3-Hydroxy Acid Dehydrogenases in Sheep Tissues

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Williamson & Kuenzel (1971) have shown that the 'soluble' 3-hydroxybutyrate dehydrogenase activity of sheep kidney cortex described by Koundakjian & Snoswell (1970) is, in fact, that of an L-3-hydroxy acid dehydrogenase (probably L-gulonate-NAD⁺ oxidoreductase, EC 1.1.1.45). Smiley & Ashwell (1961) have shown that this enzyme reacts with a large number of 3-hydroxy acids provided that the 3-hydroxyl group is in the L-configuration. It does not react with D-3-hydroxy acids (Smiley & Ashwell, 1961). Koundakjian & Snoswell (1970) had used DL-3-hydroxybutyrate as substrate in the original assay of the dehydrogenase and implied that the activity found was that of D(-)-3-hydroxybutyrate-NAD⁺ oxidoreductase (EC 1.1.1.30). In order to ascertain the contribution of L-3-hydroxy acid dehydrogenase to the overall 3-hydroxybutyrate dehydrogenase activity of sheep tissues we have examined the activity of dehydrogenases of L- and D-3-hydroxybutyrate in various tissues of the sheep. The results obtained are discussed in relation to ketone-body metabolism in sheep.

Experimental

The sheep used were 3-year-old Merino wethers, weighing about 40 kg and pen-fed on lucerne hay.

Samples of liver, kidney cortex, heart, skeletal muscle and rumen epithelium were collected, and homogenates, subcellular fractions and sonicated fractions were prepared as described previously (Koundakjian & Snoswell, 1970), except that the medium used was 0.25 M-sucrose in 23 mM-potassium phosphate buffer, pH 7.2. Phosphate-buffered sucrose was used because recovery of glutamate dehydrogenase (EC 1.4.1.3) activity is impaired in the absence of phosphate (Walter & Anabitarte, 1971).

3-Hydroxy acid dehydrogenase activities were measured as described for 3-hydroxybutyrate dehydrogenase (Koundakjian & Snoswell, 1970) with either 22 mM-sodium DL-3-hydroxybutyrate or 11 mM-sodium L-3-hydroxybutyrate as substrate. Enzyme activity was also measured in the reverse direction, the reaction mixture consisting of (final concentrations) 33 mM-tris-HCl buffer, pH 7.0, 0.2 mM-NADH, 50 mM-nicotinamide, 1 mM-CaCl₂ and 10 mM-sodium acetoacetate in a final volume of 1.0 ml. All activities recorded were corrected for blank values.

Glutamate dehydrogenase was assayed by the method of Barker *et al.* (1968), but 1.5 mM-ADP was used to ensure maximal activity, 10 μ M-rotenone replaced 3 mM-KCN and phosphate was used instead of tris-HCl buffer.

Further assessment of the activities of 3-hydroxy acid dehydrogenases was made by incubating tissue fractions with substrates and measuring the consequent changes in the concentrations of D-3-hydroxybutyrate and acetoacetate. The assay systems used were (final concentrations): for acetoacetate reduction (final vol. 3 ml), 33 mM-tris-HCl buffer, pH 7.0, 0.2 mM-NADH, 67 mM-nicotinamide, 1 mM-CaCl₂, 10 μ M-rotenone and 3.28 mM-sodium acetoacetate; for 3-hydroxybutyrate oxidation (final vol. 3 ml), 33 mM-tris-HCl buffer, pH 8.5, 1.8 mM-NAD⁺, 67 mM-nicotinamide, 1 mM-CaCl₂, 20 mM-dithiothreitol, 1 mM-KCN, 2.25 mg of asolectin (see the Materials and Methods section of Koundakjian & Snoswell, 1970) and either 22 mM-sodium DL-3-hydroxybutyrate or 11 mM-sodium L-3-hydroxybutyrate. A 0.2 ml portion of tissue fraction was used in each assay and the reaction was started by the addition of substrate. After 1 h at 20°C, 0.3 ml of 30% (w/v) HClO₄ was added to stop the reaction. The protein-free solution was neutralized, and acetoacetate and D-3-hydroxybutyrate were measured by the enzymic method of Williamson *et al.* (1962). All values presented were corrected for zero-time ketone-body concentrations.

The L-isomer of 3-hydroxybutyric acid was prepared from the DL-acid by the method of McCann & Greville (1962). This preparation of L-isomer contained only 1.2% of D-isomer. We were unable to obtain a sufficiently pure sample of D-isomer either with this method (McCann & Greville, 1962) or with several others tried.

Results and discussion

The results presented in Table 1 show that for all tissues recorded here the glutamate dehydrogenase activity was associated almost exclusively with the particulate fractions. Since glutamate dehydrogenase is used as a marker for mitochondria, the cytosol fractions prepared were considered to be free of significant contamination with mitochondrial enzymes. Values for the glutamate dehydrogenase

Table 1. *Distribution of glutamate dehydrogenase and 3-hydroxy acid dehydrogenase activities in various tissues of the sheep*

Tissue fractions were prepared and assayed as described in the text. Results are expressed in $\mu\text{mol/h}$ per g of tissue, and are given as mean values $\pm$ S.E.M. for three (glutamate dehydrogenase), five (3-hydroxy acid dehydrogenase with acetoacetate) and seven (3-hydroxy acid dehydrogenase with 3-hydroxybutyrate) observations.

	Glutamate dehydrogenase activity	Substrate ...	3-Hydroxy acid dehydrogenase activity		
			Acetoacetate	L-3-Hydroxybutyrate	DL-3-Hydroxybutyrate
Liver					
Homogenate	2450 $\pm$ 181		26.9 $\pm$ 7.3	9.6 $\pm$ 7.7	17.3 $\pm$ 4.7
Mitochondria	1990 $\pm$ 48		4.5 $\pm$ 1.6	0.16 $\pm$ 0.11	0.66 $\pm$ 0.47
Cytosol	272 $\pm$ 78		19.1 $\pm$ 7.4	7.3 $\pm$ 5.3	9.8 $\pm$ 3.7
Kidney cortex					
Homogenate	634 $\pm$ 9		116 $\pm$ 9	126 $\pm$ 23	160 $\pm$ 19
Mitochondria	555 $\pm$ 42		14 $\pm$ 2.1	2.24 $\pm$ 1.14	6.9 $\pm$ 2.9
Cytosol	69 $\pm$ 13		118 $\pm$ 5.9	110 $\pm$ 14	122 $\pm$ 14
Heart					
Homogenate	128 $\pm$ 6		25.6 $\pm$ 6.1	9.3 $\pm$ 5.7	13.9 $\pm$ 7
Cytosol	22 $\pm$ 7		11.1 $\pm$ 4.4	5.5 $\pm$ 3.6	4.5 $\pm$ 2.9
Rumen epithelium					
Homogenate	38 $\pm$ 8		65.1 $\pm$ 12	9.5 $\pm$ 5.3	15.5 $\pm$ 5.8
Cytosol	8.7 $\pm$ 2.2		10.3 $\pm$ 4.8	4 $\pm$ 2.1	7.8 $\pm$ 3.7

activity of skeletal-muscle fractions indicated extensive mitochondrial breakdown (see Snoswell & Koundakjian, 1972), so results obtained for this tissue have been omitted.

The dehydrogenase activities detected with DL-3-hydroxybutyrate as substrate (Table 1) were similar to those reported by Koundakjian & Snoswell (1970) except that those for rumen epithelium were somewhat lower. However, this tissue is extremely difficult to homogenize and fractionate, and any enzyme-activity results obtained in this manner for rumen epithelium are likely to be greatly underestimated. A major proportion of the dehydrogenase activity with DL-3-hydroxybutyrate was cytoplasmic in liver and kidney cortex, and some cytoplasmic activity was detected in heart and rumen epithelium (Table 1). When the L-isomer was used as substrate the cytoplasmic activities observed were similar to those with the DL-salt, whereas the activities of the particulate fractions were lower. Thus it would appear that the cytoplasmic activity of all the tissues examined is attributable to L-3-hydroxy acid dehydrogenase, and that the D(-)-3-hydroxybutyrate-NAD⁺ oxidoreductase activity is mitochondrial. The results shown for assays with acetoacetate as substrate indicate that measuring the activity in this direction generally gives higher apparent activities for both the D- and the L-3-hydroxybutyrate dehydrogenase, particularly for rumen epithelium.

The results of assay of reactant formation and removal when tissue fractions were incubated with the different substrates are shown in Table 2. With all the tissues examined D-3-hydroxybutyrate was formed by the particulate fractions from acetoacetate, but, with the exceptions of kidney-cortex mitochondria and liver homogenate and mitochondria, the amount formed was considerably less than the amount of acetoacetate removed. With kidney-cortex mitochondria the amount of substrate removed was roughly equal to that of D-3-hydroxybutyrate produced; and with liver homogenate and mitochondria there was an increase in the concentration of acetoacetate during the incubation period owing to concomitant oxidation of endogenous fatty acids.

When L-3-hydroxybutyrate was used as substrate (Table 2) there was very little change in D-3-hydroxybutyrate concentrations compared with the endogenous values. There was significant acetoacetate production by all tissue fractions, particularly kidney-cortex homogenate and cytosol. However, the production of acetoacetate by the liver homogenate and mitochondrial fractions in the presence of L-3-hydroxybutyrate (and also the DL-salt) was surprisingly low. It is probable that L-3-hydroxybutyrate inhibits endogenous acetoacetate formation.

The use of DL-3-hydroxybutyrate as substrate resulted in the formation of more acetoacetate by the particulate fractions than when the L-isomer was

Table 2. Oxidation of D- and L-3-hydroxybutyrate and reduction of acetoacetate by fractions prepared from various sheep tissues

Tissue fractions were prepared, incubated and assayed as described in the text. The results are in μmol and were corrected for zero-time ketone-body concentrations. They are the mean values for five to seven animals; S.E.M. values ranged from 1.5 to 40% of the mean values.

Substrate	Reactant determined	Liver				Kidney cortex				Heart		Rumen epithelium	
		Homo-generate		Mito-chondria		Homo-generate		Mito-chondria		Homo-generate		Homo-generate	
Acetoacetate	D-3-Hydroxybutyrate	+30	+24	0	+117	+400	0	+95	+15	+316	+7		
L-3-Hydroxybutyrate	D-3-Hydroxybutyrate	+2	+4	+1	+1	+4	+1	+1	+2	+2	+1		
DL-3-Hydroxybutyrate	D-3-Hydroxybutyrate	0	-28	0	-1504	-962	0	-177	0	-783	0		
Acetoacetate	Acetoacetate	+480	+460	-575	-450	-465	-860	-520	-450	-855	-250		
L-3-Hydroxybutyrate	Acetoacetate	+40	+23	+58	+155	+62	+146	+46	+33	+17	+8		
DL-3-Hydroxybutyrate	Acetoacetate	+44	+42	+87	+191	+127	+142	+90	+43	+117	+9		

used, but the amounts produced by the cytosol fractions were virtually the same (Table 2). The amount by which the D-3-hydroxybutyrate concentration was decreased during incubation with the particulate fractions was much greater than could be accounted for by acetoacetate production, especially when the fact that acetoacetate can also be formed by the homogenate fractions from the L-isomer is taken into account. There was no evidence of breakdown of 3-hydroxybutyrate during the incubation period. It seems likely that some of the formed acetoacetate was further metabolized, and that under the conditions used the L-3-hydroxy acid dehydrogenase in particular could be using the acetoacetate, thus leading to low estimates of the amount formed. The tissues showing the greatest rate of removal of D-3-hydroxybutyrate were kidney cortex and rumen epithelium (Table 2). This is in accord with the facts that the kidney cortex has been shown by Koundakjian & Snoswell (1970) to have the highest D-3-hydroxybutyrate-NAD⁺ oxidoreductase activity of sheep tissues and that since rumen epithelium produces D(-)-3-hydroxybutyrate from butyrate in the fed animal (Annison & Lewis, 1959) it is likely to have much higher D(-)-3-hydroxybutyrate-NAD⁺ oxidoreductase activity than is readily demonstrable.

The results of these tissue-incubation experiments therefore further substantiate the conclusions drawn from the results of direct measurement of the apparent tissue enzyme activities: the D(-)-3-hydroxybutyrate-NAD⁺ oxidoreductase activity of all the sheep tissues examined is associated with the mitochondria, and is very low in liver and kidney cortex, and there is cytoplasmic activity of L-3-hydroxy acid dehydrogenase, which is in some tissues (liver and kidney cortex) much greater than the activity of the D-3-hydroxybutyrate dehydrogenase. It must be emphasized that values given for both the D- and the L-hydroxybutyrate dehydrogenase activities may not be absolute values, but are comparative values between the various tissues for the two enzymes since only one aliquot size of the fractions was used in each case, and the assay conditions for the L-acid dehydrogenase may not have been optimal for this enzyme.

Although there is comparatively high activity of L-3-hydroxy acid dehydrogenase in the liver and kidney cortex of the sheep, both organs being particularly involved in ketone-body metabolism, it is not likely that this enzyme has any important role in the metabolism of ketone bodies by sheep. The high NAD⁺/NADH concentration ratio of the cytosol of sheep liver and kidney cortex (A. M. Snoswell, unpublished work) is unfavourable for acetoacetate reduction; and also Williamson & Kuenzel (1971) have shown that the K_m value of the kidney-cortex enzyme for acetoacetate is high.

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Annison, E. F. & Lewis, D. (1959) *Metabolism in the Rumen*, p. 70, Methuen and Co. Ltd., London

Barker, P. J., Fincham, N. J. & Hardwick, D. C. (1968) *Biochem. J.* **110**, 739

Koundakjian, P. P. & Snoswell, A. M. (1970) *Biochem. J.* **119**, 49

McCann, W. P. & Greville, G. D. (1962) *Biochem. Prep.* **9**, 63

Smiley, J. D. & Ashwell, G. (1961) *J. Biol. Chem.* **236**, 357

Snoswell, A. M. & Koundakjian, P. P. (1972) *Biochem. J.* **127**, 133

Walter, P. & Anabitarte, M. (1971) *FEBS Lett.* **12**, 289

Williamson, D. H. & Kuenzel, P. (1971) *Biochem. J.* **121**, 569

Williamson, D. H., Mellanby, J. & Krebs, H. A. (1962) *Biochem. J.* **82**, 90