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Ketone Body and Fatty Acid Metabolism in Sheep Tissues

3-HYDROXYBUTYRATE DEHYDROGENASE, A CYTOPLASMIC ENZYME IN SHEEP LIVER AND KIDNEY

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1. 3-Hydroxybutyrate dehydrogenase (EC 1.1.1.30) activities in sheep kidney cortex, rumen epithelium, skeletal muscle, brain, heart and liver were 177, 41, 38, 33, 27 and 17 μmol/h per g of tissue respectively, and in rat liver and kidney cortex the values were 1150 and 170 respectively. 2. In sheep liver and kidney cortex the 3-hydroxybutyrate dehydrogenase was located predominantly in the cytosol fractions. In contrast, the enzyme was found in the mitochondria in rat liver and kidney cortex. 3. Laurate, myristate, palmitate and stearate were not oxidized by sheep liver mitochondria, whereas the L-carnitine esters were oxidized at appreciable rates. The free acids were readily oxidized by rat liver mitochondria. 4. During oxidation of palmitoyl-L-carnitine by sheep liver mitochondria, acetoacetate production accounted for 63% of the oxygen uptake. No 3-hydroxybutyrate was formed, even after 10min anaerobic incubation, except when sheep liver cytosol was added. With rat liver mitochondria, half of the preformed acetoacetate was converted into 3-hydroxybutyrate after anaerobic incubation. 5. Measurement of ketone bodies by using specific enzymic methods (Williamson, Mellanby & Krebs, 1962) showed that blood of normal sheep and cattle has a high [3-hydroxybutyrate]/[acetoacetate] ratio, in contrast with that of non-ruminants (rats and pigeons). This ratio in the blood of lambs was similar to that of non-ruminants. The ratio in sheep blood decreased on starvation and rose again on re-feeding. 6. The physiological implications of the low activity of 3-hydroxybutyrate dehydrogenase in sheep liver and the fact that it is found in the cytoplasm in sheep liver and kidney cortex are discussed.

The production of ketone bodies in sheep has been investigated extensively since these animals are more prone to ketosis than non-ruminants. However, the majority of investigations have been made *in vivo*, with whole animals. Further, it has been assumed that the various enzyme reactions involved are similar to those occurring in the rat, the animal most frequently used in detailed studies on ketone-body formation. D(−)-3-Hydroxybutyrate–NAD oxidoreductase (3-hydroxybutyrate dehydrogenase, EC 1.1.1.30), the enzyme catalysing the interconversion of the two major ketone bodies, 3-hydroxybutyrate and acetoacetate, is regarded as a classical 'marker enzyme' for the inner mitochondrial membranes (e.g. see Tubbs & Garland, 1968). In attempting to use the enzyme to identify mitochondrial membrane fractions prepared from sheep liver, we failed to detect activity in any membrane fractions. Thus we have examined the intracellular distribution of this enzyme in liver and other tissues of the sheep.

Ketone bodies are produced in the mitochondria of liver cells from acetyl-CoA derived from oxidation of fatty acids (Lehninger, 1964), particularly under conditions where high concentrations of free fatty acids prevail (e.g. in starvation). However, these relationships have not previously been examined at the mitochondrial level in tissues of sheep. Thus we have investigated the oxidation of long-chain fatty acids and associated ketone-body formation in isolated sheep liver mitochondria.

In sheep, as in other ruminants, the investigation of ketone-body formation is complicated by the fact that ketone bodies are produced in two main tissues, i.e. the rumen epithelium and liver (Annison & Lewis, 1959). Work by Katz & Bergman (1969) on whole animals suggests that in normal sheep the tissues supplying the portal vascular system are the main areas for ketone-body production but in the starved animal the liver is the major site of ketone-body formation. We have determined the amounts of acetoacetate and 3-hydroxybutyrate in the blood

of sheep under different conditions in order to attempt to make some correlation between these blood concentrations, the known sites of ketone body production and the tissue distribution of 3-hydroxybutyrate dehydrogenase. Some of these results have been published in a preliminary form (Snoswell, 1968; Snoswell, Broadhead & Henderson, 1969).

MATERIALS AND METHODS

Animals. The sheep used were 4-year-old Merino or Merino × Dorset Horn wethers weighing between 35 and 45 kg. The animals were pen-fed on 1:1 (w/w) mixture of lucerne-hay chaff and wheaten-hay chaff, lucerne-hay chaff or pelleted sheep cubes (Barastoc and Co., Melbourne, Vic., Australia) as indicated. The lambs were day-old Merinos. The cattle were Aberdeen Angus steers, which were stall-fed on hay chaff. The horses were thoroughbred race-horses, which were maintained on a relatively high plane of nutrition. The rats were males of the Wistar strain (250–300 g); the pigeons were of mixed strain. Both the rats and pigeons were maintained on a pelleted diet suitable for small animals (Barastoc and Co., Melbourne, Vic., Australia).

Tissue samples. Blood samples for ketone-body assays were taken from the jugular vein of the sheep, cattle and horses, from the tail of the rats and from the pigeons after decapitation. Samples (2.0 ml) of the whole blood were immediately added to 1.0 ml of ice-cold 30% (w/v) $HClO_4$ with the sheep and lambs and 0.2–0.3 ml to 2.0 ml of 3% (w/v) $HClO_4$ for the blood from the other species.

Samples of liver, kidney, heart, rumen wall, skeletal muscle, spleen, brain and small intestine of sheep were collected into ice-cold 0.25 M-sucrose immediately after slaughter. Liver and kidney samples from rats and cattle were treated similarly. Adipose tissue from sheep was collected into 0.25 M-sucrose at 37°C.

Homogenates and subcellular fractions. Homogenates (5%, w/v) of all tissues except adipose tissue and rumen epithelium were prepared in 0.25 M-sucrose at 0°C with the aid of a Potter–Elvehjem homogenizer. Liver and kidney-cortex homogenates were centrifuged at 700 *g* for 5 min to remove cell debris and nuclei. The supernatant fractions were then centrifuged at 7000 *g* for 10 min to sediment the mitochondria. The mitochondrial pellets were washed in 0.25 M-sucrose and recentrifuged at 13000 *g* for 10 min and finally suspended in 0.25 M-sucrose. Microsome and supernatant (cytosol) fractions were prepared by centrifuging at 100000 *g* for 30 min the supernatants obtained after sedimentation of the mitochondria. The microsomal pellet was resuspended in 0.25 M-sucrose and the supernatant represented the cytosol fraction. Other tissue homogenates were centrifuged directly at 100000 *g* to give cytosol and particulate fractions.

The mitochondria prepared from sheep liver and kidney by the method outlined above appeared intact as seen under the electron microscope and had low adenosine triphosphatase activity. With palmitoyl-L-carnitine as substrate the respiratory control index was 3.8 ± 2 (4 samples) and the P/O ratio was 2.4 ± 1 (4). These results are approximately the same as those for rat liver mitochondria assayed under similar conditions, and suggest that the mitochondria isolated were not extensively damaged.

Assays of succinate–cytochrome *c* dehydrogenase, glutamate dehydrogenase and monoamine oxidase activities of sheep liver and kidney mitochondria and cytosol fractions showed that contamination of cytosol with mitochondria was less than 4%.

Rumen epithelium was stripped from the rumen wall and 5% (w/v) homogenates in 0.25 M-sucrose were prepared with a high-speed homogenizer (Edmund Buhler and Co., Tubingen, Germany). Subcellular fractions were prepared as outlined above. Some samples of rumen epithelium (and liver, for comparison) were frozen in liquid nitrogen before homogenization.

Adipose tissue was homogenized in 0.25 M-sucrose at 37°C.

Assay procedures. (*a*) Ketone bodies. Acetoacetate and 3-hydroxybutyrate were determined by the specific enzymic methods developed by Williamson, Mellanby & Krebs (1962), with 3-hydroxybutyrate dehydrogenase isolated from *Rhodopseudomonas spheroides* (Williamson *et al.* 1962) with further purification on a DEAE-Sephadex column as suggested by Williamson, Lund & Krebs (1967). 3-Hydroxybutyrate dehydrogenase activities were determined on all particulate fractions before and after a total of 2 min sonication (in 30 s periods) in an MSE sonic disintegrator (Measuring and Scientific Equipment Ltd., London S.W.1, U.K.) at 20 kHz and 1.5 A.

Sheep liver homogenates and mitochondria were also subjected to the following treatments before assay of the enzyme: addition of glycerol to 50% (w/v) concentration; freezing and thawing three times; shaking for 3 min with glass beads in the presence of 1% digitonin; addition of 5% (w/v) Tween 80; exposure to 0.125 M-, 0.05 M- and 0.025 M-sucrose for 15 min at 0°C.

The 3-hydroxybutyrate dehydrogenase activity was measured in the sonicated fractions (see above) by a modification of the method of Lehninger, Sudduth & Wise (1960). The assay mixture consisted of 33 mM-tris–HCl buffer, pH 8.5, 1.8 mM-NAD, 50 mM-nicotinamide, 20 mM-dithiothreitol, 1 mM-$CaCl_2$, 1 mM-KCN, 0.75 mg of asolectin (Associated Concentrates Inc., New York, N.Y., U.S.A.) and 22 mM-sodium DL-3-hydroxybutyrate in a final volume of 1.0 ml. KCN was added to suppress NADH oxidase activity and asolectin (purified soya phosphatides) to meet the known requirement of mitochondrial 3-hydroxybutyrate dehydrogenase for phospholipid. The tissue fraction was added to start the reaction, which was followed at 20°C and at 366 nm in semi-micro cuvettes with an Eppendorf spectrophotometer fitted with an automatic sample-changer and recording attachment (Eppendorf, Netheler und Hinz, Hamburg, Germany). All activities presented were corrected for blank values.

(*b*) Oxidation experiments. Oxidation rates were measured polarographically with a small Clark-type oxygen electrode (Titron Instrument Co., Sandringham, Vic., Australia) as described previously (Snoswell, 1966). Oxidation rates with free fatty acids and fatty acyl-carnitine derivatives were measured in a medium described by Bode & Klingenberg (1964).

Chemicals. Bovine serum albumin (fraction V; Calbiochem, Los Angeles, Calif., U.S.A.) was freed from fatty acids by the method of Chen (1967). The free fatty acids were combined with this bovine serum albumin in a molar ratio of 5:1 by the method of Björntorp (1968). Lauroyl, myristoyl, palmitoyl and stearoyl esters of L-carnitine

were kindly synthesized by Mr K. C. Reed from the corresponding acid chlorides and L-carnitine hydrochloride by the method of Bremer (1968). D- and L-Carnitine hydrochloride and *O*-acetyl-L-carnitine hydrochloride were generously supplied by the Otsuka Pharmaceutical Factory (Osaka, Japan) and were recrystallized from ethanol. Other compounds used were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A.

RESULTS

Distribution of 3-hydroxybutyrate dehydrogenase in various tissues. Kidney cortex was found to have the highest 3-hydroxybutyrate dehydrogenase activity of the various sheep tissues examined (Table 1). The activity in sheep kidney cortex was approximately the same as in rat kidney cortex (Table 2). However, the activity of this enzyme in sheep liver was less than 2% of that in rat liver (Table 2). The figures for rat liver and kidney are comparable with those reported by Lehninger *et al* (1960). Rumen epithelium also had appreciable 3-hydroxybutyrate dehydrogenase activity but considerable difficulty was encountered in preparing homogenates from this tissue. The most active homogenates were made from tissue that had been first frozen in liquid nitrogen and then powdered before homogenization. This treatment was not necessary for the softer tissues, such as liver, and indeed the activity of homogenates of liver prepared in this way was the same as that obtained by the direct homogenization of fresh tissue.

Table 1. *Distribution of 3-hydroxybutyrate dehydrogenase in various tissues of the sheep*

Homogenates (5% w/v) of the tissues were sonicated and assayed as described in the Materials and Methods section. Two values are given for rumen epithelium, one for tissue treated as described above, the other for tissue prefrozen in liquid N_2. The values are given as means ±S.E.M. when more than two assays were done.

Tissue	No. of determinations	3-Hydroxybutyrate dehydrogenase activity (μmol/h per g of tissue)
Kidney cortex	6	177 ±26
Rumen epithelium	2 (N_2)	41
	6	11 ± 4
Skeletal muscle	2	38
Brain	2	32.5
Heart	7	27.4±16.1
Liver	6	16.5± 2.2
Kidney medulla	2	12
Spleen	2	3.4
Small intestine	2	3.3
Adipose tissue	2	0

The most striking characteristic of the 3-hydroxybutyrate dehydrogenase in sheep liver and kidney cortex is that in these tissues the enzyme is found predominantly in the cytosol fraction, whereas the activity in rat liver and kidney cortex is mainly in the mitochondria (Table 2). The lack of activity in the sheep liver mitochondria is not due to the presence of an inhibitor, as addition of the liver mitochondria to cytosol fractions of both liver and kidney cortex did not diminish the activity of the enzyme in these fractions. Moreover, disruption of the sheep liver mitochondria by techniques other than the standard sonication procedure, e.g. repeated freezing and thawing, exposure to 50% glycerol and various sucrose solutions, treatment with 5% Tween 80 etc., did not result in any increase in the amount of enzyme activity detected.

Although asolectin was included as a routine in the assay mixture for all tissue fractions, the

Table 2. *Subcellular distribution of 3-hydroxybutyrate dehydrogenase in the liver and kidney cortex of the rat and the sheep*

Sonicated subcellular fractions were prepared and assayed as described in the Materials and Methods section. The values shown are the means ±S.E.M. of two determinations (rats) and six determinations (sheep). Activities are expressed in μmol/h per g of tissue.

	Rat		Sheep	
Tissue fraction	Activity	%	Activity	%
Liver				
Homogenate	1150±11	100	16.5± 2.2	100
Mitochondria	1110±13	97	0.3± 0.2	1.6
Microsomes	12± 0.8	1.4	2.2± 1.0	13.2
Cytosol	35± 3	3.1	14.3± 2.8	87.2
Kidney cortex				
Homogenate	170±15	100	177 ±26	100
Mitochondria	167± 5	98.3	9.9± 3.8	5.6
Microsomes	0	0	8.3± 2.1	4.7
Cytosol	2± 2	1.2	163 ±21.3	92

cytosol 3-hydroxybutyrate dehydrogenase did not require added phospholipid to elicit maximal activity.

In contrast with the results with sheep liver and kidney cortex the enzyme activity of rumen epithelium was mainly associated with the particulate fractions and appeared to be associated with mitochondria. However, it is difficult to prepare uncontaminated subcellular fractions from this tissue, owing to the problems mentioned above. In other sheep tissues where appreciable 3-hydroxybutyrate dehydrogenase activity was found, e.g. skeletal muscle, brain and heart (Table 1), the enzyme was also predominantly in the particulate fraction and again was probably located in the mitochondria.

The activity of 3-hydroxybutyrate dehydrogenase in bovine liver and kidney cortex was 3 and 21 μmol/h per g of tissue respectively (values are the means of two assays). In the bovine liver all the activity was present in the cytosol fraction and in the kidney cortex 80% was in this fraction.

Oxidation of fatty acids by sheep liver mitochondria and associated ketone-body production. The results presented in Table 3 indicate that long-chain fatty acids were not oxidized by sheep liver mitochondria. Subsequent additions of 4mM-L-carnitine, but not D-carnitine, resulted in quite significant rates of oxidation of long-chain fatty acids by these mitochondria (e.g. for palmitate 18ng-atoms of O/min per mg of mitochondrial protein). Butyrate and crotonate were oxidized slowly. Acetate was not oxidized but acetyl-L-carnitine was oxidized slowly (Table 3). The L-carnitine esters of the long-chain fatty acids were oxidized at appreciable rates although the overall oxidative activity of sheep liver mitochondria appeared to be only about one-third of that for rat liver mitochondria oxidizing the same substrate (Table 3).

In contrast, the results presented in Table 3 also show that free long-chain fatty acids, especially laurate and myristate, were oxidized at quite high rates by rat liver mitochondria. The results for oxidation of the L-carnitine esters of these fatty acids are also shown in Table 3.

In view of the fact that free long-chain fatty acids were not oxidized by sheep liver mitochondria in the absence of added L-carnitine, palmitoyl-L-carnitine was used as substrate in subsequent experiments. During the oxidation of this substrate by sheep liver mitochondria, 84nmol of acetoacetate was formed with a concomitant uptake of 235nmol of oxygen (Table 4). Thus acetoacetate formation accounts for 63% of the oxygen uptake observed, based on the equation:

$$\text{Palmitate} + 7O_2 \rightarrow 4 \text{ acetoacetate} + 4H_2O$$

Similarly, acetoacetate production accounted for approx. 60% of the oxygen uptake by rat liver mitochondria.

When the incubation mixtures were allowed to remain under anaerobic conditions for 10min after all the oxygen had been utilized, about half of the acetoacetate formed was reduced to 3-hydroxybutyrate in the experiments with rat liver mitochondria (Table 4). Similar results with rat liver mitochondria have been reported by Portenhauser, Schäfer & Lamprecht (1969). In contrast no 3-hydroxybutyrate was produced in the experiments with sheep liver mitochondria. This difference

Table 3. *Oxidation of free fatty acids and their corresponding* L-*carnitine esters by isolated sheep and rat liver mitochondria*

Oxygen uptake was measured polarographically by using an oxygen electrode in a volume of 2.5ml at 25°C. The incubation medium used was that described by Bode & Klingenberg (1964) including 6μmol of ADP. Free fatty acid (FFA) (0.4μmol) or L-carnitine esters (0.12μmol), both combined with bovine serum albumin (free from bound fatty acids), were used per assay. Sheep or rat liver mitochondria equivalent to 2–4mg of mitochondrial protein were used per assay. The values are corrected for the rates obtained in the absence of added substrate and are the means ±S.E.M. for three experiments.

	Oxygen uptake (ng-atoms/min per mg of mitochondrial protein)			
	Sheep liver mitochondria		Rat liver mitochondria	
Chain length of saturated fatty acid	FFA	Carnitine ester	FFA	Carnitine ester
C_2	0	9±2	—	—
C_4	4±1	—	—	—
C_6	9±1	—	—	—
C_{12}	0	29±3	76±6	84±4
C_{14}	0	26±2	65±4	80±4
C_{16}	0	23±3	51±3	77±3
C_{18}	0	21±2	8±1	74±2
Glutamate	21±2		51±4	

Table 4. *Oxidation of palmitoylcarnitine by sheep and rat liver mitochondria and the associated production of ketone bodies*

The standard mixture contained 80mM-KCl, 20mM-tris–chloride buffer, pH 7.4, 2mM-$MgCl_2$, 1mM-EDTA, 1.25mM-AMP, 1.25mM-sodium–potassium phosphate, pH 7.4, and 25mg of bovine serum albumin (free from fatty acids), in a total volume of 2.5ml. The substrate was 45μM-palmitoyl-L-carnitine and the reaction was started by the addition of 5–7mg of protein of sheep or rat liver mitochondria. Oxygen uptakes were measured as described in Table 3. The incubations were allowed to proceed until almost all of the oxygen in the solution was utilized and then the reaction was stopped by the addition of 0.2ml of 30% $HClO_4$. Acetoacetate and 3-hydroxybutyrate in the neutralized supernatants were measured by the enzymic method of Williamson *et al.* (1962). The results are means ±S.E.M. and in each case are corrected for the values obtained with no substrate. The number of experiments is shown in parentheses. N.D., not detectable. The limit of detection was considered to be 1nmol under the conditions of assay.

	Sheep			Rat		
Incubation conditions	Oxygen uptake (nmol)	Acetoacetate formed (nmol)	3-Hydroxybutyrate formed (nmol)	Oxygen uptake (nmol)	Acetoacetate formed (nmol)	3-Hydroxybutyrate formed (nmol)
Standard	235±3 (3)	84±1 (3)	N.D. (3)	180±4 (3)	62±2 (3)	5±1 (3)
Plus 10min subsequent anaerobic incubation	239±3 (3)	87±1 (3)	N.D. (3)	183±4 (3)	32±2 (3)	33±2 (3)
*Plus 3nmol of rotenone after half the oxygen utilized	220±5 (2)	96±2 (2)	N.D. (2)	239±5 (2)	38±3 (2)	141±6 (2)
Plus 1ml of liver cytosol and 10min anaerobic incubation	276±4 (2)	51±2 (2)	20±2 (2)	—	—	—

* 10mM-Malonate and 10μM-fluorocitrate were added to the standard incubation medium.

between sheep and rat liver mitochondria was further demonstrated when ketone-body production was increased to a maximum rate by the addition of fluorocitrate and malonate during the oxidation of palmitoyl-L-carnitine. Under these conditions, the addition of rotenone, after about half of the total oxygen in the reaction mixture had been utilized, resulted in the production of 3-hydroxybutyrate as the main end product with rat liver mitochondria (Table 4). Again, there was no 3-hydroxybutyrate produced with sheep liver mitochondria (Table 4).

The results shown in Table 4 indicate that, if cytosol fraction of sheep liver was added to the reaction mixture containing sheep liver mitochondria and the incubation continued for 10min under anaerobic conditions, a significant amount of 3-hydroxybutyrate was produced.

Blood ketone bodies. In Table 5 acetoacetate and 3-hydroxybutyrate concentrations in sheep blood are contrasted with those in cattle blood and in the blood of non-ruminant species. The results in this table show that cattle and sheep have a significantly higher ($P<0.01$) [3-hydroxybutyrate]/[acetoacetate] ratio in the blood than have the non-ruminants, pigeons and rats. The results for horses are intermediate between these two groups. The horse, although a non-ruminant, produces large quantities of volatile fatty acids in the caecum and colon (Dukes, 1955). The variation between the results for the two groups of sheep may be in part dietary or may be due to the difference in breed. For example, Merino wethers showed significantly lower [3-hydroxybutyrate]/[acetoacetate] ratios in the blood than did Merino × Dorset Horn wethers (see Table 6). Also, differences in diet and strain of the rats used here may well account for the variation between the results reported here and those reported for rat blood by Berry, Williamson & Wilson (1965). Eggleston & Krebs (1969) have reported strain differences in the activities of various enzymes in rat liver. Such differences could affect metabolite concentrations.

The values shown in Table 5 for the blood ketone-body concentration in lambs indicate [3-hydroxybutyrate]/[acetoacetate] ratios similar to those of non-ruminant species. The lambs were 1 day old and thus there was no active rumen fermentation. Knodt, Shaw & White (1942) found in young calves a gradual increase in ketone-body production, particularly of 3-hydroxybutyrate, which roughly paralleled the development of the rumen.

Table 5. *Acetoacetate and 3-hydroxybutyrate in the blood of various species*

The smaller group of sheep were Merino × Dorset Horn wethers, fed on pelleted sheep cubes (see the text), in contrast with the larger group, which were Merino wethers fed on an equal mixture of lucerne and oaten chaff. Acetoacetate and 3-hydroxybutyrate were determined as described in Table 4. The mean value for the acetoacetate of the cattle is shown as <0.002 as the actual values were less than 0.002 μmol/ml in a number of cases. This value was the lowest that could be detected. The results shown are means ±S.E.M.

Species	No. of animals	Acetoacetate (μmol/ml)	3-Hydroxybutyrate (μmol/ml)	[3-Hydroxybutyrate]/[acetoacetate] ratio
Cow	12	< 0.002	0.102±0.011	> 38
Sheep	12	0.058±0.004	0.623±0.034	12 ±1
Sheep	4	0.010±0.002	0.250±0.064	24 ±3
Horse	10	0.008±0.001	0.080±0.006	11 ±2
Pigeon	12	0.035±0.006	0.168±0.025	5.8±1.0
Rat	10	0.029±0.006	0.096±0.016	4.3±0.8
Lamb	6	0.044±0.006	0.207±0.032	5.0±0.2

Table 6. *Acetoacetate and 3-hydroxybutyrate in the blood of normal, starved and re-fed sheep*

Each group of four animals was stabilized on a diet of lucerne-hay chaff before the experiment and the normal values shown are the means for 14 successive daily samples for the Merino wethers, and three for the Merino × Dorset Horn wethers. The values for the starved condition are the means for six successive daily samples taken after a period of 4 days starvation and the values for the re-fed period are the means for four successive daily samples after re-feeding. Acetoacetate and 3-hydroxybutyrate were determined as described in Table 4. The values are the means ±S.E.M. for four animals in each group.

Sheep	Dietary status	Acetoacetate (μmol/ml)	3-Hydroxybutyrate (μmol/ml)	[3-Hydroxybutyrate]/[acetoacetate] ratio
Merino wethers	Normal	0.026±0.003	0.252±0.014	11.2±0.9
	Starved	0.111±0.005	0.723±0.018	6.6±0.3
	Re-fed	0.030±0.006	0.254±0.023	9.9±2.4
Merino × Dorset Horn wethers	Normal	0.010±0.001	0.241±0.009	26.8±2.4
	Starved	0.106±0.011	0.895±0.062	10.8±1.9
	Re-fed	0.024±0.003	0.423±0.040	22.7±2.8

The results presented in Table 6 show that the [3-hydroxybutyrate]/[acetoacetate] ratio in sheep blood falls on starvation and increases again on re-feeding. Total 3-hydroxybutyrate plus acetoacetate increases on starvation, as expected, but the amount of acetoacetate increases relatively more than the amount of 3-hydroxybutyrate, thus exerting a major influence on the ratio of these two ketone bodies. The effects of starvation on this ratio are more pronounced in the crossbred sheep than with the pure Merinos, the former sheep showing a higher [3-hydroxybutyrate]/[acetoacetate] ratio in the blood under normal feeding conditions.

DISCUSSION

The most striking feature of the results presented here is the fact that 3-hydroxybutyrate dehydrogenase is present in the cytosol fraction of the sheep liver and kidney cortex. This is in direct contrast with the rat, where the enzyme is found in the mitochondrial fraction of the same tissues. Of the other tissues of the sheep examined, appreciable 3-hydroxybutyrate dehydrogenase activity was found in rumen epithelium, skeletal muscle, heart and brain, and the enzyme was located predominantly in the particulate fractions, probably in the mitochondria. The absence of this enzyme in liver mitochondria of sheep is particularly unusual as this organ under certain conditions produces ketone bodies in large quantities (see Katz & Bergman, 1969) and ketone bodies are known to be produced in mitochondria (Lehninger, 1964). The experiments reported above with isolated sheep liver mitochondria show that during the oxidation of palmitoyl-L-carnitine, acetoacetate was produced but no 3-hydroxybutyrate. Even when ketone-body formation was raised to a maximal rate by the addition of malonate and fluorocitrate to block oxidation in the tricarboxylic acid cycle and an increase in reducing potential was provided (by the addition of rotenone or by anaero-

bic conditions), no 3-hydroxybutyrate was produced by the sheep liver mitochondria. Under similar conditions the major end-product of palmitoyl-L-carnitine oxidation by the rat liver mitochondria was 3-hydroxybutyrate. Thus in sheep liver mitochondria internally generated acetoacetate is not reduced to 3-hydroxybutyrate even under reducing conditions. These results thus confirm the absence of 3-hydroxybutyrate dehydrogenase in sheep liver mitochondria.

The work of Katz & Bergman (1969) shows that some 3-hydroxybutyrate is produced by sheep liver, particularly during starvation. The results presented above show that if a cytosol fraction of sheep liver is added to isolated sheep liver mitochondria oxidizing palmitoyl-L-carnitine, then some 3-hydroxybutyrate is produced under reducing conditions. This result illustrates that 3-hydroxybutyrate can be produced in sheep liver under appropriate conditions even though the enzyme is located in the cytosol fraction. However, the $[NAD^+]/[NADH]$ ratio in the cytoplasm of the liver is not particularly favourable for the reduction of acetoacetate to 3-hydroxybutyrate. Veech, Eggleston & Krebs (1969) reported an $[NAD^+]/[NADH]$ ratio of 1164 for the rat liver cytoplasm in normal animals which fell to 564 in starved rats. The ratio in normal sheep liver is slightly lower but does not decrease on starvation (A. M. Snoswell, unpublished work). Thus sheep liver would appear to have a very limited capacity for producing 3-hydroxybutyrate.

3-Hydroxybutyrate dehydrogenase is associated only with the inner mitochondrial membrane, at least in rat liver (Schnaitman, Erwin & Greenawalt, 1967). This fact has been used by Williamson *et al.* (1967) to assess the $[NAD^+]/[NADH]$ ratio in the mitochondrial cristae from the [3-hydroxybutyrate]/[acetoacetate] ratio determined in freeze-clamped rat liver. This technique for determining redox states in intracellular compartments from a knowledge of the amounts of metabolites of a redox pair is very useful (see Williamson *et al.* 1967) but the intracellular distribution of the appropriate enzyme in the tissue being examined must be known. Quite obviously knowledge of the $[NAD^+]/[NADH]$ ratio in the mitochondrial cristae could not be obtained from the [3-hydroxybutyrate]/[acetoacetate] ratio in sheep liver since the 3-hydroxybutyrate dehydrogenase in this tissue is mainly found in the cytosol. Ballard, Hanson & Kronfeld (1968) have used the [3-hydroxybutyrate]/[acetoacetate] ratio to determine the $[NAD^+]/[NADH]$ ratio for the mitochondrial cristae in bovine liver. However, this approach would appear invalid because in bovine liver, as in sheep liver, the 3-hydroxybutyrate dehydrogenase is predominantly in the cytosol fraction.

A further feature of the results presented here is the profound difference in the activity of 3-hydroxybutyrate dehydrogenase in different tissues of sheep when compared with those of the rat. Lehninger *et al.* (1960) have reported that the activity of this enzyme in rat liver is some eight times greater than the activity in any other rat tissues. The activity in sheep liver, however, is less than 2% of that in rat liver and is about 10% of that in sheep kidney cortex, the tissue with the highest activity in the sheep. Lehninger *et al.* (1960) found considerable differences in the activity of 3-hydroxybutyrate dehydrogenase in the liver of various species although no ruminant species were examined. Baird *et al.* (1968) report that the activity in bovine liver is less than one-thirtieth of that in rat liver. It is probable that the relatively high activity of 3-hydroxybutyrate dehydrogenase in the kidney is indicative of the kidney being a major tissue for the utilization of 3-hydroxybutyrate in the sheep. Leng & Annison (1964) found that kidney had the highest rate of uptake of 3-hydroxybutyrate when slices from a number of sheep tissues were examined. Weidemann & Krebs (1969) have shown that ketone bodies are a preferred metabolic fuel for rat kidney. The reason for the occurrence of 3-hydroxybutyrate dehydrogenase in the cytosol fraction of sheep kidney cortex is obscure but may be related to efficient utilization of 3-hydroxybutyrate by this tissue.

The results presented above show that sheep, like cattle, have a high [3-hydroxybutyrate]/[acetoacetate] ratio in the blood under normal conditions. This has been generally accepted to be the case for cattle since Thin & Robertson (1952) found that 3-hydroxybutyrate was the only ketone body that could be detected in the blood of normal dairy cattle. However, the results published for individual ketone-body concentrations in sheep blood have been variable. Values reported for the [3-hydroxybutyrate]/[acetoacetate] ratio in normal sheep blood have ranged from 0.25:1 (Procos, 1962) to 2:1 (Reid, 1960), and Leng & Annison (1963) implied that in normal sheep 3-hydroxybutyrate represents about 85% of the blood ketone bodies. This wide range of values is undoubtedly due to the difficulty in determining accurately the concentration of individual ketone bodies in sheep blood by the older chemical methods (see Roe, Bergman & Kon, 1966, for a discussion of this point). The values reported above were determined by the specific enzyme procedures developed by Williamson *et al.* (1962) and appear to be the first values published for sheep blood obtained by these methods.

The high [3-hydroxybutyrate]/[acetoacetate] ratio in the blood is probably a general characteristic of ruminant animals, as it seems to be associated with active rumen fermentation because it

is not observed in young lambs (the present study) or in young calves (Knodt *et al.* 1942). The results presented above show that the rumen epithelium has significant 3-hydroxybutyrate dehydrogenase activity and it is known that butyrate absorbed from the rumen is substantially converted into ketone bodies (Hungate, 1966). Oxidation of fatty acids leads to a low $[NAD^+]/[NADH]$ ratio within rat heart mitochondria (Klingenberg & Kröger, 1966), and it is reasonable to assume that fatty acid oxidation in rumen epithelium mitochondria has the same effect. Thus a favourable environment is provided within the mitochondria of the rumen epithelium for the conversion of acetoacetate (derived from oxidation of butyrate) into 3-hydroxybutyrate by 3-hydroxybutyrate dehydrogenase. In this tissue the enzyme is probably associated with mitochondria.

In starved sheep the [3-hydroxybutyrate]/[acetoacetate] ratio in the blood falls markedly, particularly in crossbred animals. This is in direct contrast with the results for the rat where the blood ratio rises on starvation (Berry *et al.* 1965). In the rat this increase in the ratio is attributed to increased fatty acid oxidation in the liver and the subsequent effect on the concentration of NADH in the mitochondria of hepatic cells (Williamson & Wilson, 1965). Katz & Bergman (1969) have shown that in the sheep there is a close correlation between the concentration of free fatty acids in the blood and ketone production in the liver, particularly in starvation, and the present results show that isolated sheep liver mitochondria readily form acetoacetate, but not 3-hydroxybutyrate, during the oxidation of palmitoyl-L-carnitine. Thus the reason for the decrease in the [3-hydroxybutyrate]/[acetoacetate] ratio in the blood of sheep on starvation appears to be twofold. First, we have suggested that the normal high ratio is mainly due to the production of 3-hydroxybutyrate in the mitochondria of the rumen epithelium; this production will diminish greatly on prolonged starvation. Secondly, on starvation ketone-body production occurs in sheep liver (Katz & Bergman, 1969) and since sheep liver has relatively low 3-hydroxybutyrate dehydrogenase activity, and the enzyme is in the cytosol fraction, it is probable that the conversion of the primary ketone body, acetoacetate, into 3-hydroxybutyrate is severely limited.

The presence of 3-hydroxybutyrate dehydrogenase in the cytosol of sheep liver cells rather than in the mitochondria, and its comparatively low activity in sheep liver, may well have some important physiological implications. It appears that it is the accumulation of acetoacetate that depresses the functioning of the central nervous system in ketosis, as the acidosis of 3-hydroxybutyrate accumulation is readily compensated (Behnke, 1964). The conversion of acetoacetate into 3-hydroxybutyrate in the liver therefore may be regarded as a detoxification process. Thus, as cattle and sheep liver contain only relatively small amounts of 3-hydroxybutyrate dehydrogenase, and this small amount is present in the cytosol, this may be one reason why these animals are more prone to severe ketosis than non-ruminants.

The experimental conditions chosen in the present work for the study of the oxidation of long-chain fatty acids by mitochondria are known (for the rat) to result in appreciable oxidation of these fatty acids independent of L-carnitine (see Greville & Tubbs, 1968, for discussion of this point). Yet under the same conditions the oxidation of long-chain fatty acids by sheep liver mitochondria was completely dependent on the presence of L-carnitine. This could indicate that a different system operates for the activation of fatty acids in sheep liver mitochondria from that in rat liver mitochondria (see Van Den Bergh, 1967; Garland & Yates, 1967). An alternative explanation may be that the high activity of carnitine acetyltransferase in sheep liver mitochondria, in contrast with the low activity of carnitine palmitoyltransferase (Snoswell & Henderson, 1970) results in the former enzyme competing more effectively for endogenous L-carnitine, thus severely limiting the oxidation of long-chain fatty acids in the absence of added carnitine. The fact that sheep liver contains only small amounts of long-chain carnitine esters relative to acetylcarnitine, and this long-chain ester fraction increases only slightly on starvation (Snoswell & Henderson, 1970), would support this view.

In the final stages of the preparation of this manuscript a report by Nielsen & Fleischer (1969) appeared indicating that ruminant liver mitochondria lack 3-hydroxybutyrate dehydrogenase. Thus the present work confirms this point and also establishes that homogenates of sheep liver (and bovine liver) do have demonstrable enzyme activity, the enzyme being present in the cytosol. This is also the case for sheep kidney cortex, which has much higher 3-hydroxybutyrate dehydrogenase activity.

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