FACTORS CONTROLLING THE CONCENTRATION OF MITOCHONDRIAL OXALOACETATE IN LIVER DURING SPONTANEOUS BOVINE KETOSIS*

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It has been suggested that a major factor in the increased synthesis of acetoacetate and β-hydroxybutyrate in ketosis is a lower level of mitochondrial oxaloacetate (Wieland et al.,1964) perhaps owing to an increase in gluconeogenesis (Krebs,1966). Citrate formation by citrate synthase would thereby be impaired and would be accompanied by an increase in acetyl CoA concentration (Wieland et al.,1964). Although measurements of intermediates in the liver of ketotic rats may show an increase in acetyl CoA and a decrease in oxaloacetate (Wieland et al.,1964), some caution in interpretation is appropriate as these determinations do not distinguish between the mitochondria and other cellular compartments, and furthermore, calculations of the mitochondrial oxaloacetate levels from the β-hydroxybutyrate-acetoacetate couplet indicate an increase rather than a decrease during ketosis (Williamson et al., 1967). It is useful to examine this hypothesis in bovine ketosis, not only because the disorder occurs spontaneously, but since the main gluconeogenic precursor in these animals is propionate rather than lactate.

The results of this investigation imply that ketosis is associated with diminished citrate synthesis, as indicated by lower citrate and higher lactate, pyruvate and malate levels. These findings together with measurements of the activities of enzymes involved in the mitochondrial formation and removal of oxaloacetate are best explained by an inhibition of citrate synthase.

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MATERIALS AND METHODS

Six cows diagnosed to have primary uncomplicated ketosis were used for these studies. A liver biopsy was taken and then the animal was treated with dexamethasone by intramuscular injection to relieve the ketosis. A second liver biopsy was taken when the packed cell volume, plasma concentrations of ketone bodies, free fatty acids and glucose, and the milk production indicated that the cow was completely recovered.

Liver samples were homogenized in buffered isotonic sucrose (Henning et al., 1966). Supernatant and particulate fractions were prepared and pyruvate carboxylase and phosphoenolpyruvate carboxykinase were assayed as described previously (Ballard & Hanson, 1967). Citrate synthase (Ochoa, 1955a) and NAD-malate dehydrogenase (Ochoa, 1955b) were assayed spectrophotometrically.

A portion of the biopsy sample was immediately placed in liquid dichlorodifluoromethane for the determination of metabolic intermediates. Perchloric acid extracts of the frozen tissue were prepared and neutralized as recommended by Hohorst (1965a) and used for the measurements of lactate (Hohorst, 1965a), malate (Hohorst 1965b), citrate and pyruvate. Citrate and pyruvate were measured sequentially by a fluorometric modification of the method of Dagley (1965). Oxaloacetate and acetoacetate were assayed according to Kalnitsky and Tapley (1958) and β-hydroxybutyrate by the method of Williamson et al. (1962).

NAD and NADH were measured as described by Bassham et al. (1959). The exact procedure for the extraction of nucleotides from liver, and minor modifications in the assays have been described previously (Kronfeld & Raggi, 1964).

RESULTS AND DISCUSSION

Krebs (1966) has suggested that bovine ketosis may be explained in part by an increase in the activity of hepatic phosphoenolpyruvate carboxykinase relative to pyruvate carboxylase. This would result in a depletion of oxaloacetate levels as well as an increase in gluconeogenesis. The theory was supported by data derived from alloxan diabetic rat liver in which phosphoenolpyruvate carboxykinase activity is markedly increased. In the present study we have sought to establish the importance of changes in activity of this enzyme as well as several others involved in the synthesis or utilization of oxaloacetate and have measured these enzymes in the livers of spontaneously ketotic and normal cows. The results of these measurements (Table 1) indicate that neither pyruvate carboxylase nor phosphoenolpyruvate carboxykinase in either the soluble or particulate fractions increases significantly in

activity during spontaneous ketosis (Table 1). It is not surprising that phosphoenolpyruvate carboxykinase activity does not increase in the spontaneously ketotic cow liver since the activity is already substantially higher than that noticed for the combined particulate and soluble fractions of rat liver (2.7 units/g for the rat as against 10.2 for the cow, Ballard and Hanson, 1967).

Table I

Enzyme activities in liver from spontaneously ketotic and normal cows.

Enzyme	Spontaneously Ketotic	Normal
NAD-malate dehydrogenase, particulate	85.5* ± 30.5	242 ± 74
Citrate synthase, particulate	1.20 ± 0.04	0.99 ± 0.21
Phosphoenolpyruvate carboxykinase, particulate	4.24 ± 0.82	5.46 ± 0.83
Phosphoenolpyruvate carboxykinase, supernatant	4.36 ± 0.96	4.73 ± 0.69
Pyruvate carboxylase, particulate	6.96 ± 0.47	8.28 ± 2.29
Pyruvate carboxylase, supernatant	2.45 ± 0.73	2.62 ± 0.45

Activities are expressed as μmoles per min. per g. liver plus or minus the standard error of the mean. All assays were carried out at 37°. Values significantly different (5% probability level) from the normal are indicated by an asterisk.

The only enzyme that changes significantly during spontaneous ketosis and that could also contribute to the levels of mitochondrial oxaloacetate was NAD malate dehydrogenase. The activity of this enzyme markedly decreased. Our findings thus suggest that if oxaloacetate levels are involved in the etiology of bovine ketosis only the conversion of malate to oxaloacetate via NAD-malate dehydrogenase would be affected by changes in the activity of the four enzymes involved in its synthesis or utilization. It should be pointed out, however, that the rat and the cow differ in the source of mitochondrial oxaloacetate. In the rat, pyruvate carboxylation via pyruvate carboxylase is the major pathway of synthesis, while in the ruminant liver the conversion of propionate to oxaloacetate intra-mitochondrially is the major pathway. Thus a reduction in the activity of NAD-malate dehydrogenase in cow liver during ketosis could lead to a reduction in the production and possibly also the concentration of oxaloacetate.

As shown in Table II, there was an increase in lactate and pyruvate, no change in malate and oxaloacetate and a sharp decrease in citrate concentration

in the ketotic as compared with the normal cow liver. The finding that oxaloacetate concentrations in whole liver remain unchanged during ketosis is in agreement with the work of Shaw and Tapley (1958) and in contrast to the results of Wieland et al. (1964) with normal and diabetic rat liver.

The important point to be resolved is whether or not the mitochondrial rather than the total cellular concentration of oxaloacetate changes during ketosis. If the β-hydroxybutyrate-acetoacetate couplet is used to calculate the intramitochondrial oxaloacetate levels as described by Williamson et al. (1967) there is no difference between spontaneously ketotic and normal animals (Table II).

Table II

Levels of intermediates in liver from spontaneously ketotic and normal cows.

Intermediates	Spontaneously Ketotic	Normal
Citrate	71.6 ± 10.3	209 ± 21
Oxaloacetate	14 ± 5	12 ± 2
Malate	463 ± 62	319 ± 55
Pyruvate	51.8 ± 5.7	33.7± 5.1
Lactate	1774 ± 183	1178 ±140
β-hydroxybutyrate	1831* ± 315	409 ± 71
Acetoacetate	280* ± 18	68.2± 16.1
NAD^+	230 ± 37	267 ± 43
NADH	183 ± 38	237 ± 55
NAD^+/NADH (from lactate/pyruvate)	269	261
NAD^+/NADH (from β-hydroxybutyrate/ acetoacetate)	3.1	3.4
NAD^+/NADH (observed)	1.4	1.2

Concentrations are expressed as mμmoles per g. liver plus or minus the standard error of the mean. Values significantly different from the normal are indicated by an asterisk. The ratios of NAD^+ to NADH determined from the lactate pyruvate or β-hydroxybutyrate-acetoacetate couplets are calculated using the respective equilibrium constants reported by Williamson et al (1967).

If the assumptions upon which these calculations are based are correct, and the levels of oxaloacetate in the mitochondria do not change in spontaneous ketosis then the reduction in the citrate concentration cannot be explained by a lower concentration of oxaloacetate available for condensation with acetyl CoA. Since the citric acid cycle is not impaired during bovine ketosis (Ballard et al., unpublished observations), and the alternate pathway for removal of citrate is not functional since the cow liver lacks significant activity of ATP-citrate

lyase (Hanson and Ballard, 1967), it is conceivable that control of citrate formation could be exerted directly on the enzyme citrate synthase. Although the maximum velocity of citrate synthase was not impaired in bovine ketosis, it is possible that in vivo the enzyme is inhibited by long chain acyl CoA esters (Wieland et al., 1964). If this were the case one would predict that during ketosis there would be no decrease in oxaloacetate, a decrease in citrate, and higher lactate and pyruvate levels. This prediction is consistant with our findings.

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