3-Hydroxybutyrate Dehydrogenase in Tissues from Normal and Ketonaemic Sheep

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1. In liver, rumen epithelium and kidney cortex of the sheep, a dehydrogenase active against DL-3-hydroxybutyrate occurred in both the cytosol and particulate fractions of the tissues. In brain, heart, skeletal and smooth muscles, the enzyme occurred only in the particulate fraction. 2. Enzyme activity in the cytoplasmic fraction of liver and rumen epithelium was similar with either D(-)-3-hydroxybutyrate or DL-3-hydroxybutyrate, but was less with acetoacetate as the substrate. The cytosol fraction of kidney cortex showed very little activity with D(-)-3-hydroxybutyrate, confirming that most of the activity with DL-3-hydroxybutyrate was with the L(+) isomer in this tissue. 3. 3-Hydroxybutyrate dehydrogenase activities in the cytosol and particulate fractions of liver, rumen epithelium and kidney cortex and in the particulate fraction of brain tissue were not stimulated by phosphatidylcholine, unlike the enzyme in sheep muscle and in tissues of other species. 4. The activity of 3-hydroxybutyrate dehydrogenase was not increased significantly in any of the tissues of ketonaemic sheep. 5. Comparison of rates of 3-hydroxybutyrate production *in vivo* with the enzyme activity in ketogenic tissue suggested that in sheep the maximum rate of production might be limited by this activity.

D(-)-3-Hydroxybutyrate-NAD oxidoreductase (EC 1.1.1.30) catalyses the reversible NAD-linked oxidation of 3-hydroxybutyrate to acetoacetate. Lehninger *et al.* (1960) showed that it was present in many rat tissues. In the mitochondria of rat liver (Gotterer, 1967) and ox heart (Sekuzu *et al.*, 1961) the enzyme is tightly bound to the mitochondrial membrane, and when freed from this membrane has a requirement for phosphatidylcholine. However, Neilson & Fleischer (1970) found very low activity in sheep liver mitochondria, whereas Koundakjian & Snoswell (1970) found appreciable activity in the cytoplasmic fraction of sheep liver and kidney. In sheep kidney, this 'cytoplasmic 3-hydroxybutyrate dehydrogenase' has been shown to react readily with L(+)-3-hydroxybutyrate (Williamson & Kuenzel, 1971) and these authors have suggested that this enzyme is identical with L-gulonate-NAD oxidoreductase (EC 1.1.1.45).

It is generally supposed (see, e.g., Wieland, 1968) that D(-)-3-hydroxybutyrate dehydrogenase is essential for the last step in the production and the first step in the utilization of D(-)-3-hydroxybutyrate. Leng & Annison (1964) showed that there was substantial production of D(-)-3-hydroxybutyrate in

sheep and that it was readily oxidized by a number of tissues. Katz & Bergman (1969) showed that it was produced in fed sheep mainly by the tissues of the gastrointestinal tract (especially the rumen mucosa) and in starved sheep mainly by the liver. They also showed that in underfed pregnant ketonaemic sheep there was a substantial increase in production of 3-hydroxybutyrate by the liver.

In view of the metabolic significance of 3-hydroxybutyrate for sheep, we have measured the activity, distribution and substrate specificity of 3-hydroxybutyrate dehydrogenase in a number of sheep tissues. In addition, since Smith *et al.* (1969) found that there was an increase in 3-hydroxybutyrate dehydrogenase activity in the brain of ketonaemic rats, and Neilson & Fleischer (1970) postulated an increase in this enzyme in the liver of ketonaemic sheep, we have also measured the enzyme activity in the tissue of ketonaemic sheep.

Methods

Two groups of Merino ewes were used: (a) non-pregnant sheep given 800 g of lucerne once daily; these sheep were killed 1 h after feeding; (b) pregnant sheep given a sub-maintenance daily ratio of 250 g of lucerne+250 g of wheat chaff; these sheep were starved for 24 h immediately before slaughter, which

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occurred at about 130 days gestation (10 days before parturition). Clun Forest wethers were used for the specificity studies shown in Table 2. These animals were fed on 500g of chaffed hay and 150g of oats once daily.

Sheep were killed by exsanguination and severing of the spinal cord. Tissues were removed from the animals within 2min of death and placed in ice-cold 0.25M-sucrose. Portions were minced and homogenized by hand by using an all-glass Potter-Elvehjem-type homogenizer in a sucrose buffer at 4°C as described by Henning *et al.* (1966). Rumen epithelium was separated from underlying connective and muscle tissue before it was homogenized. The homogenates were centrifuged at 100000g for 45min and the pellet was washed once in the buffer and centrifuged again at 100000g for 30min. The supernatants that were removed after each centrifugation were pooled and were considered to contain the soluble components of the cell. The remaining pellet was resuspended in the buffer and while in an ice-bath was subjected to ultrasonication at 20kHz for 30s. This suspension was centrifuged for 30min at 100000g; the supernatant, which contained the proteins solubilized by sonication, was used to assess enzyme activity in the particulate components of the cell. This procedure was followed because it was found that higher activity was obtained from the supernatant of the centrifuged sonicated particulate fraction than from the entire sonicated particulate fraction.

Glutamate dehydrogenase activity (EC 1.4.1.2) was used as a marker to assess possible mitochondrial

damage during preparation. It was assayed by the method of Barker *et al.* (1968) except that 80 μ M-ADP was used.

3-Hydroxybutyrate dehydrogenase activity was assayed by following the rate of reduction of NAD⁺. The assay conditions used have been described (Gotterer, 1967), except that phosphatidylcholine was omitted from the assay except where indicated. The assay system was at pH 8.5, with excess concentrations of substrate. Correction was made for the change in E_{340} of a substrate-free control, and the initial rates were proportional over a tenfold range of enzyme concentration.

The conditions for the assay of 3-hydroxybutyrate dehydrogenase activity with acetoacetate as substrate were: 25mM-disodium hydrogen phosphate buffer, pH 7.0, 2mM-acetoacetate (lithium salt), 50mM-nicotinamide, 1mM-KCN, 0.2mM-NADH.

The pH optimum of the enzyme was measured by using 0.1M-tris-HCl buffers (pH 7.5–9.0). Phosphatidylcholine was prepared from sheep liver (Rhodes & Lea, 1957). A micellar form was prepared by sonication as described by Fleischer & Fleischer (1967). D(-)-3-Hydroxybutyrate was a gift from the late G. D. Greville. Enzymic assay showed that it was substantially free of L(+)-3-hydroxybutyrate (purity 102 $\pm$ 5%). Other chemicals were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A.

Blood samples were taken 1h before slaughter from a catheter implanted the previous day in the jugular vein of the sheep. Blood was collected in heparinized tubes, cooled and deproteinized with an equal volume of 6% (w/v) HClO₄. Acetoacetate and

Table 1. *Distribution of DL-3-hydroxybutyrate dehydrogenase and glutamate dehydrogenase activities in various tissues from non-pregnant sheep given 800g of lucerne chaff daily*

Units are μ mol of substrate converted/min per g of tissue, measured at 30°C. The values for DL-3-hydroxybutyrate dehydrogenase in skeletal and smooth muscle are the means of three observations, as is the value for glutamate dehydrogenase in rumen epithelium. Other values are the means of five observations $\pm$ S.E.M.

Tissue	DL-3-Hydroxybutyrate dehydrogenase activity			Glutamate dehydrogenase activity		
	(units)		Percentage activity in the cytoplasm	(units)		Percentage activity in the cytoplasm
	Soluble fraction	Particulate fraction		Soluble fraction	Particulate fraction	
Liver	0.30 $\pm$ 0.09	0.07 $\pm$ 0.01	81.1	1.81 $\pm$ 0.45	5.87 $\pm$ 0.87	23.5
Rumen epithelium*	0.28 $\pm$ 0.06	0.22 $\pm$ 0.10	56.0	0.406	0.465	46.0
Kidney cortex*	1.82 $\pm$ 0.11	0.35 $\pm$ 0.04	83.9	0.17 $\pm$ 0.03	2.77 $\pm$ 0.92	5.8
Brain	0	0.13 $\pm$ 0.06				
Heart	0	0.12 $\pm$ 0.06				
Skeletal muscle	0	0.07				
Smooth muscle	0	0.07				

* Phosphatidylcholine omitted from the assay.

Table 2. Activity of 3-hydroxybutyrate dehydrogenase in the soluble fraction of sheep tissues with DL-3-hydroxybutyrate, D(-)-3-hydroxybutyrate and acetoacetate as substrates

Activities are expressed in μmol of substrate converted/min per g of tissue, measured at 20°C . Each value is the mean of three preparations.

Soluble fraction from	Substrate ...	Enzyme activity		
		DL-3-Hydroxybutyrate	D(-)-3-Hydroxybutyrate	Acetoacetate
Liver		0.40	0.32	0.12
Rumen epithelium		0.15	0.14	0.12
Kidney cortex		0.91	0.08	0.26

3-hydroxybutyrate were determined enzymically (Williamson *et al.*, 1962).

Results

In liver, kidney cortex and rumen epithelium of sheep, DL-3-hydroxybutyrate dehydrogenase activity was observed in both the soluble and particulate fractions of the cell (Table 1). In liver and kidney cortex the activity was significantly higher ($P < 0.001$ and 0.01 respectively) in the soluble fraction of the cell than in the particulate, whereas in rumen epithelium the activities were similar in the two fractions. In heart, brain and muscle tissues the enzyme activity was confined to the particulate fraction.

Only 6% of the total glutamate dehydrogenase activity was found in the cytosol fraction of kidney, suggesting little mitochondrial leakage. In liver, the comparable value was 23.5%. This is similar to the value reported by Taylor *et al.* (1971) for sheep liver preparations, showing little sign of mitochondrial damage. For rumen epithelium, however, nearly half the glutamate dehydrogenase activity was in the cytoplasmic fraction, which might be due to significant damage to mitochondria. Rumen epithelium is more resistant to homogenization than is liver or kidney. Baird *et al.* (1970) reported that 58% of glutamate dehydrogenase activity was in the cytosol fraction of bovine rumen epithelium.

The activity of 3-hydroxybutyrate dehydrogenase from the soluble fractions of liver and rumen epithelium is similar with either DL- or D(-)-3-hydroxybutyrate as substrate (Table 2). With the soluble kidney enzyme, however, the activity is very much less with D(-)-3-hydroxybutyrate, suggesting that most of the activity with the DL mixture was due to reaction with the L(+) isomer. However, the small amount of activity with the D(-) isomer was not due to contamination of the substrate with L(+) isomer, as the activity remained constant when the concentration of D(-) isomer in the assay was

increased from 1 to 20 mM. According to Williamson & Kuenzel (1971) the K_m of the sheep kidney enzyme with respect to the L(+) isomer is about 6 mM. The activity of the liver enzyme was also independent of the concentration of the D(-) isomer in the range 0.5–10 mM. All three soluble enzymes reacted with acetoacetate; for the liver enzyme, the rate was appreciably lower than with D(-)-3-hydroxybutyrate.

Enzyme activity was not stimulated by phosphatidylcholine in soluble or particulate fractions of liver, or rumen epithelium (Table 3). On the contrary, there was some indication that it was inhibitory. There was no stimulation of the particulate enzyme from sheep brain, in contrast to that reported for rat brain (Smith *et al.*, 1969). However, activity in the particulate fractions of heart and muscle was increased by the presence of phosphatidylcholine.

The optimum pH for assay of the cytoplasmic enzyme in sheep liver was 8.5, which is the same as for the particulate enzyme in rat liver (Gotterer, 1967).

Table 4 shows the blood ketone concentrations in control and underfed pregnant sheep. The latter group were severely ketonaemic, whereas the control (fed, non-pregnant) sheep showed normal concentrations of blood ketones. The 3-hydroxybutyrate/acetoacetate ratio in the ketonaemic sheep fell from 10:1 to 4:1. Whereas the ratio in fed, non-pregnant sheep was similar to that observed by Koundakjian & Snoswell (1970), the ratio in underfed pregnant sheep was lower than they found in non-pregnant starved sheep. There was no increase, indeed an apparent decrease, in the mean value of 3-hydroxybutyrate dehydrogenase activity in the cytoplasmic fraction of liver, and in the particulate fraction of kidney, brain and heart of ketonaemic sheep (Table 5), where the values were compared with those of non-pregnant fed sheep determined at the same time (Table 1). Perhaps because there was considerable individual variation in the values obtained in ketonaemic sheep, none of these differences was statistically significant.

Table 3. *Effect of inclusion of phosphatidylcholine in the assay medium on the activity of DL-3-hydroxybutyrate dehydrogenase in extracts of various tissues from normal sheep*

Activities are expressed in μmol of substrate converted/min per g of tissue, measured at 30°C . Phosphatidylcholine was added to a final concentration of approx. 0.02mg/ml .

Tissue	Enzyme activity			
	Soluble fraction		Particulate fraction	
	Control	Plus phosphatidylcholine	Control	Plus phosphatidylcholine
Liver	0.499	0.274	0.083	0.043
Rumen epithelium	0.463	0.451	0.427	0.402
Kidney cortex	—	—	0.340	0.301
Brain	—	—	0.073	0.042
Heart	—	—	0.045	0.181
Skeletal muscle	—	—	0.049	0.070
Smooth muscle	—	—	0.048	0.70

Table 4. *Concentrations of 3-hydroxybutyrate and acetoacetate in blood taken 1 h before death from non-pregnant fed sheep and ketonaemic pregnant sheep.*

Results are means $\pm$ S.E.M. for five sheep in each group.

	Acetoacetate ($\mu\text{mol/ml}$ of blood)	3-Hydroxybutyrate ($\mu\text{mol/ml}$ of blood)
Fed sheep	0.019 ± 0.007	0.18 ± 0.002
Ketonaemic sheep	0.55 ± 0.15	2.01 ± 0.21

Table 5. *Activity of DL-3-hydroxybutyrate dehydrogenase in the soluble and particulate fractions of tissues from ketonaemic pregnant sheep*

Activities are expressed as μmol of substrate converted/min per g of tissue, measured at 30°C . Values are means $\pm$ S.E.M. for five sheep in each group.

Tissue	Enzyme activity	
	Soluble fraction	Particulate fraction
Liver	0.138 ± 0.020	0.076 ± 0.026
Rumen epithelium	0.209 ± 0.055	0.215 ± 0.134
Kidney cortex	1.478 ± 0.343	0.220 ± 0.089
Brain	—	0.054 ± 0.019
Heart	—	0.036 ± 0.007

Discussion

As 3-hydroxybutyrate dehydrogenase is generally considered a mitochondrial enzyme, the apparent activity in the cytosol fraction in sheep liver, kidney and rumen mucosa warrants critical examination.

In kidney the low activity measurable with D(-)-3-hydroxybutyrate strongly supports the conclusion

of Williamson & Kuenzel (1971) that the sheep kidney cytosol enzyme is unlikely to play any significant role in ketone-body interconversions. The cytosol enzymes of liver and rumen epithelium, however, are clearly active with respect to the D(-) isomer; in these cases, however, the presence of significant cytosol glutamate dehydrogenase activity suggests that the 3-hydroxybutyrate dehydrogenase may simply have leaked from mitochondria. This is plausible for rumen epithelia, but less likely in liver, as so little is found in the mitochondria; in one preparation, with 90% of glutamate dehydrogenase activity in the mitochondria, more than 90% of 3-hydroxybutyrate dehydrogenase activity was present in the cytosol. Moreover, Neilson & Fleischer (1970) could barely detect 3-hydroxybutyrate activity in sheep liver mitochondria by their normal method of assay.

In other species, 3-hydroxybutyrate dehydrogenase is firmly bound to the inner mitochondrial membrane, so that even damaged mitochondria would lose very little enzyme (Norum *et al.*, 1966). However, the lack of response to phosphatidylcholine by 3-hydroxybutyrate dehydrogenase from all sheep tissues except muscle suggests that in these tissues there is not the intimate association with membrane lipid

that is characteristic of the enzyme in other species; conceivably this could result in ready detachment of the enzyme from the mitochondrial membrane.

In support of the presence of a true cytosol 3-hydroxybutyrate dehydrogenase in rumen mucosa is the fact that other enzymes involved in ketone formation are also found in the cytosol in this tissue (H. Watson, unpublished work). Baird *et al.* (1970) found that in bovine rumen epithelium, 3-hydroxy-3-methylglutaryl-CoA synthase and lyase were both recovered predominantly in the cytosol fraction (74%). The presence of ketogenic enzymes in the cytosol of rumen epithelium may be connected with the fact that butyrate is the normal carbon source for ketone formation in this tissue.

Irrespective of the intracellular distribution of 3-hydroxybutyrate dehydrogenase, one of the most striking characteristics of ketogenic tissues in ruminants is the relatively low activity of 3-hydroxybutyrate dehydrogenase. The activity in sheep liver is nearly one order of magnitude less than the lowest of the values reported by Lehninger *et al.* (1960) in eight non-ruminant species, and the total activity in rumen epithelium is only twice this. The low activity is particularly noteworthy in comparison with the rates of 3-hydroxybutyrate production that can occur *in vivo*. From the results of Katz & Bergman (1969) one may calculate that production of 3-hydroxybutyrate can be up to 0.4 $\mu\text{mol/min}$ per g of liver in starved sheep, and up to 0.6 $\mu\text{mol/min}$ per g of liver in ketonaemic sheep. These values are of the same order as the total 3-hydroxybutyrate dehydrogenase activity reported in the present paper. For rumen mucosa, by using also the results of Weekes (1971) for weight of mucosa per sheep, the production rate of 3-hydroxybutyrate in fed animals is about 0.5 $\mu\text{mol/min}$ per g of mucosa, which is also of the order of total 3-hydroxybutyrate dehydrogenase activity in rumen mucosa. In contrast, in rat liver, Krebs & Hems (1970) found maximum production of 3-hydroxybutyrate of about 2 $\mu\text{mol/min}$ per g, whereas 3-hydroxybutyrate dehydrogenase activity is about 20 $\mu\text{mol/min}$ per g of liver (Lehninger *et al.*, 1960). It would seem that in ketogenic tissues of sheep, in contrast to non-ruminant species, the 3-hydroxybutyrate dehydrogenase activity may limit the maximum rate of 3-hydroxybutyrate production; alternatively some other enzyme system may be involved in the production of 3-hydroxybutyrate.

The lack of change in 3-hydroxybutyrate dehydrogenase activity in several tissues of ketonaemic sheep is in contrast with the finding of Smith *et al.* (1969) in rats. It may be noted, however, that Williamson *et al.* (1971) found no increase in the enzyme in the brain of ketonaemic rats. Wood *et al.* (1970) found that minces prepared from ketonaemic sheep brain had decreased ability to oxidize D(-)-3-hydroxy[^{14}C]-butyrate.

Although there was no statistically significant change in 3-hydroxybutyrate dehydrogenase activity in any tissues of ketonaemic sheep, the mean values were decreased in every tissue compared with normal tissue. Such a decrease, if genuine, may reflect depletion of tissue protein to meet the needs of foetal growth, as the animals were underfed in late pregnancy.

Whether such a decrease occurred or not, it seems likely that in ketonaemic sheep, as Williamson *et al.* (1971) have suggested for ketonaemic rats, the increases *in vivo* in ketone production (Katz & Bergman, 1969) and ketone utilization (Leng, 1965) are mediated not by variations in enzymic capacity, but are functions of the increased availability of substrate for these processes.

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