Activity of Selected Gluconeogenic and Lipogenic Enzymes in Bovine Rumen Mucosa, Liver and Adipose Tissue

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(Received 11 February 1969)

The activities of phosphoenolpyruvate carboxykinase, 'malic enzyme', citrate-cleavage enzyme and glucose 6-phosphate dehydrogenase were assayed in homogenates of rumen mucosa, liver and adipose tissue of cattle. Rumen mucosa cytoplasm contained activities of 'malic enzyme' approximately sevenfold those of phosphoenolpyruvate carboxykinase, suggesting that the conversion of propionate into lactate by rumen mucosa involves 'malic enzyme'. Neither starvation for 8 days nor feeding with a concentrate diet for at least 3 months before slaughter produced enzyme patterns in the tissues different from those in cattle given only hay, except that the all-concentrate diet caused increased activities of glucose 6-phosphate dehydrogenase and 'malic enzyme' in adipose tissues. Rumen mucosa, liver and adipose tissue contained phosphoenolpyruvate carboxykinase activity. 'Malic enzyme' was absent in liver. Citrate-cleavage enzyme activity was present in liver and adipose tissue but was quite low in rumen mucosa. Liver contained much less glucose 6-phosphate dehydrogenase activity than rumen mucosa or adipose tissue.

Although the general metabolic capabilities of rumen mucosa have been much studied, only a limited amount of research has been done on the enzyme composition of this tissue. Incubations of rumen mucosa with propionate in an atmosphere of oxygen + carbon dioxide (95:5) were shown to lead to a major accumulation of lactate (Pennington & Sutherland, 1956; Taylor & Ramsey, 1965). Results of preliminary work in our laboratory, which agree with these findings, show a linear relationship between propionate uptake and lactate production, with lactate accounting for approx. 50% of the propionate disappearance at any given time. After the metabolic formation of succinyl-CoA from propionate, lactate could be synthesized by the decarboxylation of either oxaloacetate or malate. Hence two different pathways could be involved: decarboxylation of oxaloacetate to form phosphoenolpyruvate, requiring the enzyme phosphoenolpyruvate carboxykinase (EC 4.1.1.32), a key gluconeogenic enzyme (Scrutton & Utter, 1968), or decarboxylation of malate to form pyruvate, requiring 'malic enzyme' (EC 1.1.1.40), which is involved in extramitochondrial lipogenesis (Goodridge, 1968; Hanson & Ballard, 1968; Wise & Ball, 1964; Young, Shrago & Lardy, 1964).

The first objective of the present research was to evaluate the activity of phosphoenolpyruvate carboxykinase and 'malic enzyme' in rumen

mucosa and the second was to compare the activity of these and other enzymes in rumen mucosa, liver and adipose tissue from cattle on widely differing diets.

MATERIALS AND METHODS

Experimental animals. Tissues were collected from 20 cattle in two independent experiments. Five animals were 14–17-month-old Holstein females that had received an alfalfa-hay diet for at least 5 months (group 1), and five others (group 2) were similar except for having been starved 8 days before slaughter (Cook, McGilliard & Richard, 1968). Ten animals were 8–10-month-old Holstein steers, of which five had received an alfalfa-hay diet (group 3) and the other five (group 4) had received a diet of concentrates for at least 3 months before slaughter (Weigand, Young & Jacobson, 1967). The concentrate ration was composed of 80% ground corn, 15.5% soya-bean meal and 4.5% limestone and contained 14% (calculated) protein.

Tissue preparation. At slaughter a sample of adipose tissue, attached to the rumen, and a sample of liver were removed and placed in cold 0.25 M-sucrose. A segment of heavily papillated rumen wall was removed, washed in warm tap water, placed in Krebs-Ringer bicarbonate buffer (Hodson, Thomas, McGilliard, Jacobson & Allen, 1967) at 39° and immediately transported to the laboratory, where papillae were removed by shaving.

The liver and adipose tissue were homogenized in 0.25 M-sucrose (10%, w/v) with a glass homogenizer and Teflon pestle. The rumen papillae were frozen in liquid N₂ and pulverized in a stainless-steel mortar and pestle cooled in

liquid N₂ (Iehhponani & Johnson, 1965). The frozen powder was sifted through a 30-mesh stainless-steel screen and homogenized as described for liver and adipose tissue. All homogenates were centrifuged at 1° for 1 hr. at 105000g in a Spinco model L-2 ultracentrifuge (no. 40 rotor). The resulting supernatant solutions were stored at -15° for several weeks before analysis. All the enzymes had been shown to be stable to the storage conditions.

Enzyme assays. 'Malic enzyme' was assayed by the procedure of Ochoa (1955), glucose 6-phosphate dehydrogenase by that of Kornberg & Horecker (1955) and the citrate-cleavage enzyme by that of Srere (1962). Controls without substrate were run for all assays. Phosphoenolpyruvate carboxykinase was assayed by the method of Nordlie & Lardy (1963) with minor modifications (Young *et al.* 1964).

Liver and rumen mucosa supernatants were analysed for protein by the biuret method (Layne, 1957). Protein in adipose-tissue supernatants was determined by the method of Lowry, Rosebrough, Farr & Randall (1951) because sucrose interfered with the biuret method at low protein concentrations.

Purification of phosphoenolpyruvate carboxykinase from rumen mucosa. To verify the presence of phosphoenolpyruvate carboxykinase in rumen mucosa, a procedure for partial purification was developed. Rumen papillae, obtained from an abattoir, were prepared as described above (but diluted 1:5, w/v) or by homogenizing freshly shaved papillae for 2 min. in a VirTis 45 homogenizer operated at top speed. All subsequent procedures were at 1-4°. The 105000g supernatant solution was treated with calcium phosphate gel as described by Holten & Nordlie (1965) for soluble phosphoenolpyruvate carboxykinase. For each 3.6 mg. of protein 1 mg. of gel was added. The pH was adjusted to 6.8 with 1 M-acetic acid and the suspension centrifuged at 5000g for 20 min. To this supernatant 1 mg. of additional gel was added for each 8.6 mg. of protein present in the initial 105000g supernatant. The pH was adjusted to 6.5 and the centrifugation was repeated. The supernatant was dialysed against 2 mM-tris-HCl buffer, pH 7.5, for a minimum of 64 hr. After dialysis the preparation was concentrated by freeze-drying.

Results of the purification procedure are summarized in Table 1. The treatment with calcium phosphate gel

Table 1. *Purification of rumen mucosa phosphoenolpyruvate carboxykinase*

The assay mixture contained: GSH, 1.6 μ moles; oxaloacetate, 6.7 μ moles; ITP, 9.0 μ moles; MgCl₂, 22.5 μ moles; KF, 20.0 μ moles; tris, 44.0 μ moles. The final volume was either 1.5 or 2.0 ml. depending on the volume of enzyme preparation used. Incubations were for 7 min.

Enzyme preparation	Enzyme volume for assay (ml.)	P _i (μ moles/assay)			Protein (mg./ml.)	Specific activity (nmoles of phosphoenolpyruvate/min./mg. of protein)
		Background plus phosphoenolpyruvate	Background	Phosphoenolpyruvate		
105000g supernatant from rumen mucosa	0.5	3.54	3.40	0.14	7.7	5.2
Gel 1 supernatant	0.5	2.95	2.78	0.17	5.9	8.2
Gel 2 supernatant	0.5	2.89	2.72	0.17	5.0	9.7
Remains after 20 hr. dialysis	0.5	1.47	1.35	0.12	3.8	9.0
Remains after 64 hr. dialysis	1.0	1.25	1.04	0.21	3.5	8.6

Table 2. *Dependence of concentrated rumen mucosa phosphoenolpyruvate carboxykinase on substrate composition*

The assay mixture was as described for Table 1 with total volume 2.0 ml. The enzyme used was 1 ml. of purified rumen mucosa phosphoenolpyruvate carboxykinase that had been concentrated by freeze-drying to contain 25.8 mg. of protein/ml.

Omitted reaction components	Sources of P _i in assays ...	P _i (μ moles/assay)		
		Total	Background	Phosphoenolpyruvate
No omissions		2.35	1.88	0.47
Oxaloacetate		1.72	1.71	0.01
ITP		0.54	0.52	0.02
Oxaloacetate and ITP		0.24	0.27	0
MgCl ₂		1.14	1.08	0.06
Enzyme		0.73	0.73	0

approximately doubled the specific activity but had little effect on the 'background' inorganic phosphate values. Extended dialysis decreased the 'background' phosphate value without appreciably altering specific activity.

As shown in Table 2, the formation of phosphoenolpyruvate is completely dependent on oxaloacetate and ITP, thus proving that the enzyme activity is phospho-

enolpyruvate carboxykinase. The reaction requires Mg^{2+} . The results also show that the high background phosphate value was related to an 'inosine triphosphatase' activity because the background value was much lower when ITP was omitted from the assay mixture.

The relationship of enzyme activity to pH is shown in Fig. 1. The optimum pH is approx. 8.4, which agrees with the values reported for guinea-pig liver phosphoenolpyruvate carboxykinase by Holten & Nordlie (1965). However, there was very low activity below pH 8.0, resulting in a relatively narrow band of activity in the pH-activity curve. The cattle liver cytoplasmic phosphoenolpyruvate carboxykinase was also studied, and exhibited a broad pH-activity curve with maximum activity at pH 8.4.

Fig. 1. Phosphoenolpyruvate carboxykinase activity and pH of the reaction mixture. The basic assay mixture was that given for Table 2. The enzyme source was 1 ml. of dialysed preparation.

RESULTS

The average activities for each of the enzymes in each of the tissues are summarized for the four treatments in Table 3. Although groups 1 and 3 received the same diet, there were age and sex differences, so the groups were not combined.

Mucosal conversion of propionate into lactate. The results in Table 3 show that rumen mucosa cytoplasm had 'malic enzyme' activity averaging approx. seven times that of phosphoenolpyruvate carboxykinase. This suggests that the following metabolic pathway predominates *in vivo*:

Table 3. Activities of phosphoenolpyruvate carboxykinase, 'malic enzyme', citrate-cleavage enzyme and glucose 6-phosphate dehydrogenase in rumen mucosa, liver and adipose tissue of cattle on different diets

Each value represents an average from five animals, with the exception of results for rumen and liver samples from animals of group 1, which represent four animals. An analysis of variance for effects of treatment was carried out for each enzyme in each tissue. If *F* values were significant at the 5% level, Duncan's multiple range comparisons were made (Steel & Torrie, 1960). In the horizontal lines values with the same superscripts (a or b) are not significantly different from each other. Absence of superscripts indicates that there were no significant effects of treatment. Each value is given as mean $\pm$ S.E.M.

Enzyme	Organ	Group 1 (females given hay)	Group 2 (females given hay and starved)	Group 3 (steers given hay)	Group 4 (steers given concentrates)
Phosphoenolpyruvate carboxykinase (nmol of phospho- enolpyruvate/min./ mg. of protein)	Rumen	3.72 $\pm$ 0.88 ^{a,b}	1.50 $\pm$ 0.35 ^a	4.74 $\pm$ 0.98 ^b	5.70 $\pm$ 1.09 ^b
	Liver	62.15 $\pm$ 4.42	48.26 $\pm$ 4.07	56.56 $\pm$ 4.50	49.74 $\pm$ 4.43
	Adipose tissue	69.84 $\pm$ 19.92	69.57 $\pm$ 29.64	46.33 $\pm$ 34.44	53.85 $\pm$ 8.06
'Malic enzyme' (nmol of NADPH/ min./mg. of protein)	Rumen	33.93 $\pm$ 6.15 ^a	18.75 $\pm$ 3.62 ^b	28.21 $\pm$ 0.89 ^{a,b}	24.47 $\pm$ 3.08 ^{a,b}
	Liver	0.14 $\pm$ 0.08 ^a	0.21 $\pm$ 0.03 ^a	0.44 $\pm$ 0.13 ^b	0.63 $\pm$ 0.15 ^b
	Adipose tissue	1.92 $\pm$ 0.94 ^a	3.43 $\pm$ 1.11 ^a	4.13 $\pm$ 1.11 ^a	10.07 $\pm$ 3.16 ^b
Citrate-cleavage enzyme (nmol of NADP/ min./mg. of protein)	Rumen	1.63 $\pm$ 0.64	2.38 $\pm$ 0.41	2.89 $\pm$ 0.50	1.89 $\pm$ 0.22
	Liver	5.74 $\pm$ 0.54	6.87 $\pm$ 0.48	5.93 $\pm$ 0.33	6.63 $\pm$ 2.70
	Adipose tissue	21.7 $\pm$ 8.90	12.1 $\pm$ 3.1	11.6 $\pm$ 1.8	6.76 $\pm$ 4.1
Glucose 6-phosphate dehydrogenase (nmol of NADPH/ min./mg. of protein)	Rumen	20.21 $\pm$ 3.13	19.80 $\pm$ 2.82	12.88 $\pm$ 0.83	15.81 $\pm$ 1.30
	Liver	2.67 $\pm$ 0.05	3.26 $\pm$ 0.12	3.24 $\pm$ 0.28	2.59 $\pm$ 0.27
	Adipose tissue	5.52 $\pm$ 4.95 ^a	13.06 $\pm$ 4.55 ^a	26.29 $\pm$ 9.44 ^a	171.90 $\pm$ 60.64 ^b

However, higher apparent activity for the 'malic enzyme' is not conclusive proof that this enzyme has the dominant role.

Enzyme adaptations. (a) Phosphoenolpyruvate carboxykinase. Neither liver nor adipose tissue phosphoenolpyruvate carboxykinase activity was altered significantly by starvation or by the high-carbohydrate diet. In rats, either starvation or variations in diet usually produce marked changes in liver phosphoenolpyruvate carboxykinase values. The presence of phosphoenolpyruvate carboxykinase in adipose tissue from rats and mice was only recently reported (Ballard, Hanson & Leveille 1967; Chakrabarty & Leveille, 1968). Because the activity of phosphoenolpyruvate carboxykinase in rumen mucosa was relatively low and difficulties were encountered in assaying the crude preparations, no biological significance can be attached to differences in values for rumen mucosa phosphoenolpyruvate carboxykinase.

(b) 'Malic enzyme'. Rumen mucosa cytoplasm had much higher 'malic enzyme' activity than either liver or adipose tissue. Heifers of group 1, which were not starved, gave values that were higher than those of group 2, which were starved. Starvation of rats, causing a decreased lipogenesis, also results in lower 'malic enzyme' activity for liver and adipose tissue (Young *et al.* 1964).

'Malic enzyme' activity was exceptionally low or absent in liver, confirming other work (Ballard, Hanson, Kronfeld & Raggi, 1968; Hanson & Ballard, 1968; Somasundaram, Hibbs & Conrad, 1965). Hence the differences would seem to be of little biological importance. Values for 'malic enzyme' in adipose tissue were relatively low, in contrast with activities in rats (Young, 1968; Young *et al.* 1964), and these differences might be even greater if the biuret method, overestimating the protein, had not been used in the earlier work. The significant point for adipose tissue, however, is that values for 'malic enzyme' in animals of group 4 (all-concentrate diet), were significantly higher than those for animals in other groups.

(c) Citrate-cleavage enzyme. The various dietary treatments caused no significant changes in the activity of citrate-cleavage enzyme in any of the tissues. However, we found definite activity for this enzyme in the liver preparation, whereas Hardwick (1966) and Hanson & Ballard (1967, 1968) found that citrate-cleavage enzyme activity in the liver of lactating cows is very low or absent.

(d) Glucose 6-phosphate dehydrogenase. There were no statistically significant differences among groups in the activity for glucose 6-phosphate dehydrogenase in either rumen mucosa or liver. However, glucose 6-phosphate dehydrogenase activity in the rumen mucosa was much greater than in the liver. The adipose-tissue samples of animals in

group 4 (all-concentrate diet) had much higher glucose 6-phosphate dehydrogenase activity than animals in any of the other three groups.

DISCUSSION

The presence of 'malic enzyme' (NADP-dependent malate dehydrogenase) was demonstrated histochemically in bovine rumen mucosa by de Lahunta (1965), but its potential role in propionate metabolism was not evaluated. Our present study strongly suggests that this enzyme plays a major role in converting propionate into lactate in rumen mucosa. Leng, Steel & Luick (1967) have suggested extensive conversion of propionate into lactate *in vivo*, probably by rumen mucosa. The advantage of the conversion, however, is difficult to understand. A summary of the reactions involved is:

Assuming that two high-energy phosphates can be produced from the FADH_2 , the conversion of propionate into lactate then gives a net yield of one high-energy phosphate bond. This process would be less efficient in supplying energy to the rumen tissue than the conversion of butyrate into β -hydroxybutyrate, which produces the equivalent of three high-energy phosphates in one enzymic step. Possibly this conversion provides a means of conserving C_3 compounds for gluconeogenesis, since lactate is not oxidized by muscle, whereas propionate probably is oxidized as effectively as acetate and butyrate (Young, Tove & Ramsey, 1965).

An alternative for 'malic enzyme' in rumen mucosa might be suggested. In both liver and adipose tissue of some species 'malic enzyme', malate dehydrogenase and the citrate-cleavage enzyme are involved in providing extramitochondrial acetyl-CoA for fatty acid synthesis (Hanson & Ballard, 1968). However, as shown in Table 3, the activity of citrate-cleavage enzyme in rumen mucosa averaged less than 10% of that of 'malic enzyme'. This suggests that there is only a limited possibility for such a pathway of fatty acid synthesis in rumen mucosa. The activities of these two enzymes are more nearly equal in liver and adipose tissue of rats (Young, 1968).

The citrate-cleavage enzyme activity that we observed in liver is surprising since Hanson & Ballard (1967, 1968) reported a virtual absence of this enzyme from liver from adult cows, and Hardwick (1966) reported its absence from both liver and mammary tissue of adult cows. However, these workers used lactating animals whereas the

present work was with non-lactating cattle. We found no significant activity of 'malic enzyme' in liver, which agrees with the results of Hanson & Ballard (1967).

The results with adipose tissue (Table 3) show that animals given the all-concentrate ration (group 4) had significantly higher activities of both 'malic enzyme' and glucose 6-phosphate dehydrogenase. This suggests that long-term feeding of concentrates alone to ruminants promotes an enzymic adaptation to provide for an increased lipogenesis in adipose tissue (Opstvedt, Baldwin & Ronning, 1967). This idea, however, is not supported by the fact that this same group of animals had citrate-cleavage enzyme values that were lower than the other groups, although not significantly so.

Phosphoenolpyruvate carboxykinase was present in rumen mucosa even though the activity was much lower than that of 'malic enzyme'. It is not likely that this enzyme is involved in propionate metabolism by rumen mucosa, and its actual role remains unclear; it is normally present at higher activities in liver and kidney, which are the major gluconeogenic tissues. Opie & Newsholme (1967) observed this enzyme in white muscle and suggested a relationship to the α -glycerophosphate-dihydroxyacetone phosphate and malate-oxaloacetate cycles. In adipose tissue, Ballard *et al.* (1967) and Chakrabarty & Leveille (1968) demonstrated its presence and a role in providing glyceride glycerol for triglyceride synthesis.

None of the assayed enzymes was greatly influenced by the dietary changes. In contrast, the same enzymes in rats are easily influenced by dietary modifications. Goetsch (1966) studied liver enzyme changes during rumen development of calves and suggested that the slight decrease in activity of the glycolytic enzymes was due to decreased rates of glucose absorption as a consequence of rumen development. Baldwin & Ronning (1966) found no dramatic changes for several enzymes in livers of calves subjected to various manipulations of fat and carbohydrate contents of the diet. Howarth, Baldwin & Ronning (1968) have demonstrated that liver enzyme changes are relatively small when a calf receiving a milk diet matures to an adult ruminant, which absorbs little carbohydrate. However, dietary changes in ruminants result in alterations in the rumen micro-organism population and these altered microbiological patterns result in a relatively constant composition of absorbed nutrients; in the non-ruminant, dietary alterations may drastically change the composition of absorbed nutrients. Consequently the constituent enzymes of ruminant tissues have to adapt to less change in composition of absorbed nutrients.

This is Journal Paper no. J-6164 of the Iowa Agricultural and Home Economics Experiment Station, Ames, Iowa (Projects no. 1671 and no. 1324). The work was supported in part by funds provided by Grants AM-10706 and HE-04969, U.S. Department of Health, Education and Welfare. Technical assistance in various phases of the work was provided by Mrs Marlene Richard, Mr Emmanuel Akowuah, Mr John Peters and Mr Edgar Weigand. Preparation of the manuscript was aided by Dr N. L. Jacobson, Dr D. C. Beitz, Miss Kathy Stageman and Mrs Debbie Schwarz.

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