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Effect of Diet on the Activity of Several Enzymes in Extracts of Rumen Microorganisms

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Abstract

Baldwin, R. L. (University of California, Davis), and D. L. Palmquist. Effect of diet on the activity of several enzymes in extracts of rumen microorganisms. Appl. Microbiol. **13**:194–200. 1965.—The use of enzymatic techniques to characterize rumen metabolism was investigated. Assays were developed to estimate the activities of 14 enzymes in cell-free extracts of microorganisms collected from rumen contents of cows fed two diets, selected to produce widely different proportions of fermentation end products. The results reflected the differences between the two diets in metabolic potential, fermentation patterns, and microbial populations. The differences between the diets in the relative activities of succinic dehydrogenase and fumaric reductase, for example, indicated a shift in the microbial population favoring organisms of the *Veillonella alcalescens* type on the concentrate diet. The data presented indicate that, if employed carefully, enzymatic criteria can be utilized effectively in studies of rumen metabolism.

Several aspects of carbohydrate metabolism in the rumen have been studied extensively, whereas others have received very little attention. Many of the rumen microorganisms have been identified and partially characterized (Bryant, 1959; Hungate, 1960), and their numbers have been determined on a variety of diets (Warner, 1962). The relationships among the composition of the ruminant diet, the products of rumen fermentation, and animal performance have received considerable attention (Barnett and Reid, 1961). Very little information is available regarding the contributions of individual microorganisms or types of microorganisms to the total fermentation and the relative contributions of different metabolic pathways. The lack of progress in these latter areas has been due, in part, to a lack of techniques adequate for the study of metabolism in mixed culture. Several techniques, including differential counts (Bryant, 1959), measurement of substrate and intermediate turnover rates and pool sizes (Jayasuriya and Hungate, 1959), and the use of specifically labeled substrates (Baldwin, Wood, and Emery, 1963) have been useful in partially assessing the contributions of various microorganisms and metabolic pathways. The present study was initiated to investigate the possibility that enzymatic criteria can be utilized effectively in evaluating the nature of changes in the rumen fermentation caused by changes in diet. As is the case with all the techniques employed thus far, the results obtained through the use of enzymatic techniques must be interpreted with care. However, it appears that, when properly employed in conjunction with other measurements, enzymatic techniques are useful.

Materials and Methods

Animals. Two rumen-fistulated Holstein cows, fitted with rubber fistula plugs, were used in the investigation. One cow was maintained on chopped barley straw (average daily intake, 13.1 lb), and the other was fed 18.7 lb daily of a concentrate mix composed of (in per cent) barley, 45; milo, 30; wheat-mixed feed, 14; beet pulp, 10; and salt, 1. The proximate analyses of the diets are presented in Table 1. The animals were fed twice daily at 7 AM and 7 PM. Water was restricted for a 2-hr period between 9 AM and 11 AM, to reduce sampling errors.

Sample preparation. Representative samples of approximately 1 kg of rumen contents were removed at 7 AM (prefeeding) and 11 AM (4 hr postfeeding) and transported to the laboratory. The fluids were removed by squeezing the samples in a fruit press, and the residue was washed in a volume of 0.05 M $KHPO_4$ buffer (pH 7.5), equal to the volume of fluids extracted during the first squeezing. The extracts were strained through one layer of cheesecloth and combined. A portion of the combined fluids (about 800 ml) was centrifuged at 320 × *g* (International centrifuge, model 2) for 3 min to remove particulate material, and a 300-ml sample of the supernatant from this step was centrifuged at 35,000 × *g* for 30 min in a Servall refrigerated centrifuge (RC-2). The supernatant fluid was discarded, and the bacterial cells were

TABLE 1. *Proximate analysis of the two diets fed (as 100% dry matter)*

Diet	Protein	Crude fiber	Ether extract	Ash	Nitrogen-free extract
Concentrate.....	10.8	7.2	3.7	5.3	72.8
Barley straw....	6.4	34.6	1.6*	8.7	48.7

* Estimated from Morrison (1957).

washed once in 300 ml of 0.05 M $KHPO_4$ buffer (*p*H 7.5). The washed cells were resuspended in a minimal volume of buffer and disrupted in a Raytheon 10-kc sonic oscillator (15 min) under a hydrogen atmosphere. The cellular debris were removed by centrifugation at 35,000 × *g* for 30 min. The cell-free extract thus prepared was employed for the enzyme determinations.

Weight and volume of the rumen contents were determined by emptying. Dry matter (per cent) was determined by drying the whole contents at 70 C for 48 hr.

Glucose disappearance. A 100-g amount of the combined rumen fluid and wash was incubated with 0.500 g of dextrose under a nitrogen atmosphere at 39 C. Samples (1.0 ml) removed at zero time and after 15, 30, 60, 120, and 180 min of incubation were mixed with 1.0 ml of 1 N H_2SO_4 to stop the fermentation.

Volatile fatty acids. Volatile fatty acids were analyzed by adding 20 ml of 10 N H_3PO_4 to a 100-g sample of whole rumen contents. This was then diluted to 200 ml with distilled water and allowed to stand at least 24 hr at 4 C. A portion was centrifuged at 35,000 × *g* for 15 min, and the clear supernatant liquid was analyzed.

Chemical and analytical. Safranine, 2,6-dichlorophenol-indophenol, 3-(p-nitrophenyl)-2-(*p*-iodophenyl)tetrazolium chloride, coenzyme A (CoA), acrylic anhydride, crotonic anhydride, fructose-1,6-diphosphate (sodium salt), lactic dehydrogenase, and glyceraldehyde-3-phosphate dehydrogenase were purchased from commercial sources. CoA esters of acrylate, crotonate, propionate, and butyrate were synthesized and purified according to the methods described by Stadtman (1957). The esters were analyzed enzymatically by arsenolysis with phosphotransacetylase and CoA transphorase.

Spectrophotometric measurements were performed either in a Beckman DU Monochromator fitted with a Gilford model 2000 automatic cuvette positioner and recording system or in a Bausch & Lomb Spectronic-20 colorimeter. Protein was determined by the method of Lowry et al. (1951). Acetyl-phosphate was determined by the method of Lipmann and Tuttle, as presented by Rose (1955). Glucose was determined by the phenol-sulfuric method (Dubois et al., 1956). Volatile fatty acids were analyzed by gas-liquid chromatography with an Aerograph HyFI equipped with a hydrogen flame ionization detector. The stainless-steel column (182.8 by 0.32 cm) was packed with 15% terephthalic acid on 80/90 Anakrom A.

Enzymatic. The assays developed for estimation of the activity of the enzymes studied are presented in Table 2. Conditions for each enzyme assay were developed which approach zero-order measurements within the limitations inherent in the estimation of enzyme activity in extracts prepared from mixed cultures. All assays were linear with respect to enzyme concentration over the ranges employed in the experiment. Initial rates were used in cases where the assays were linear for limited periods of time; for example, the assay for phosphoroclastic activity was linear for only 15 to 30 sec. Values were corrected for endogenous activity in the absence of substrate. Enzyme activities are presented in arbitrary units because the measurements reflect the activities of enzymes from a number of different species, assayed under conditions which may not have been optimal for all enzymes. Further, it was not feasible under the conditions of these experiments to carry out absolute maximal velocity measurements, as proposed by Bernath and Singer (1962) for dehydrogenase assays employing artificial electron acceptors.

RESULTS AND DISCUSSION

The relative proportions of the rumen volatile fatty acids of the two diets are shown in Table 3. The acetate-propionate ratios indicate that the diets produced the desired result, namely, two drastically different fermentation patterns, one representative of high levels of concentrates and the other of poor quality roughages. Also reflective of the difference between the two fermentations are the lower proportions of *n*- and *iso*-valeric acids observed on the straw diet.

Data relative to the characteristics of the rumen contents are shown in Table 4. Based upon the estimate of net weight of cells per kilogram of rumen contents, the protein released per 100 g of cells was 4.4 and 9.5 g on the straw and concentrate diets, respectively. This difference may be due to (i) a systematic error in the estimation of net weight of cells, (ii) consistently incomplete breakage of cells from the straw diet, (iii) a difference in the composition of cells on the two diets, possibly due to the low nitrogen content of the straw diet, or (iv) an unknown factor. There were no apparent differences between the diets in either the estimate of wet weight of cells, or the extent of cell breakage by sonic oscillation.

The results of the enzyme determinations are presented as specific activities (units per milligram of protein) in Table 5 and as activity per kilogram of rumen contents in Table 6. The enzymes studied were selected as representatives of their respective pathways, and, where suffi-

TABLE 2. *Enzymatic methods*

Enzyme	Assay mixture[a]	Vol	Wavelength	Reference
		ml	*mμ*	
Aldolase	Glycine, 10 μmoles; $KAsO_4$, 7 μmoles; GSH, 2 μmoles; fructose-1,6-diphosphate, 5 μmoles; NAD, 1 μmole; glyceraldehyde-3-phosphate-dehydrogenase, 4.5 units; pH, 7.6	0.30	340	Taylor, 1955
Lactic dehydrogenase[b]	$KHPO_4$, 10 μmoles; INT, 25 μg; potassium lactate, 10 μmoles; *p*H, 7.5	0.30	500	Baldwin et al., 1963
Lactic dehydrogenase[c]	$KHPO_4$, 12.5 μmoles; $NADH_2$, 0.09 μmole; sodium pyruvate, 1 μmole; *p*H, 7.5	0.30	340	Kornberg, 1955
Phosphoroclastic activity	$KHPO_4$, 8 μmoles; INT, 20 μg; sodium pyruvate, 10 μmoles; CoA, 0.15 μmole; *p*H, 7.5	0.30	500	Baldwin et al., 1964
Phosphotransacetylase	Tris-HCl, 20 μmoles; CoA, 0.10 μmoles; acetyl-phosphate, 3 μmoles, *p*H 8.0	0.30	232	Stadtman, 1955
Acetokinase	Tris-HCl, 40 μmoles; hydroxylamine, 500 μmoles; $MgCl_2$, 5 μmoles; ATP, 10 μmoles; potassium acetate, 50 μmoles; *p*H 7.5	1.00	540	Rose, 1955
Diaphorase	$KHPO_4$, 10 μmoles; DCP, 5 μg; $NADH_2$, 0.18 μmoles; *p*H, 7.5	0.30	600	Baldwin et al., 1964
Malic dehydrogenase	$KHPO_4$, 8 μmoles; INT, 20 μg; NAD, 0.15 μmole; potassium malate, 2 μmoles; *p*H, 7.5	0.30	500	
Succinic dehydrogenase	$KHPO_4$, 10 μmoles; INT, 25 μg; potassium succinate, 10 μmoles; *p*H 7.5.	0.30	500	Bernath and Singer, 1962
Fumaric reductase	$KHPO_4$, 25 μmoles; safranine, 0.15 mg; potassium fumarate, 20 μmoles; $Na_2S_2O_4$, 300 μg; *p*H, 7.5; Thunberg cuvette	4.25	540	Bernath and Singer, 1962
Lactyl-CoA dehydrase	Tris-HCl, 10 μmoles; INT, 0.40 μg; NAD, 0.15 μmole; lactic dehydrogenase, 0.02 ml (1 → 10 dilution, Worthington slurry of crystals); acrylyl-CoA, 0.04 μmole; *p*H, 8.0.	0.30	500	Baldwin et al., 1964
Acyl-CoA dehydrogenase[d]	$KHPO_4$, 10 μmoles; DCP, 5 μg; propionyl-CoA, 0.06 μmole; *p*H, 7.5.	0.30	600	Baldwin et al., 1964
Crotonase:β-hydroxybutyryl-CoA dehydrogenase	$KHPO_4$, 8 μmoles, INT, 20 μg; NAD, 0.15 μmole; crotonyl-CoA, 0.04 μmole; *p*H, 7.5.	0.30	500	
Acyl-CoA dehydrogenase[e]	$KHPO_4$, 10 μmoles; DCP, 5 μg; butyryl-CoA, 0.04 μmoles; *p*H 7.5.	0.30	600	Baldwin et al., 1964

[a] INT = 3-(*p*-nitrophenyl)-2-(*p*-iodophenyl) tetrazolium chloride; NAD = nicotinamide adenine dinucleotide; $NADH_2$ = reduced NAD; Tris = tris(hydroxymethyl)aminomethane; ATP = adenosine triphosphate; DCP = 2,6-dichlorophenol-indophenol; GSH = reduced glutathione.

[b] Pyridine nucleotide nonlinked.

[c] Pyridine nucleotide linked.

[d] Substrate propionyl-CoA.

[e] Substrate butyryl-CoA.

TABLE 3. *Ratios (molar per cent) of the rumen volatile fatty acids when cows were fed concentrates or barley straw*

Diet	Acetic	Propionic	Butyric	*iso*-Valeric	*n*-Valeric	Acetic-propionic
Concentrate	59.80	24.58	11.65	1.67	2.27	2.43
Barley straw	71.44	17.67	9.15	0.73	0.98	4.04

TABLE 4. *Characteristics of rumen contents*

Determination	Barley straw		Concentrate	
	7:00 AM	11:00 AM	7:00 AM	11:00 AM
Total weight (kg)	59.7 ± 0.9[a]	79.0 ± 1.9	32.8 ± 2.1	34.5 ± 3.6
Per cent dry matter (whole contents)	8.5 ± 0.14	9.4 ± 0.90	10.3 ± 0.56	14.0 ± 0.41
Glucose utilization (half-life, min)	41.5 ± 3.1	42.0 ± 3.9	25.8 ± 2.8	49.7 ± 8.2
Wet wt of cells (g/kg of rumen contents)	15.4 ± 2.5	15.1 ± 1.8	26.1 ± 3.7	17.2 ± 2.5
Protein (g extracted/kg)	0.74 ± 0.06	0.61 ± 0.03	2.2 ± 0.30	1.8 ± 0.05

[a] Mean ± standard error.

cient information was available, to represent different types of microorganisms. Since only limited data are available with respect to the selection of enzymes whose activities are truly reflective of a specific pathway, these data must be interpreted with care. Further, the exact nature of these enzymes has not been investigated in many of the rumen bacteria; hence, changes in the characteristics of an enzyme cannot be strictly interpreted as reflecting a specific change in the rumen microbial population.

Aldolase and the pyridine nucleotide-linked and pyridine nucleotide-nonlinked lactic dehydrogenases were selected as representatives of glycolysis. Phosphoroclastic activity, phosphotransacetylase, and acetokinase were measured as participants in acetate formation. Diaphorase activity is a characteristic of several types of enzymes involved in electron transport. Malic dehydrogenase, succinic dehydrogenase, and fumaric reductase were chosen as representatives of the dicarboxylic acid pathway of propionate formation, and lactyl-CoA dehydrase and acyl-CoA dehydrogenase (with propionyl-CoA as substrate) as representatives of the direct reductive pathway of propionate formation. Both succinic dehydrogenase and fumaric reductase were measured because of species differences in their relative activities (Singer and Lara, 1957). The crotonase:β-hydroxybutyryl-CoA dehydrogenase assay and the acyl-CoA dehydrogenase assay with butyryl-CoA as substrate were selected as representatives of the pathway of butyrate synthesis.

The largest differences between the two diets were observed in the activities of the enzymes in the pathways of propionate formation, in aldolase, and in the pyridine nucleotide-nonlinked lactic dehydrogenase. Lesser differences were observed in the enzymes of the pathways of acetate and butyrate formation. In general, the differences between the activities of the enzymes are consistent with the overall differences suggested by the proportions of the volatile fatty acids formed on the two diets. Enzyme activity per kilogram of rumen contents reflects the metabolic potential of the rumen contents as indicated by rate of glucose utilization (Table 4). The calculation of enzyme activity per kilogram of rumen contents involved several assumptions: (i) that a constant and representative proportion of the rumen microorganisms were recovered during the extraction procedure, (ii) that the microorganisms were equally distributed between the supernatant and residue fractions obtained during the low-speed centrifugation step, and (iii) that a constant proportion of the cells were broken during sonic oscillation. As indicated previously, these assumptions may not be completely valid. The presentation of the data in this form does have a certain advantage, however, in that differences in total metabolic potential not represented by specific activities become apparent. The differences in metabolic potential between the two

TABLE 5. *Specific activity*[a] *of several enzymes in cell-free extracts of rumen microorganisms*

Enzyme	Straw		Concentrate		Ratio of concentrate-straw
	7:00 AM	11:00 AM	7:00 AM	11:00 AM	
Aldolase	2.9 ± 0.21	2.4 ± 0.23	6.2 ± 0.52	4.5 ± 1.10	2.0[b]
Lactic dehydrogenase[c]	0.87 ± 0.03	0.71 ± 0.02	1.8 ± 0.20	1.8 ± 0.11	2.3
Lactic dehydrogenase[d]	12.0 ± 4.1	42.4 ± 4.8	26.3 ± 3.7	34.8 ± 7.8	1.1
Phosphoroclastic activity	7.2 ± 0.57	18.9 ± 2.8	23.7 ± 2.2	24.5 ± 1.7	1.8
Phosphotransacetylase	150 ± 12.0	165 ± 18.0	184.5 ± 14.0	206 ± 4.0	1.2
Acetokinase	4.8 ± 0.30	4.9 ± 0.74	5.6 ± 1.35	10.6 ± 0.4	1.7
Diaphorase	28.7 ± 1.4	28.9 ± 1.7	66.6 ± 4.2	45.1 ± 5.9	1.9
Malic dehydrogenase	3.7 ± 0.21	5.0 ± 1.15	9.2 ± 1.24	8.4 ± 1.06	2.0
Succinic dehydrogenase	1.5 ± 0.11	1.4 ± 0.02	6.1 ± 0.78	6.5 ± 0.90	4.3
Fumaric reductase	0.11 ± 0.007	0.12 ± 0.02	0.70 ± 0.05	0.75 ± 0.09	6.3
Lactyl-CoA dehydrase[e]	0.28 ± 0.03	0.12 ± 0.06	0.73 ± 0.08	0.44 ± 0.03	2.9
Acyl-CoA dehydrogenase[e]	13.6 ± 2.5	20.0 ± 1.7	25.5 ± 3.4	29.7 ± 1.3	1.6
Crotonase:β-hydroxybutyryl-CoA dehydrogenase	2.4 ± 0.19	1.9 ± 0.33	2.7 ± 0.22	2.2 ± 0.11	1.1
Acyl-CoA dehydrogenase[f]	8.8 ± 1.14	16.6 ± 1.1	24.6 ± 2.5	26.6 ± 1.9	2.0
Succinic dehydrogenase/fumaric reductase	14.4 ± 1.6	13.2 ± 1.6	8.7 ± 0.17	8.8 ± 0.45	0.63

[a] Units per milligram of protein.
[b] Mean concentrates/mean straw.
[c] Pyridine nucleotide nonlinked.
[d] Nicotinamide adenine dinucleotide linked.
[e] Propionyl-CoA substrate.
[f] Butyryl-CoA substrate.

diets appear to be due to both a higher specific activity and a greater quantity of bacterial cells in the rumen contents of the cow fed the concentrate diet.

The lack of large changes in enzyme activity during the 4-hr period postfeeding is consistent with the slow rate of growth of rumen microorganisms, dilution by feed and saliva, and the increased rate of passage during this period. These effects are especially notable on the concentrate diet, where the total weight of rumen contents was approximately the same 4 hr postfeeding as immediately prefeeding; while the percentage dry matter had increased 40%, the rate of glucose utilization had almost halved and the net weight of cells per kilogram of rumen contents had decreased 34%.

The succinic dehydrogenase-fumaric reductase ratios were significantly different between the two diets (concentrate/straw = 0.63, $P < 0.01$), indicating a difference in the rumen microbial population favoring microorganisms of the *Viellonella alcalescens* type on the concentrate diet. The proportionate changes in the activities of alternate metabolic pathways (see below) indicate a difference in the rumen microbial population between the two diets. The fact that the specific activities of some enzymes are lower 4 hr postfeeding than immediately prefeeding (i.e., aldolase, lactyl-CoA dehydrase, crotonase:β-hydroxybutyryl-CoA dehydrogenase) while others remain constant (i.e., fumaric reductase, succinic dehydrogenase) or increase (i.e., phosphotransacetylase, nicotinamide adenine dinucleotide-linked lactic dehydrogenase) suggests that a shift in microbial population occurred during the 4-hr postfeeding period. This suggestion is based on the assumption that the rumen microorganisms do not form significant amounts of inducible enzymes.

TABLE 6. *Enzyme activity*[a] *per kilogram of rumen contents*

Enzyme	Straw		Concentrate		Ratio of concentrate-straw
	7:00 AM	11:00 AM	7:00 AM	11:00 AM	
Aldolase	2.1 ± 0.21[a]	1.5 ± 0.14	14.0 ± 2.8	10.1 ± 2.6	6.7[b]
Lactic dehydrogenase[c]	0.63 ± 0.03	0.42 ± 0.02	4.0 ± 0.70	3.1 ± 0.19	6.7
Lactic dehydrogenase[d]	8.1 ± 2.30	25.8 ± 2.7	58.0 ± 12.3	70.2 ± 28.0	3.8
Phosphoroclastic activity	5.3 ± 0.34	11.6 ± 1.7	53.0 ± 9.9	43.2 ± 0.86	5.7
Phosphotransacetylase	111 ± 15.0	101 ± 7.0	415 ± 81	371 ± 37	3.7
Acetokinase	3.55 ± 0.30	3.0 ± 0.40	12.0 ± 3.3	18.8 ± 2.4	4.7
Diaphorase	21.2 ± 2.1	17.6 ± 1.1	148 ± 25	76.3 ± 16.6	8.4
Malic dehydrogenase	2.7 ± 0.14	3.0 ± 0.60	21.2 ± 5.2	14.2 ± 3.1	6.2
Succinic dehydrogenase	1.16 ± 0.16	0.87 ± 0.03	13.8 ± 2.7	10.4 ± 1.7	11.9
Fumaric reductase	0.08 ± 0.008	0.07 ± 0.010	1.6 ± 0.29	1.2 ± 0.29	18.7
Lactyl-CoA dehydrase	0.21 ± 0.03	0.07 ± 0.003	1.58 ± 0.25	0.79 ± 0.15	8.5
Acyl-CoA dehydrogenase[e]	10.0 ± 2.0	12.3 ± 1.4	55.2 ± 9.0	54.6 ± 9.2	4.9
Crotonase:β-hydroxybutyryl-CoA dehydrogenase	1.80 ± 0.26	1.1 ± 0.20	6.1 ± 1.10	3.8 ± 0.33	3.4
Acyl-CoA dehydrogenase[f]	6.6 ± 1.01	10.2 ± 0.7	52.2 ± 2.3	46.8 ± 8.1	5.9

[a] Enzyme activity expressed in arbitrary units. Mean of four determinations ± standard error, except concentrate 11:00 AM represents three determinations.
[b] Mean concentrate/mean straw.
[c] Pyridine nucleotide-nonlinked lactic dehydrogenase.
[d] Nicotinamide adenine dinucleotide-linked lactic dehydrogenase.
[e] Propionyl-CoA substrate.
[f] Butyryl-CoA substrate

$$\text{activity/kg} = \frac{\text{units/ml of extract} \times \text{volume of extract} \times (\text{volume of combined wash}/300)}{\text{sample weight (kg)}}$$

Considerable amounts of additional work characterizing the enzymes of representative rumen microorganisms in pure culture, resolving some of the biochemical problems related to anaerobic metabolism and anaerobic electron transport, and developing new enzyme assays must be carried out before enzymatic measurements can be employed effectively in defining the metabolic changes associated with changes in the rumen microbial population on various diets. The data presented indicate that, if employed carefully in combination with other measurements, enzymatic criteria can be utilized effectively in studies of rumen metabolism.

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