Biochemical Aspects of Bovine Ketosis

By G. D. BAIRD, K. G. HIBBITT AND G. D. HUNTER

*Department of Biochemistry, Agricultural Research Council Institute for Research on Animal Diseases,
Compton, Berks.*

AND PATRICIA LUND,* MARION STUBBS* AND H. A. KREBS*

*Medical Research Council Unit for Research in Cell Metabolism, Department of Biochemistry,
University of Oxford*

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1. The concentrations of acetoacetate, β -hydroxybutyrate and metabolites related to gluconeogenesis were determined in biopsy samples of the livers of ketotic, normal lactating and normal non-lactating cows. Key enzymes of gluconeogenesis in the liver were also assayed. 2. Significant decreases were found in the ketotic liver in the concentrations of glucogenic amino acids (glutamate, glutamine, alanine) and of glucogenic oxo acids (α -oxoglutarate, pyruvate, oxaloacetate). 3. The β -hydroxybutyrate/acetoacetate concentration ratios were generally much higher than in rat liver. 4. The concentration of total fat was sevenfold higher in the ketotic liver, and that of glucose plus glycogen fourfold lower than in normal liver. 5. The blood of ketotic cows showed a marked rise in the concentration of free fatty acids. 6. The activities of pyruvate carboxylase, propionyl-CoA carboxylase, phosphopyruvate carboxylase and fructose 1,6-diphosphatase showed no clear-cut differences between normal and ketotic animals. 7. Glucose injection promptly relieved the ketotic condition with respect to both the clinical and biochemical signs. The fall in the concentrations of the ketone bodies in the blood was preceded by a fall in the concentrations of free fatty acids and glycerol. 8. The findings are taken to be consistent with the concept that an increased rate of gluconeogenesis, causing a decrease in the concentration of oxaloacetate, is a major causal factor in ketogenesis.

The newer methods of enzyme assay and metabolite analysis, together with the clarification of the concepts on the nature of excessive ketogenesis, have made it possible to approach the biochemical problems of bovine ketosis from new angles. This paper reports measurements of the concentrations of ketone bodies and other metabolites in blood and liver of ketotic and normal lactating and non-lactating dairy cows together with assays of the activity of key liver enzymes. The selection of enzymes and metabolites was determined by the concept that excessive ketogenesis is related to excessive gluconeogenesis (see Krebs, 1966*a,b*). The results obtained are consistent with this concept.

EXPERIMENTAL

Materials. Substrates for enzyme assays and crystalline enzymes were obtained from Boehringer Corporation (London) Ltd. (London, W. 5), except for β -hydroxy-

butyrate dehydrogenase, which was prepared from *Rhodospseudomonas spheroides* by the method of Bergmeyer, Gawehn, Klotzsch, Krebs & Williamson (1967).

Animals. Healthy lactating and non-lactating stall-fed dairy cows were provided from the normal herds maintained at the Institute at Compton. They had been through at least one previous lactation. The non-lactating cows were not in calf.

Ketotic cows, if satisfying the following criteria, were purchased from farms in the neighbouring area, and transported to the Institute: clinical signs consistent with primary spontaneous ketosis, uncomplicated by endometritis, mastitis, or other pathogenic conditions likely to cause a secondary ketosis; a strongly positive Rothera test in the milk; a raised concentration of ketone bodies in the blood. In every case, the diagnosis was confirmed by the fact that the animals responded to glucose-infusion therapy.

Liver tissue. Liver tissue was removed through an incision in the right sub-lumbar fossa. The operation was carried out in the standing position under local anaesthesia provided by an infiltration of the tissues with Xylotox (diethylamino-2,6-dimethylacetanilide; Willows Francis Ltd., Epsom, Surrey). To obtain rapidly frozen liver tissue the following procedure was adopted. A hand was passed through the incision and forward into the peritoneal cavity to reach the caudate lobe of the liver, which projects

* Present address: Metabolic Research Laboratory, Nuffield Department of Clinical Medicine, Radcliffe Infirmary, Oxford.

posteriorly. After the liver capsule had been broken a small piece (about 3–5 g.) of tissue was grasped in the hand, quickly removed and clamped in aluminium tongs (Wollenberger, Ristau & Schoffa, 1960) that had previously been cooled in liquid N_2 . Between 2 and 3 sec. elapsed from removing the piece of liver to clamping it in the tongs. Further pieces of liver removed for the assay of enzymic activity (Table 4) and for the determination of some more stable liver constituents (Table 3) were placed in an ice-cooled container.

Tissue extraction. The metabolites listed in Table 2 were determined in extracts prepared from the rapidly frozen tissue. The freeze-clamped tissue was treated as described by Williamson, Lund & Krebs (1967a), except that Florisil treatment was omitted and several extracts were prepared from the freeze-clamped tissue. The supernatants from the extracts were filtered through glass wool to remove fatty material. Metabolite concentrations were then determined on the combined supernatants. Values are expressed as μ moles/g. fresh wt. of tissue.

The concentration of oxaloacetate in these extracts was found to be too low for determination by the micro-method of Löffler & Wieland (1963). A special procedure, involving preparation of a more concentrated extract, was therefore used, and is described below.

Blood. In many instances two blood samples were taken from the cow at the conclusion of the biopsy operation. For the first blood sample, approx. 10 ml. of blood was allowed to flow directly from the jugular vein into a weighed bottle containing 10 ml. of ice-cold 6% (w/v) $HClO_4$. The bottle was thoroughly shaken and placed in ice. Subsequently the bottle and contents were reweighed and centrifuged. The supernatant was filtered through paper into a graduated centrifuge tube and the volume noted before and after neutralization with 20% (w/v) KOH. Metabolite concentrations were measured in the neutralized supernatant after removal of the $KClO_4$ precipitate. Concentrations were expressed as μ moles/ml. of whole blood (i.e. mm), assuming that 1 ml. of blood weighed 1.06 g.

For the second blood sample, approx. 20 ml. of jugular-vein blood was collected in a bottle containing 4 mg. of heparin. The blood was mixed with the heparin and then kept ice-cold until the bottle and contents were centrifuged. The plasma was used for the determination of free fatty acids and glycerol.

Determination of metabolites. The methods used for determinations of metabolites were the following: lactate and pyruvate (Hohorst, Kreutz & Bücher, 1959); β -hydroxybutyrate and acetoacetate (Williamson, Mellanby & Krebs, 1962); α -oxoglutarate (Bergmeyer & Bernt, 1963); malate (Hohorst, 1963a); α -glycerophosphate (Hohorst, 1963b); fructose diphosphate and triose phosphate (Bücher & Hohorst, 1963). Pyruvate, α -oxoglutarate and acetoacetate were determined in the same cuvette by successive additions of lactate dehydrogenase, glutamate dehydrogenase and β -hydroxybutyrate dehydrogenase. Similarly, malate and α -glycerophosphate were determined in the same cuvette. For assay of amino acids the extracts were pretreated as described by Williamson, Lopes-Vieira & Walker (1967b). Aspartate was determined by the method of Pfeleiderer (1963), glutamate by the method of Bernt & Bergmeyer (1963), glutamine as glutamate after hydrolysis at 100° for 5 min. in the presence of 2N- H_2SO_4 followed by heating to 100° in the presence of N-KOH for 45 min., and alanine by

the method of Williamson *et al.* (1967b). Glucose and the sum of glucose and glycogen were measured by the glucose oxidase method (see Krebs, Bennett, de Gasquet, Gascoyne & Yoshida, 1963) as modified by Krebs, Dierks & Gascoyne (1964). Oxaloacetate was determined essentially by the method of Löffler & Wieland (1963) with the following modifications: 1 part of powdered freeze-clamped tissue was homogenized with 2 parts of frozen 6% $HClO_4$. A portion of the supernatant was adjusted directly to pH 12 with 2N-KOH and 1014 internal indicator (British Drug Houses Ltd., Poole, Dorset), and left for 30 min. in ice to precipitate the $KClO_4$. The supernatant was then incubated for 2 hr. at 30° to destroy endogenous acetyl-CoA; 30 min. at room temperature was found to be inadequate. For the conversion of oxaloacetate into citrate the incubation mixture was as follows: 1 ml. of 0.1 M-tris-HCl buffer, pH 7.4; 3 μ moles of [^{14}C]acetyl-CoA; 0.4–1.6 ml. of sample; 30 μ l. of citrate synthase (2 mg./ml.). Incubation was for 30–40 min. at 30°. Unchanged acetyl-CoA was again decomposed at pH 12 under the conditions described above (2 hr. at 30°). It proved inadequate for the removal of acetic acid to bubble the acidified solutions for 30 min. at 50° with N_2 . The temperature was raised to 65° and gassing was continued to dryness. This step considerably decreased the radioactivity in the blank. The two modifications of the method of Löffler & Wieland (1963), namely longer incubation for the decomposition of endogenous acetyl-CoA and more effective removal of acetic acid, considerably improve the accuracy of the method.

Free fatty acids in plasma were assayed by the method of Dole (1956) as modified by Salaman & Robinson (1961) and glycerol was assayed by the method of Garland & Randle (1962).

Enzyme assays. The CO_2 -fixing enzymes pyruvate carboxylase (EC 6.4.1.1), phosphopyruvate carboxylase (EC 4.1.1.32) and 'propionyl-CoA carboxylase' (i.e. $^{14}CO_2$ incorporation in the overall reaction propionate $\rightarrow$ succinate in which the latter enzyme catalyses the sole CO_2 -fixing reaction) were assayed by a method elaborated by L. V. Eggleston, W. Gevers, P. Lund & H. A. Krebs (unpublished work). Preliminary experiments to determine phosphopyruvate carboxylase in cow liver obtained from the abattoir indicated that the enzyme was largely present in the mitochondria. Homogenates were therefore prepared in distilled water and 1 ml. was treated ultrasonically for 1 min. in an MSE 100 w ultrasonic disintegrator; this was followed by centrifugation at 3000g for 5 min. to remove cell debris. The enzyme was assayed in the supernatant. There remained less than 10% of the activity in the sediment. Fructose diphosphatase was determined by the method of Krebs & Woodford (1965), and malate dehydrogenase and glutamate-oxaloacetate transaminase were determined essentially as described by Delbrück, Zebe & Bücher (1959). Activities are expressed as μ moles of substrate removed or product formed/min./g. wet wt. at 25°. Because of great differences in appearance and composition of liver from ketotic and normal animals, a direct comparison on the basis of wet weight alone was not immediately justified. Therefore various other criteria were taken into account. The dry weight of the tissue was determined to obtain the wet wt./dry wt. ratio, and the dried tissue was then repeatedly extracted with ether to constant weight, from which the total fat content could be calculated. Total liver nitrogen was determined by the micro-Kjeldahl method.

Table 1. *Physiological status, breed and blood ketone-body concentrations of the cows studied*

The normal animals were studied during the winter when the ketotic cows were available. 'Blood ketone bodies' refers to the sum of acetoacetate and β -hydroxybutyrate. Abbreviations used for the breed of cows are the following: A, Ayrshire; F, Friesian; G, Guernsey; A $\times$ F, Ayrshire $\times$ Friesian cross. Blood samples were taken immediately after the biopsy. The asterisk indicates that the cow was within the period of susceptibility to acetonaemia at the time of liver biopsy (i.e. within 10 weeks after calving; see Pearce, 1960). N.D., Not determined; —, not known.

Non-lactating cows				Normal lactating cows				Ketotic cows			
Serial no.	Interval after calving (weeks)	Breed	Blood ketone bodies (mm)	Serial no.	Interval after calving (weeks)	Breed	Blood ketone bodies (mm)	Serial no.	Approx. interval after calving (weeks)	Breed	Blood ketone bodies (mm)
1	42	A	0.26	1	26	A $\times$ F	N.D.	1	6	A	5.4
2	70	A	0.20	2	17	A	0.19	2	4	G	5.7
3	43	A	0.38	3*	3	A	N.D.	3	8	A	4.9
4	34	A	0.27	4*	6	A	0.36	4	3	F	2.3
				5	16	A	0.36	5	—	A	6.0
				6*	7	A	0.43	6	4	F	4.5
				7*	8	A	0.44				

Table 2. *Concentrations of metabolites in normal and ketotic bovine livers*

Concentrations of metabolites are expressed as μ moles/g. wet wt. of liver. The values are means $\pm$ S.D. with the numbers of observations in parentheses. In cases where only two values were obtained, both are given.

	Non-lactating cows	Normal lactating cows	Ketotic cows	Significance of difference between means of normal lactating and ketotic cows
β -Hydroxybutyrate	0.50 $\pm$ 0.14 (4)	0.51 $\pm$ 0.10 (7)	2.84 $\pm$ 0.88 (6)	$P < 0.001$
Acetoacetate	< 0.01 (2)	< 0.01 (7)	0.53 $\pm$ 0.30 (6)	$P < 0.001$
L-Malate	0.51 $\pm$ 0.13 (4)	0.53 $\pm$ 0.28 (7)	0.53 $\pm$ 0.31 (6)	$P > 0.9$
Oxaloacetate	2.25 $\times 10^{-3}$ $\pm$ 0.44 $\times 10^{-3}$ (4)	1.95 $\times 10^{-3}$ $\pm$ 0.10 $\times 10^{-3}$ (4)	0.65 $\times 10^{-3}$ $\pm$ 0.18 $\times 10^{-3}$ (3)	$P < 0.001$
L-Lactate	1.64 $\pm$ 0.40 (4)	1.37 $\pm$ 0.73 (7)	1.44 $\pm$ 0.88 (6)	$P > 0.8$
Pyruvate	0.065 $\pm$ 0.019 (4)	0.045 $\pm$ 0.015 (7)	0.026 $\pm$ 0.007 (6)	$P < 0.02$
α -Glycerophosphate	0.68 $\pm$ 0.09 (4)	0.73 $\pm$ 0.22 (7)	0.78 $\pm$ 0.18 (6)	$P > 0.6$
Triose phosphate	0.057, 0.072	0.040 $\pm$ 0.010 (3)	0.047 $\pm$ 0.007 (4)	$P > 0.4$
L-Glutamate	3.47 $\pm$ 1.34 (4)	3.06 $\pm$ 0.63 (7)	1.82 $\pm$ 0.55 (6)	$P < 0.01$
α -Oxoglutarate	0.112 $\pm$ 0.053 (4)	0.092 $\pm$ 0.028 (7)	0.044 $\pm$ 0.029 (5)	$P < 0.01$
L-Aspartate	0.46 $\pm$ 0.07 (4)	0.80 $\pm$ 0.53 (7)	0.76 $\pm$ 0.60 (6)	$P > 0.9$
L-Alanine	1.64 $\pm$ 0.66 (4)	1.57 $\pm$ 0.57 (7)	0.53 $\pm$ 0.39 (6)	$P < 0.01$
L-Glutamine	3.28 $\pm$ 0.24 (4)	2.56 $\pm$ 2.07 (7)	0.59 $\pm$ 0.37 (6)	$P < 0.05$
Fructose 1,6-diphosphate	0.060, 0.065	0.039 $\pm$ 0.019 (3)	0.026 $\pm$ 0.004 (4)	$P > 0.2$
β -Hydroxybutyrate/ acetoacetate ratio (mean)	> 50	> 51	5.3	
Lactate/pyruvate ratio (mean)	25	31	55	

Nitrogen was distilled as NH_3 into HCl and titrated against NaOH , with Tashiro indicator (Conway & Byrne, 1933). The method of Lowry, Rosebrough, Farr & Randall (1951) was used to determine tissue protein, with bovine serum albumin as standard. As protein/g. wet wt. was the same in the different physiological states, wet weight was adopted as the standard for comparison.

RESULTS

Metabolite concentrations in the liver. All determinations, with the exception of oxaloacetate in normal lactating and ketotic cows, were carried out

on the livers of the animals listed in Table 1. Oxaloacetate was determined separately in liver from a further four normal and three ketotic cows. The four normal lactating cows used for this determination were all within the period of susceptibility to ketosis. The concentrations of metabolites in the three physiological states are given in Table 2. No significant differences were detected between livers from non-lactating and normal lactating cows. For this reason 'normal' in the text refers to the normal lactating cow. Highly significant differences ($P < 0.001$) between normal

Table 3. *Carbohydrate, fat, protein and water content of livers from normal lactating and ketotic cows*

The concentrations of the constituents are expressed as mg./g. wet wt. of liver except for glucose + glycogen, which is expressed as mg. of glucose equivalent/g. wet wt. of liver. The values are means $\pm$ S.D. with the numbers of observations in parentheses, except for non-lactating cows where only two values were obtained and both are given.

	Non-lactating cows		Normal lactating cows	Ketotic cows	Significance of difference between means of normal lactating and ketotic cows
Glucose	3.76,	4.86	4.37 $\pm$ 0.95 (7)	2.47 $\pm$ 1.45 (6)	$P < 0.02$
Glucose + glycogen	19.5,	36.3	39.4 $\pm$ 22.5 (7)	10.1 $\pm$ 5.4 (6)	$P < 0.02$
Fat	10.1,	11.2	15.4 $\pm$ 5.5 (5)	112.4 $\pm$ 35.6 (6)	$P < 0.001$
Nitrogen	27.9,	30.7	29.7 $\pm$ 2.3 (7)	30.6 $\pm$ 4.65 (6)	$P > 0.9$
Protein	200,	210	185 $\pm$ 23.9 (7)	186 $\pm$ 26.2 (6)	$P > 0.9$
Wet wt./dry wt. ratio	3.5,	3.61	3.80 $\pm$ 0.30 (7)	3.05 $\pm$ 0.38 (6)	$P < 0.01$

and ketotic livers were found for acetoacetate, β -hydroxybutyrate and oxaloacetate. As the liver is the main site of ketone-body formation the large increase in the concentration of the ketone bodies was to be expected. A decrease in the concentration of oxaloacetate in the ketotic liver, on average to one-third of the normal value, is also expected in view of the parallel findings of Wieland, Weiss & Eger-Neufeldt (1964) on ketotic alloxan-diabetic rat liver. No significant differences ($P > 0.2$) were found for malate, lactate, α -glycerophosphate, triose phosphates, aspartate and fructose 1,6-diphosphate. Significant decreases ($P < 0.05$) were found for three amino acids, glutamate, glutamine and alanine, and for two α -oxo acids, pyruvate and α -oxoglutarate. A decrease in the concentration of glucogenic precursors and of amino acids and α -oxo acids has already been reported for alloxan-diabetes by Kirsten, Kirsten, Hohorst & Bücher (1961), but was contradicted by Roberts & Simonsen (1962). It was definitely established for alloxan-diabetic rats by Williamson *et al.* (1967b).

Whereas in the normal and in the ketotic alloxan-diabetic rat liver the β -hydroxybutyrate/acetoacetate concentration ratio is between 2 and 3 (Williamson *et al.* 1967a), the mean ratio in acetonaemic cow liver was higher, at 5.3. It was much lower than in the normal controls though the calculated value of at least 51 must be regarded as approximate because the determination of acetoacetate was not accurate on account of its very low concentration.

Carbohydrate, fat, protein and water content of the liver. In accordance with the earlier work of Shaw (1956) a highly significant difference ($P < 0.001$) in the fat content of normal and ketotic liver was found (Table 3). The mean fat content was sevenfold higher in the ketotic liver, and presumably as a direct consequence of this the wet wt./dry wt. ratio was significantly ($P < 0.01$) lower in the ketotic

liver (3.05) than in the normal liver (3.80). The carbohydrate content was also significantly ($P < 0.02$) lower in the ketotic liver, the mean value for glucose plus glycogen being 10.1, against 39.4 mg./g. fresh wt. in the normal liver. There were no differences in the protein and nitrogen content.

Key enzymes of gluconeogenesis. Propionyl-CoA carboxylase, pyruvate carboxylase, phosphopyruvate carboxylase and fructose 1,6-diphosphatase were assayed because they catalyse stages of the pathway of gluconeogenesis. Malate dehydrogenase and aspartate aminotransferase were included because they are taken to play a special role in gluconeogenesis in connexion with the transfer of reducing equivalents from mitochondria to cytoplasm (Borst, 1963; Lardy, Paetkau & Walter, 1965; Krebs, Gascoyne & Notton, 1967). Though the mean values for all the enzymes except for fructose diphosphatase (Table 4) were somewhat higher in the ketotic cows the significance of the differences between the normal lactating cows and the ketotic cows is doubtful, with the possible exception of pyruvate carboxylase ($P < 0.05$).

As the values for non-lactating cows refer to two animals only, further assays are required to assess the significance of the apparent rise in the activities of the enzymes, other than fructose diphosphatase, above the activities observed in normal lactating cows.

Metabolites in the blood. The concentrations in the blood of most of the metabolites tested (Table 5) were similar to those in the liver, and the differences between the normal and ketotic liver were also to some extent encountered between normal and ketotic blood. Thus the concentration of the ketone bodies was about 13-fold higher in the ketotic than in the normal blood and the concentration of glucose was somewhat lower in the ketotic blood than in normal blood. The lactate/pyruvate concentration ratio and the β -hydroxybutyrate/

Table 4. *Activities of enzymes related to gluconeogenesis in normal and ketotic livers*

Enzyme activity is expressed as μ moles of substrate consumed or product formed/min./g. wet wt. of liver at 25°. The values are means $\pm$ s.d. with the numbers of observations in parentheses, except for non-lactating cows, where only two values were obtained. 'Endogenous' values represent $^{14}\text{CO}_2$ incorporation in the absence of added substrate in the propionyl-CoA carboxylase and pyruvate carboxylase assays.

Enzyme	Non-lactating cows		Normal lactating cows		Ketotic cows	Significance of difference between means of normal lactating and ketotic cows
Endogenous	0.25,	0.25	0.23 $\pm$	0.08 (6)	0.35 $\pm$ 0.18 (6)	$P > 0.1$
Propionyl-CoA carboxylase	1.04,	1.36	0.72 $\pm$	0.43 (6)	1.05 $\pm$ 0.22 (6)	$P > 0.1$
Pyruvate carboxylase	1.18,	1.35	0.76 $\pm$	0.37 (6)	1.34 $\pm$ 0.46 (6)	$P < 0.05$
Phosphopyruvate carboxylase	1.17,	1.43	0.99 $\pm$	0.34 (7)	1.05 $\pm$ 0.50 (6)	$P > 0.7$
Fructose 1,6-diphosphatase	1.40,	2.53	4.86 $\pm$	1.06 (6)	4.00 $\pm$ 2.50 (6)	$P > 0.4$
Malate dehydrogenase	85.0,	123	81.7 $\pm$	46.3 (6)	94.0 $\pm$ 40.6 (6)	$P > 0.6$
Glutamate-oxaloacetate transaminase	71.0,	67.0	64.3 $\pm$	14.0 (6)	70.0 $\pm$ 19.7 (6)	$P > 0.6$

Table 5. *Metabolites in the blood of normal and ketotic cows*

The concentrations of free fatty acid and glycerol are expressed as μ moles/ml. of plasma. The concentrations of the other substances are expressed as μ moles/ml. of whole blood. The values are means $\pm$ s.d. with the numbers of observations in parentheses. In cases where only two values were obtained, both are given.

	Non-lactating cows	Normal lactating cows	Ketotic cows	Significance of difference between means of normal lactating and ketotic cows
β -Hydroxybutyrate	0.27 $\pm$ 0.07 (4)	0.34 $\pm$ 0.09 (5)	3.95 $\pm$ 1.05 (6)	$P < 0.001$
Acetoacetate	0.011 $\pm$ 0.006 (4)	0.019 $\pm$ 0.011 (5)	0.85 $\pm$ 0.32 (6)	$P < 0.001$
L-Lactate	1.55 $\pm$ 0.19 (4)	1.70 $\pm$ 1.08 (3)	1.10 $\pm$ 0.84 (6)	$P > 0.3$
Pyruvate	0.088 $\pm$ 0.012 (4)	0.091 $\pm$ 0.034 (3)	0.046 $\pm$ 0.03 (6)	$P > 0.05$
α -Oxoglutarate	0.011 $\pm$ 0.002 (4)	0.014 $\pm$ 0.003 (4)	0.017 $\pm$ 0.0044 (6)	$P > 0.3$
Glucose	3.64, 6.78	4.50 $\pm$ 0.93 (3)	3.22 $\pm$ 0.79 (6)	$P > 0.05$
Free fatty acids	1.33, 1.49	0.39, 1.20	2.25 $\pm$ 0.27 (6)	
Glycerol	0.08, 0.11	0.078, 0.120	0.153 $\pm$ 0.033 (5)	

Table 6. *Changes in the concentration of blood constituents on glucose treatment of a ketotic cow*

The values given are those obtained for ketotic cow no. 2. The concentrations of glucose and ketone bodies are expressed as μ moles/ml. of whole blood and the concentrations of free fatty acids and glycerol as μ moles/ml. of plasma.

Time after start of glucose treatment (hr.) ...	0	24	48	72
β -Hydroxybutyrate + acetoacetate	5.65	3.72	0.74	0.52
β -Hydroxybutyrate/acetoacetate ratio	5.48	7.85	8.25	51
Glucose	2.81	2.56	3.02	3.5
Free fatty acids	2.32	0.51	0.43	0.22
Glycerol	0.164	0.04	0.05	0.04

acetoacetate concentration ratio differed between normal and ketotic blood in a manner similar to that found in the liver. The concentration of the free fatty acids was increased in the blood of ketotic cows, as it is in other forms of ketosis.

Effect of glucose treatment. The ketotic cows were given subcutaneous injections of glucose beginning on the day of biopsy. A single daily dose of 900 ml. of aq. 20% (w/v) glucose solution (Willows Francis Ltd.) was given until the cows became normal, as

judged by clinical signs and blood ketone-body concentrations. Usually they were normal after 4 days, but some improvement was already evident after 24 hr. With two of the ketotic cows (nos. 2 and 5) the concentrations of ketone bodies, glucose, free fatty acids and glycerol in the blood were measured daily. The blood was collected before the administration of glucose. The results obtained with one of the ketotic cows are recorded in Table 6. They were very similar in the other case. The most rapid change, already very striking after 24 hr., was in the concentrations of free fatty acids and glycerol, which fell below the concentrations found in the normal lactating cow (cf. Table 5). The concentration of ketone bodies had also fallen after 24 hr., but the normal concentration was approached only after 48 hr. The concentration of glucose rose slowly. The β -hydroxybutyrate/acetoacetate concentration ratio rose gradually and reached the range of the normal lactating cow after 72 hr.

DISCUSSION

Intermediary metabolites in the ketotic liver. The special features observed in the ketotic liver fall into two categories. One is the increased concentration of ketone bodies, which, of course, is expected as a part of the acetonæmic syndrome. The other is the fall in the concentration of glucogenic amino acids (glutamate, glutamine, alanine) and glucogenic oxo acids (oxaloacetate, pyruvate, α -oxoglutarate). This is in accordance with the concept that gluconeogenesis is increased in bovine ketosis, as it is in the ketosis accompanying severe alloxan-diabetes. The striking fall in the concentration of oxaloacetate in the ketotic liver to, on average, one-third of the controls, together with the fact that the oxaloacetate concentrations lie in the region ($1\mu\text{M}$) where the rate of reaction of citrate synthase is almost directly proportional to the oxaloacetate concentration, is consistent with the view that lack of oxaloacetate, by diversion to gluconeogenesis, is a cause of ketosis (see Krebs, 1966*a,b*). Why it is the exception rather than the rule that ketosis occurs in lactating cows depends on factors, such as diet and hormonal balance, that are not the concern of this paper.

Capacity of key enzymes in the ketotic liver. There is little difference in the capacities of the key enzymes of gluconeogenesis. However, the activity of these enzymes may be controlled by substrate availability and allosteric effectors, such as acetyl-CoA, which is known to regulate the activity of pyruvate carboxylase (Utter & Keech, 1963).

β -Hydroxybutyrate/acetoacetate concentration ratio. The values of this ratio found in blood and liver are much higher than those of normal and ketotic rats. As the activity of β -hydroxybutyrate dehydro-

genase is very low in cattle liver (less than one-thirtieth of that in rat liver; M. Stubbs, unpublished work) the substrates of the enzyme are in all probability not in equilibrium with the mitochondrial NAD couple in the liver. Other cattle tissues, e.g. rumen epithelium, heart muscle, kidney cortex, skeletal muscle, contain β -hydroxybutyrate dehydrogenase and therefore influence the β -hydroxybutyrate/acetoacetate concentration ratio in the blood.

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