondrial damage. The results are consistent with ketogenesis proceeding exclusively through the mitochondrial pathway.

Materials and methods

Materials

Chemicals and biochemicals were from sources given previously (Smith & Pogson, 1980; Smith *et al.*, 1981) or from standard suppliers. 3-Hydroxy-3-methylglutaryl-CoA was synthesized by the method of Goldfarb & Pitot (1971). (–)-Hydroxycitrate was a gift from Dr. A.C. Sullivan, Hoffman La Roche Inc., Nutley, NJ, U.S.A.

Preparation of extracts

Rumens were obtained from sheep slaughtered at a local abattoir. The contents were removed and the rumen was washed in buffer (Krebs & Henseleit, 1932) at room temperature, before being transported to the laboratory (approx. 30 min). The papillae exceeding 5 mm in length were removed with a sharp blade. They were then washed in buffer, blotted and homogenized. Papillae were added to 2–3 vol. of extraction buffer [0.25 M-sucrose/10 mM-Mops(4-morpholinepropanesulphonic acid)/3 mM-EDTA, pH 7.0 at 0°C]; they were homogenized with an Ystral homogenizer ('Polytron-type') (Ystral GmbH,

Ballrechten-Dottingen, Germany, through the Scientific Instrument Centre, Liverpool, U.K.) for 30s at low speed (setting 3–4) followed by 10s at higher speed (setting 7–8). The homogenate was then filtered through nylon (150 μm mesh); filtrate and washings were diluted with extraction buffer to give a final suspension of 1g/10ml. The homogenate was centrifuged at 600g for 10 min at 4°C (Beckman J2-21 centrifuge). The resulting supernatant was recentrifuged at 40000g for 15 min at 4°C; the pellet was designated the 'mitochondrial fraction', the new supernatant the 'cytoplasmic fraction'. The mitochondrial pellet was resuspended in 5ml of extraction buffer containing 0.2% (w/v) Triton X-100; a sample of the original homogenate was diluted 1:1 with the same solution. Both fractions were then passed through three cycles of freezing in liquid N₂ followed by thawing at 37°C, before being centrifuged at 15000g for 5 min at 4°C (Sarstedt microfuge). All samples were kept on ice before assay.

Preliminary experiments revealed that the homogenization gave full extraction of all activities and minimized mitochondrial breakage. Experiments with Potter–Elvehjem homogenizers and digitonin-containing buffers were markedly less successful.

Enzyme assays

Enzymes were assayed at 30°C by the following methods: ATP citrate lyase (EC 4.1.3.8), Srere (1959) as modified by Stanley (1980); citrate synthase (EC 4.1.3.7), Srere *et al.* (1963); glutamate dehydrogenase (EC 1.4.1.3), Longshaw *et al.* (1972); lactate dehydrogenase (EC 1.1.1.27), Longshaw *et al.* (1972).

Acetoacetyl-CoA thiolase (acetyl-CoA acetyltransferase, EC 2.3.1.9) in gel-filtered extracts without K⁺ was assayed in the presence of 50 mM-KCl as described by Middleton (1973). The intracellular distribution was calculated from these assays and assays in which Na⁺ replaced K⁺ with the formula devised by Middleton (1973).

3-Hydroxy-3-methylglutaryl-CoA lyase was assayed as described by Stegink & Coon (1968) as modified by Kramer & Mizioroko (1980) and discussed by Barth (1978).

3-Hydroxy-3-methylglutaryl-CoA synthase was assayed by two methods. The first, used for crude extracts by Clinkenbeard *et al.* (1975) and Patel & Clark (1978), gave the same results as the second, that of Williamson *et al.* (1968), which measures the formation of acetoacetate. In the latter case, the presence of excess lyase was ensured by addition of crude mitochondrial extracts; appropriate controls were performed throughout. Succinyl-CoA:3-oxoacid CoA-transferase (EC 2.8.3.5) and acetoacetyl-CoA hydrolase ('deacylase'; EC 3.1.2.11) were assayed essentially as described by Tisdale &

Brennan (1983) with acetoacetyl-CoA as substrate; the molar absorption coefficient for acetoacetyl-CoA at 303 nm was taken as $20.5 \times 10^3 \text{ litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (Fenselau & Wallis, 1974).

Soluble 3-hydroxybutyrate dehydrogenase was assayed in the direction of 3-hydroxybutyrate formation as described by Watson & Lindsay (1972). Activities of the same enzyme in suspensions were measured as outlined by Chandrasena *et al.* (1979) in the same direction; the formation of 3-hydroxybutyrate was measured enzymically in neutralized extracts after termination of incubations (5 min) with HClO₄.

Tissue incubations

Pieces of rumen epithelium were treated and incubated at 39°C (Pennington, 1952; Smith *et al.*, 1978). Ketone bodies in neutralized extracts were assayed by standard procedures (Mellanby & Williamson, 1974; Williamson & Mellanby, 1974).

Results and discussion

Rumen epithelium preparations contain associated bacteria even after extensive washing (McCowan *et al.*, 1978); at least a proportion of these have penetrated between the cells (Rahman & Decker, 1966). It has, however, been shown that the degree of contamination of epithelial cell extracts with bacterial enzymes is quantitatively negligible (Lenartova *et al.*, 1981; Bush, 1982). We have therefore disregarded this factor throughout.

Values for the activities of lactate and glutamate dehydrogenase were similar to those reported elsewhere (Table 1; Watson & Lindsay, 1972; Ash & Baird, 1973; Weekes, 1974; Bush, 1982). The homogenization procedure, which gave recoveries of better than 90% for all enzymes, resulted in 80% of the lactate dehydrogenase activity being in the cytoplasmic fraction; only 9% of the glutamate dehydrogenase was found as a contaminant. Measurement of the citrate synthase in the two compartments revealed the expected similarity in the distribution of this enzyme and glutamate dehydrogenase (Table 1). The apparent degree of mitochondrial breakage was therefore considerably less than in previous studies (Baird *et al.*, 1970; Watson & Lindsay, 1972).

Appreciable activities of enzymes of the 3-hydroxy-3-methylglutaryl-CoA pathway were found in homogenates. These are considerably higher than activities found for acetoacetyl-CoA hydrolase (Baird *et al.*, 1970; not detectable in the present study) and are similar to those of succinyl-CoA:3-oxoacid CoA-transferase (Bush & Milligan, 1971; Emmanuel & Milligan, 1983; Table 1). Table 1 shows that, after correction for cross-contamination of fractions (as judged from lactate dehydrogenase and glutamate dehydrogenase distributions), 3-

Table 1. *Enzyme activities in sheep rumen epithelium*

Tissue was homogenized and fractionated as described in the Materials and methods section. Enzyme activities were measured at 30°C; results are means \pm s.e.m. with the number of independent observations shown in parentheses. All percentage values have been corrected for cross-contamination of fractions; it was assumed that lactate and glutamate dehydrogenases are respectively exclusively cytoplasmic and mitochondrial.

Enzyme	Total activity in homogenate ($\mu\text{mol}/\text{min}$ per g wet wt. of tissue)	Percentage of total activity in:	
		Cytoplasmic fraction	Mitochondrial fraction
Lactate dehydrogenase	56.7 \pm 4.6 (15)	(100)	(0)
Glutamate dehydrogenase	0.85 \pm 0.08 (14)	(0)	(100)
Citrate synthase	2.36 \pm 0.54 (3)	5.0 \pm 2.5	104.4 \pm 4.7
Acetyl-CoA thiolase	2.84 \pm 0.30 (9)	34.1 \pm 7.0	65.5 \pm 6.9
3-Hydroxy-3-methylglutaryl-CoA synthase	0.22 \pm 0.04 (3)	43.2 \pm 12.9	56.8 \pm 12.7
3-Hydroxy-3-methylglutaryl-CoA lyase	0.88 \pm 0.19 (3)	4.4 \pm 1.6	95.6 \pm 9.9
3-Hydroxybutyrate dehydrogenase			
(a) Soluble	0.10 \pm 0.02 (3)	5.4 \pm 0.8	94.6 \pm 9.0
(b) Total	1.61 \pm 0.28 (3)	4.4 \pm 0.7	95.6 \pm 3.3
ATP citrate lyase	0.12 \pm 0.01 (5)	90.7 \pm 2.8	9.3 \pm 2.7
Succinyl-CoA : 3-oxoacid CoA-transferase	0.40 \pm 0.11 (3)	11.8 \pm 1.1	88.4 \pm 1.9

hydroxy-3-methylglutaryl-CoA lyase was, within experimental error, exclusively mitochondrial. Some activity of acetoacetyl-CoA thiolase and 3-hydroxy-3-methylglutaryl-CoA synthase was found in the cytoplasmic fraction. The activity of acetoacetyl-CoA thiolase was measured at 10 μM -acetoacetyl-CoA for reasons discussed in detail by Middleton (1973). It is difficult to assess maximal thiolase activities in crude extracts, but assay at 50 μM substrate (Williamson *et al.*, 1968) yielded values of 3.81 \pm 0.59 $\mu\text{mol}/\text{min}$ per g wet wt. of tissue (mean \pm s.e.m. from three observations).

The activity of the synthase is surprisingly low. That in the mitochondrial compartment is only just sufficient to account for the maximal rates of ketogenesis *in vitro*; this raises the possibility that this enzyme may play a role in regulating ketogenic flux in this tissue. ATP citrate lyase activity is relatively low but measurable (as also noted by Young *et al.*, 1969). A pathway may therefore exist whereby acetyl units generated from butyrate in the mitochondria can be converted to cytoplasmic 3-hydroxy-3-methylglutaryl-CoA. This would be consistent with a low rate of synthesis of lipid in the tissue (B. Leighton, unpublished work).

We have confirmed the observation of Patel & Clark (1978) that 3-hydroxy-3-methylglutaryl-CoA synthase may be assayed as suggested by Clinkenbeard *et al.* (1975), at least in these crude extracts. We have not, however, found any change in enzyme activity with variation in Mg^{2+} concentration (0–20 mM), unlike Clinkenbeard *et al.* (1975) but in agreement with results for the chicken liver enzyme (Allred, 1973).

The apparent activity of 3-hydroxybutyrate dehydrogenase in soluble extracts of rumen epi-

thelium is very low (Table 1). This has stimulated some speculation about the role of this enzyme in ketogenesis (Watson & Lindsay, 1972; Bush, 1982). In our experiments, measurement in whole homogenates gave much greater activity; this was associated with the particulate mitochondrial fraction. Watson & Lindsay (1972) reported a rate of production of 3-hydroxybutyrate in fed sheep of about 0.5 $\mu\text{mol}/\text{min}$ per g of mucosa. This would be entirely consistent with our present values for 3-hydroxybutyrate dehydrogenase activity, especially when the effects of temperature are taken into consideration. Similar values for the activity of this enzyme have been recorded elsewhere (Weekes, 1974; Chandrasena *et al.*, 1979).

The enzyme distributions shown in Table 1 are as predicted by the hypothesis that ketogenesis may proceed in rumen epithelium by a pathway similar to that in liver, although some contribution by the succinyl-CoA : 3-oxoacid CoA-transferase is not unlikely; the importance of the recently described butyrate:acetoacetyl-CoA transferase should also be considered (Emmanuel & Milligan, 1983). The absence of any significant cytoplasmic component was emphasized by experiments with (–)-hydroxycitrate, an inhibitor of ATP citrate lyase (Watson *et al.*, 1969); this compound should inhibit ketogenesis if ATP citrate lyase plays any function in the process. No such inhibition was observed; the rates of ketogenesis (in $\mu\text{mol}/\text{h}$ per g dry wt. of tissue) were 11.6, 112 and 110 (averages of two independent observations) respectively for incubations without substrate, with 12 mM-butyrates and with butyrate plus 2 mM-(–)-hydroxycitrate. The ratio of 3-hydroxybutyrate to acetoacetate was also unaffected.

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