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Enzymatic Studies of Pure Cultures of Rumen Microorganisms[1]

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ABSTRACT

JOYNER, A. E., JR. (University of California, Davis), AND R. L. BALDWIN. Enzymatic studies of pure cultures of rumen microorganisms. J. Bacteriol. 92:1321–1330. 1966.—The activities of enzymes representing the major pathways of carbohydrate metabolism and anaerobic electron transport in cell-free extracts of whole rumen contents have been reported. The effects of diet upon the activities of several enzymes suggested that enzymatic measurements might prove useful for the study of rumen metabolism. In the present study, the distribution and characteristics of aldolase, succinate dehydrogenase, glutamate dehydrogenase, lactyl-coenzyme A dehydrase, lactate dehydrogenase, and other enzymes were measured in cell-free extracts of pure cultures of *Ruminococcus flavefaciens*, *R. albus*, *Bacteroides succinogenes*, *B. ruminicola*, *B. amylophilus*, *Butyrivibrio fibrisolvens*, *Peptostreptococcus elsdenii*, *Streptococcus bovis*, and *Selenomonas ruminantium*. Some enzymes were widely distributed (aldolase, glutamate dehydrogenase), whereas others were observed in one or two species (lactyl-coenzyme A dehydrase). The cofactor requirements and kinetic characteristics of enzymes varied considerably with species. Enzymes that vary with species might be employed as indices for estimating the activities of various groups of microorganisms in whole rumen contents.

Studies of pure cultures of rumen microorganisms have been largely restricted to characterization of nutritional requirements and end products (10, 23), and the investigation of unusual metabolic pathways (1, 2, 27). These studies have usually been carried out with whole cells, and enzymes involved in various metabolic pathways have not been characterized. Only in a few cases have the actual enzymes, and their associated cofactors and electron carriers, been studied (5, 6, 16). Palmquist and Baldwin (31) attempted to characterize shifts in the rumen microbial population by using enzymatic measurements of cell-free extracts of the mixed microbial population. The lack of information on the metabolic pathways and their associated enzyme systems in individual species made it difficult to interpret their results with regard to the contribution of the individual species to the end products formed on different diets.

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The application of enzymatic measurements in conjunction with other techniques may prove useful in the study of rumen metabolism. However, to utilize this technique effectively, the various species must be studied individually. In the present study, some enzymes representing the major pathways of carbohydrate fermentation and anaerobic electron transport in individual species of rumen microorganisms were determined. The characteristics of several enzymes suggest that enzymatic measurements may prove useful for the estimation of the activities of various groups of microorganisms in whole rumen contents.

MATERIALS AND METHODS

Microorganisms and culture procedures. *Ruminococcus flavefaciens* C94, *Bacteroides succinogenes* S85, *Ruminococcus albus* strain 7, *Bacteroides ruminicola* strain 23, *Bacteroides amylophilus* strain 78, *Selenomonas ruminantium* HD4, *Peptostreptococcus elsdenii* B159, *Streptococcus bovis* 2281, and *Butyrivibrio fibrisolvens* 8/4-46 were kindly provided by M. P. Bryant, K. A. Pittman, and R. E. Hungate. Stock cultures of *R. flavefaciens*, *R. albus*, and *B. succinogenes* were maintained on the rumen fluid-glucose-cellobiose medium of Bryant and Burkey (11). *B. ruminicola* was

maintained on the rumen fluid medium of Pittman and Bryant (33). *B. amylophilus* was grown on the same medium, except that maltose was substituted for glucose. *S. ruminantium* and *P. elsdenii* were kept on glucose-yeast extract media (20, 26). *S. bovis* was maintained on the rumen fluid-glucose medium of Hungate (22). *B. fibrisolvens* was grown on hay extract medium. This medium contained 40% of an aqueous extract prepared by heating 4 g of ground alfalfa hay in 100 ml water at 100 C for 5 min, 0.1% yeast extract, 0.5% glucose, 0.0001% (w/v) resazurin, 7.5% of each of the inorganic salt solutions of Bryant and Burkey (11), 0.4% Na_2CO_3, and 0.05% cysteine·HCl. Stock cultures were grown in culture tubes (16 by 150 mm) fitted with semisolid rubber stoppers and were transferred by the technique of Smith and Hungate (37). Batch cultures were grown in 5-liter glass-stoppered bottles. The media used are listed in Table 1. All cultures were transferred at least three times on the growth media before they were grown in large batches. The cells were collected 12 to 24 hr after inoculation (in a Servall RC-2 centrifuge equipped with a Szent-Gyorgy and Blum continuous-flow system), washed twice, and resuspended in 0.05 M $KHPO_4$ buffer (*p*H 7.5). *S. ruminantium* was washed only once because additional washing caused erratic results. The washed cells were disrupted by sonic oscillation in a Raytheon 10-kc sonic oscillator for 15 min. The suspensions were centrifuged at 30,000 × *g* for 30 min, and the cell-free extracts were used immediately for enzymatic measurements.

Chemicals. All chemicals and biological materials were purchased from commercial sources. Coenzyme A (CoA) esters of propionate, butyrate, crotonate, and acrylate were synthesized according to the method of Stadtman (39). Protein was measured by the method of Lowry et al. (28).

Enzymatic. The assays employed for estimation of the activities of the enzymes studied are presented in Table 2. Ketolase activity may be either transketolase or phosphoketolase, since the assay did not differentiate these enzymes. Malate dehydrogenase and malic enzyme were defined on the basis of pyridine nucleotide specificity and not identification of end products. Assays in which the rate of absorbancy change was measured were carried out in either a Bausch & Lomb Spectronic-20 colorimeter or a Beckman monochromator fitted with a Gilford model 2000 automatic recording system. Acetyl phosphate was determined by the hydroxamate method of Lipmann and Tuttle, as presented by Rose (34). Conditions for each assay were selected wherein the enzyme activities were determined under close to optimal conditions, and all assays were linear with respect to enzyme concentrations. The *p*H optimum for each enzyme in each organism was determined before the measurements reported were carried out, and the *p*H of the reaction mixtures was determined at the end of each assay. Initial rates were used in all cases. Wherever possible, or appropriate, estimates were corrected for endogenous rates measured in the absence of substrates. No corrections for the effect of pyridine oxidase activity upon estimates involving measurement of rates of pyridine nucleotide reduction were applied to the data. The activities of acetokinase were expressed as micromoles of acetyl-phosphate produced per minute per milligram of protein. All other enzyme activities were expressed as micromoles of substrate utilized per minute per milligram of protein at 30 C. The millimolar extinction coefficients used were: reduced pyridine nucleotides, $E_{340} = 6.2 \times 10^3$; 2,6-dichlorophenolindophenol (DCP), $E_{600} = 21.0 \times 10^3$; 3-(P-nitrophenyl)-2-(*p*-iodophenyl) tetrazolium chloride (INT), $E_{500} = 20.4 \times 10^3$; safranine, $E_{490} = 15.5 \times 10^3$; and acetyl-CoA, $E_{232} = 4.5 \times 10^3$.

TABLE 1. *Growth media*

Microorganisms	Energy substrate	Reference for growth medium
Ruminococcus flavefaciens	Cellobiose	1
R. albus	Glucose, cellobiose	1
Bacteroides succinogenes	Glucose	13
B. ruminicola	Glucose	33
B. amylophilus	Maltose	33
Peptostreptococcus elsdenii	Lactate	27
Streptococcus bovis	Glucose	42
Selenomonas ruminantium	Glucose	20
Butyrivibrio fibrisolvens	Glucose	—[a]

[a] See Materials and Methods.

RESULTS AND DISCUSSION

The microorganisms employed in this study have been maintained in pure culture for a number of years and were grown on laboratory media. Hence, it should be recognized that the activities of the various enzymes may not reflect the activities in the various species as they would exist in the rumen. The present study was undertaken, primarily, to establish the presence or absence of enzymes in the various species and to describe briefly the general characteristics of several important enzymes. A number of the enzymes studied were present in all of the microorganisms tested, but wide differences in specific activities between the various species were observed.

The specific activities of the enzymes studied are presented in Table 3. These enzymes were selected because of their relationships to various metabolic functions. Isotopic and enzymatic studies have indicated that the major pathway of hexose metabolism in the rumen is the Embden-Meyerhof pathway and that the major pathway of pentose utilization involves hexose synthesis (2, 5, 32, 41). In the present study, aldolase activity was selected as an index of the Embden-Meyerhof pathway. Aldolase was present in all species but its activity was quite variable among species and

TABLE 2. *Conditions of enzymatic assays*

Enzyme	Assay components[a]	Vol	Wavelength	Reference
		ml	*mμ*	
Aldolase	Glycine, 7 μmoles; K-arsenate, 4 μmoles; GSH, 6 μmoles; $MgCl_2$, 1 μmole; NAD, 0.15 μmole; F-1,6-P, 6 μmoles; G3PD, 4 units.	0.3	340	40
Diaphorase	$KHPO_4$, 32 μmoles; DCP, 4 μg; $NADH_2$, 0.14 μmole	0.3	600	6
Succinate dehydrogenase	$KHPO_4$, 32 μmoles; INT, 20 μg; K-succinate, 4 μmoles	0.3	500	8
Crotonase, β-hydroxybutyryl-CoA dehydrogenase	$KHPO_4$, 32 μmoles; INT, 20 μg; crotonyl-CoA, 0.06 μmole; NAD, 0.15 μmole	0.3	500	4
Lactyl-CoA dehydrase	Tris, 32 μmoles; INT, 20 μg; NAD, 0.15 μmole; acrylyl-CoA, 0.15 μmole, LDH, 0.02 ml (1:10 dilution, Worthington slurry of crystals)	0.3	500	4
Lactate dehydrogenase (pyridine nucleotide-nonlinked)	$KHPO_4$, 32 μmoles; INT, 20 μg; DL-, L(+)-, or D(−)-lactate, 3 μmoles	0.3	500	4
Lactate dehydrogenase (pyridine nucleotide-linked)	$KHPO_4$, 40 μmoles; $NADH_2$, 0.9 μmole; Na-pyruvate, 4 μmoles	0.3	340	24
Malate dehydrogenase	Tris, 40 μmoles; NAD, 0.15 μmole; K-malate, 4 μmoles	0.3	340	
Malic enzyme	Tris, 40 μmoles; NADP, 0.12 μmole; K-malate, 4 μmoles	0.3	340	
Ketolase	Glycine, 7 μmoles; K-arsenate, 4 μmoles; R-5-P, 20 μmoles; GSH; 6 μmoles; $MgCl_2$, 1 μmole; NAD, 0.15 μmole	0.3	340	18
Glutamate dehydrogenase (α-ketoglutarate + NH_4Cl → Glutamate)	$KHPO_4$, 40 μmoles; NH_4Cl, 2 μmoles; K-α-ketoglutarate, 2 μmoles; $NADH_2$, 0.09 μmole, or $NADPH_2$, 0.08 μmole	0.3	340	
Glutamate dehydrogenase (glutamate → α-ketoglutarate + NH_3)	$KHPO_4$, 40 μmoles; K-glutamate, 3 μmoles; NAD, 0.15 μmole, or NADP, 0.12 μmole	0.3	340	31
Glutamic oxaloacetic transaminase	$KHPO_4$, 40 μmoles; $NADH_2$, 0.09 μmole; K-aspartate, 2 μmoles; K-α-ketoglutarate, 2 μmoles; MDH, 0.48 unit	0.3	340	
Isocitrate dehydrogenase	Tris, 40 μmoles; K-isocitrate, 9 μmoles; $MnCl_2$, 1 μmole; NADP, 0.12 μmole, or NAD, 0.15 μmole	0.3	340	
Phosphotransacetylase	Tris, 40 μmoles; CoASH; 0.08 μmole; acetyl-PO_4, 2 μmoles	0.3	340	38
Fumarate reductase	$KHPO_4$, 100 μmoles; Safranine, 0.15 μg; K-fumarate, 12 μmoles; $Na_2S_2O_4$, 1.4 μmoles	4.5	490	8
Acetokinase	Tris, 40 μmoles; NH_2OH, 500 μmoles; $MgCl_2$, 5 μmoles; K-ATP, 10 μmoles; K-acetate, 50 μmoles	1.0	540	34

[a] GSH, reduced glutathione; F-1,6-P, fructose-1,6-diphosphate; G3PD, glyceraldehyde-3-phosphate dehydrogenase; INT, 3-C-*p*-nitrophenyl-2-(*p*-iodophenyl) tetrazolium chloride; LDH, lactate dehydrogenase; Tris, tris-(hydroxymethyl)aminomethane hydrochloride; R-5-P, ribose-5-phosphate, MDH, malate dehydrogenase; CoASH, coenzyme A; DCP, 2,6-dichlorophenol-indophenol.

TABLE 3. *Enzyme activities*[a] *in extracts of rumen microorganisms*

Enzyme	Microorganism								
	Ruminococcus flavefaciens	*R. albus*	*Bacteroides succinogenes*	*B. ruminicola*	*B. amylophilus*	*Peptostreptococcus elsdenii*	*Streptococcus bovis*	*Selenomonas ruminantium*	*Butyrivibrio fibrisolvens*
Aldolase	14.5	20.6	365	11	63	43.6	470	16	12.3
Ketolase[b]	0.29	0.06	6.49	0.51	1.69	0.47	11	1.14	3.3
Phosphotransacetylase	12,800	4,780	131	765	2,290	2,107	700	—	329
Acetokinase	9.4	39.4	23.4	50	4.5	244	4.6	—	23.2
Lactate dehydrogenase[c]	1.36	—	33.2	0.37	0.76	—	579	409	12.3
DL-Lactate dehydrogenase[d]	—[e]	—	0.1	0.04	—	1.88	1.65	0.85	0.1
L(+)-Lactate dehydrogenase[d]	—	—	0.15	0.02	—	0.92	2.25	0.93	0.1
D(−)-Lactate dehydrogenase[d]	—	—	0.18	0.02	—	1.47	2.4	0.69	0.1
Propionyl-CoA dehydrogenase	—	—	—	—	—	0.03	—	0.05	—
Butyryl-CoA dehydrogenase	—	—	—	—	—	0.03	—	0.05	0.06
Crotonase, β-hydroxybutyryl-CoA dehydrogenase	—	—	—	0.09	—	2.72	1.79	0.16	0.3
Glutamate dehydrogenase[f]	59.1	18.7	7.35	1.86	41.8	18.7	35.1	0.35	28.8
Glutamate dehydrogenase[g]	13.0	22.5	3.56	3.37	150	22.5	156.0	0.42	10.8
Glutamic oxaloacetic transaminase	25.2	52.3	—	34	84.3	8.32	9.4	3.2	—
Succinate dehydrogenase	0.02	—	5.1	0.21	0.21	—	0.16	0.16	4.1
Fumarate reductase	8	—	490	0.27	57.3	—	—	36.1	13.3
Lactyl-CoA dehydrase	—	—	—	2.8	0.42	1.2	—	—	—
Malate dehydrogenase	—	—	—	64.0	71.5	8.32	—	—	15.9
Malic enzyme	0.93	3.04	0.96	—	—	—	—	2	1.8
Diaphorase	51.5	32.5	34.1	24	144	28	255	20	74.2
Isocitrate dehydrogenase	80.8	26.5	6.38	—	3.29	2.7	—	—	60.5

[a] (Units/mg of protein) × 10^2.
[b] See Materials and Methods.
[c] Pyridine nucleotide-linked.
[d] Pyridine nucleotide-nonlinked.
[e] Not detectable.
[f] Glutamate substrate.
α-Ketoglutarate substrate.

no apparent correlation of aldolase activity with growth rate within species was observed. In view of the fact that most of the pentoses appear to be metabolized via hexose, transketolase might be expected to be the major ketolase measured, but the data must be interpreted as indicating the general capacity for pentose utilization via either the hexose-synthesizing pathway involving transketolase or by direct cleavage as by phosphoketolase. Some of the species studied do not utilize pentoses (10), but ketolase activity was observed in all cases.

Microbial species that produce lactate as well as species capable of utilizing lactate have been isolated from the rumen and studied in pure culture (10, 23). At least two types of lactate dehydrogenase are known, one being nicotinamide adenine dinucleotide (NAD)-linked (35) and the other(s) containing cytochrome or flavins (30). Both types were measured to characterize the systems of electron transport in the various species. The NAD-linked lactate dehydrogenase was observed in all species except *P. elsdenii* and *R. albus*.

P. elsdenii contained a cytochrome-like lactate dehydrogenase. No lactate dehydrogenase was detected in *R. albus*. Most NAD-linked lactate dehydrogenase-containing species, with the exception of *R. flavefaciens* and *B. amylophilus*, also exhibited low levels of cytochrome-like lactate dehydrogenase activity. This cytochrome-like activity may be due to a diaphorase-type reaction, with lactate reducing endogenous NAD, which then reduces the dye. On the basis of kinetic data, the NAD-linked lactate dehydrogenase reaction favors reduction of pyruvate (35), whereas the reaction catalyzed by the cytochrome-containing enzyme has an equilibrium which favors lactate oxidation (30). These observations suggest that lactate utilizers, such as *P. elsdenii* and *S. ruminantium*, might profitably have an enzyme similar to the enzyme containing cytochrome. This is apparently true for *P. elsdenii* and appears to hold also for *S. ruminantium*. NAD-linked lactate dehydrogenase activity was also observed in *S. ruminantium*. This strain has been shown to produce no lactate on a glucose medium (9).

Presumably, the phosphoroclastic reactions are the major reactions involved in acetate synthesis. Two types of phosphoroclastic reaction are known: the coli-type which yields acetyl-phosphate and formate from pyruvate, and the clostridial type which yields acetyl-phosphate, CO_2, and hydrogen (29). The two types can be differentiated by measuring the rates of exchange of labeled carbon dioxide and formate into pyruvate. Based on the exchange reaction, *P. elsdenii* had the clostridial type phosphoroclastic system, as reported previously by Ladd (26). All the other microorganisms appeared to have coli-type phosphoroclastic systems. Phosphotransacetylase and acetokinase, two auxiliary enzymes involved in acetate synthesis, were measured also. The activities of these two enzymes, which were observed in almost all species, were not correlated with one another, nor did they appear to be correlated with the amounts of acetate produced by intact cells (14, 15, 19).

The enzymes involved in butyrate formation may have been present in low levels in some of the species even though no data are reported. Deacylase activity was observed in most of the microorganisms, and the dye (DCP) employed in the butyryl-CoA dehydrogenase assay is reduced by reduced sulfhydryl groups. Thus, it was not possible to establish firmly the presence or absence of acyl-CoA dehydrogenase when the activities were low. The presence of acyl-CoA dehydrogenase activity in *P. elsdenii*, *S. ruminantium*, and *B. fibrisolvens* was confirmed by the use of an alternate assay procedure which employed INT as the electron acceptor. This assay was not as sensitive as the DCP assay. Crotonase-β-hydroxybutyryl-CoA dehydrogenase activity was observed in *B. ruminicola*, *S. ruminantium*, *P. elsdenii*, *B. fibrisolvens*, and *S. bovis*.

The activity of isocitrate dehydrogenase was quite variable and was not detected in *B. ruminicola*, *S. bovis*, or *S. ruminantium*. The activity of this enzyme was high in *R. flavefaciens*, *B. fibrisolvens*, and *R. albus*, and at least 10-fold lower in *B. succinogenes*, *B. amylophilus*, and *P. elsdenii*. In assaying this enzyme in *P. elsdenii*, it was observed that Mg^{++} and Mn^{++} were inhibitory.

Diaphorase activity was present in all species studied. The level of activity was especially high in *B. amylophilus* and *S. bovis*.

Lactyl-CoA dehydrase, an enzyme of the direct reductive pathway of propionate synthesis, was observed in three species. Of these species, only *P. elsdenii* has been shown to utilize this pathway (6, 27). The observation of this activity in *B. ruminicola* and *B. amylophilus* was unexpected, since both produce large amounts of succinate. *B. ruminicola* was recently reported to produce propionate under certain conditons (17), whereas *B. amylophilus* apparently does not produce propionate (21). Lactyl-CoA dehydrase activity in *B. ruminicola* was higher than in *P. elsdenii*. These observations indicate that caution should be used when interpreting the results of enzymatic measurements of rumen fluid extracts, such as those reported by Palmquist and Baldwin (31). The low lactyl-CoA dehydrase activity observed in rumen fluid extracts by these workers may have been due either to microorganisms such as *P. elsdenii*, which possess the acrylate pathway, or to

microorganisms such as *B. ruminicola* and *B. amylophilus*, which may or may not produce propionate, or both. The latter two possibilities would overemphasize the role of the acrylate pathway and *P. elsdenii*.

Succinate dehydrogenase and fumarate reductase were observed in all species except *R. albus* and *P. elsdenii*. The activities of these enzymes indicated wide interspecies differences. The ratios of succinate oxidation to fumarate reduction have been used to distinguish between types of succinate dehydrogenases isolated from various organisms. Singer (36) reported that the ratios of the rates of succinate oxidation to fumarate reduction in beef heart, *Micrococcus lactilyticus*, and *Proteus vulgaris* were 9.0, 0.03, and 0.11, respectively. Beef heart succinate dehydrogenase was considered representative of the aerobic type, the *M. lactilyticus* enzyme, of the anaerobic, fumarate reductase type, and the *P. vulgaris* enzyme, an intermediate type. The ratios of the specific activities of succinate dehydrogenase to fumarate reductase are shown in Table 4. In all species, the activity of fumarate reductase was greater than that of succinate dehydrogenase. Several species appear to have the anaerobic type of succinate dehydrogenase similar to the enzyme found in *M. lactilyticus*. *B. ruminicola* and *B. fibrisolvens* apparently have the intermediate type of enzyme. Palmquist and Baldwin (31) reported that the intermediate type of succinate dehydrogenase was prominent in the rumen.

TABLE 4. *Succinic dehydrogenase-fumaric reductase ratios*[a]

Microorganism	Ratio
Ruminococcus flavefaciens	0.002
Bacteroides succinogenes	0.01
B. ruminicola	0.78
B. amylophilus	0.004
Selenomonas ruminantium	0.004
Butyrivibrio fibrisolvens	0.304

[a] Specific activity of succinate dehydrogenase/specific activity of fumaric reductase.

The species studied make up about 40% of the rumen microbial population (12). Using the proportions given in Table 5 for each species, we calculated a weighted estimate of the combined activity of succinate dehydrogenase and fumarate reductase in this portion of the rumen population (Table 5). The ratio of succinic dehydrogenase (SDH) to fumaric reductase (FR) calculated with these weighted activities (Table 6) indicates that, on the basis of the species studied to date, an anaerobic-type SDH would be predicted to predominate in the rumen. Palmquist and Baldwin (31) observed an intermediate type. If the proportions of the various species in Table 5 are correct, it must be assumed that either the 60% of the rumen population not studied contains species with high SDH-FR ratios, thus yielding the ratios reported by Palmquist and Baldwin, that the 40% of the rumen population studied represented considerably less than 40% of the SDH present in rumen extracts, or that the SDH-FR ratios of the organisms studied are different in the rumen than in pure culture.

NAD or NAD phosphate (NADP) reduction in the presence of malate was observed in all

TABLE 5. *Comparison of calculated and observed activities of succinate dehydrogenase (SDH), fumaric reductase (FR), and glutamate dehydrogenase*[a]

Microorganism	Percentage of total population	SDH	FR	Glutamate dehydrogenase	
				NAD	NADP
Bacteroides ruminicola	12	0.00048	0.00033	—	0.0022
Butyrivibrio fibrisolvens	9	0.00369	0.01200	—	0.0259
Ruminococcus flavefaciens	3	0.00005	0.00240	—	0.01773
R. albus	3	—[b]	—	0.0056	—
Bacteroides succinogenes	5	0.0025	0.2450	—	0.00735
Streptococcus bovis	1	0.00002	—	—	0.00351
Selenomonas ruminantium	1	0.00002	0.0036	—	0.00004
B. amylophilus	1	0.00002	0.0057	—	0.00418
Peptostreptococcus elsdenii	1	—	—	0.00187	—
Total		0.00674	0.269	0.00748	0.0607
Rumen fluid extracts[c]		0.00589	0.0289	0.110	0.0159

[a] Units per milligram of protein times percentage of total population.
[b] No activity detected.
[c] Palmquist and Baldwin (31).

species except *S. bovis*. Present knowledge of the pyridine nucleotide specificity of enzymes oxidizing malate suggests that malate dehydrogenase was present in some species and malic enzyme in the others (25). This interpretation is not consistent with the absence of malate dehydrogenase in microorganisms which produce propionate via the dicarboxylic acid pathway. Flavin- or NADP-linked malate dehydrogenases may be present in these microorganisms. A malate-vitamin K reductase which contains flavin adenine dinucleotide (FAD) as a cofactor has been isolated from *Acetobacter xylinium* (7).

Many rumen microorganisms can utilize ammonia as their sole source of nitrogen when grown in pure culture (23). The activities and pyridine nucleotide specificities of glutamate dehydrogenases were investigated as a potential index of capacity for ammonia assimilation. An NADP-linked glutamate dehydrogenase had previously been reported in *S. bovis* (16). Several of the other species, in addition to *S. bovis*, contained the NADP-linked enzyme. In the present study, the NAD-linked glutamate dehydrogenase was observed only in *R. albus* and *P. elsdenii*. Enzyme activity within species was not correlated with the nitrogen source in the medium. Both *B. ruminicola* and *B. amylophilus* were grown in media with NH_4^+ as the nitrogen source. The glutamate dehydrogenase activity of *B. amylophilus* was one of the highest observed, and that of *B. ruminicola* was one of the lowest. Both NAD- and NADP-linked glutamate dehydrogenase were observed in rumen fluid by Palmquist and Baldwin (31). The activities of NAD-linked glutamate dehydrogenase were much higher than that of the NADP-linked enzyme on all diets studied, although the ratio of these activities decreased as the amount of concentrate in the diet increased. The weighted activities of the NAD- and NADP-linked glutamate dehydrogenases and the ratios of these weighted activities have been calculated (Tables 5 and 6). They are much different than those reported for rumen fluid extracts by Palmquist and Baldwin (31), also presented in Tables 5 and 6. The activities of enzymes in various species probably do not accurately reflect those which occur in the rumen. The rumen environment might be expected to modify enzyme activities and, hence, partially nullify the comparative value of the weighted estimates used in the calculation of the ratio of NAD- to NADP-linked glutamate dehydrogenases. However, it seems reasonable to suggest that such effects could not entirely account for the gross differences between the calculated and observed ratios of NAD-linked to NADP-linked glutamic dehydrogenase, and that a number of the species not studied must contain active NAD-linked glutamate dehydrogenases.

TABLE 6. *Comparison of calculated and observed ratios*

Ratio	SDH/FR[a]	Glutamate dehydrogenase, NAD/NADP
Calculated[b]	0.0251	0.123
Rumen fluid extracts	0.204	6.92

[a] SDH, succinate dehydrogenase; FR, fumaric reductase.

[b] Ratios of total calculated activities given in Table 5.

The pyridine nucleotide specificities of several enzymes are presented in Table 7. The pyridine nucleotide preferences were highly specific; activity with the alternate pyridine nucleotide was nil in each case. The pyridine nucleotide-linked lactate dehydrogenases were reduced NAD ($NADH_2$) specific in all species. *R. albus* and *P. elsdenii* contained NAD-specific glutamate dehydrogenases. The glutamate dehydrogenases of the other species were NADP-specific. In view of the fact that, under the assay conditions employed, malic enzyme activity could not be distinguished from NADP-linked malate dehydrogenase, and malate dehydrogenase activity could not be distinguished from NAD-linked malic enzyme activity, the distinctions made in Table 7 must be considered arbitrary. The cellulolytic species and *S. ruminantium* had malic enzyme-like activity, whereas *P. elsdenii*, *B. ruminicola*, and *B. amylophilus* had malate dehydrogenase-like activity. *S. bovis* contained neither type of activity, whereas *B. fibrisolvens* appeared to contain both. Isocitrate dehydrogenase activity was not observed in all species. *P. elsdenii* contained an NAD-specific isocitrate dehydrogenase.

The apparent *p*H optima for most of the enzymes are shown in Table 8. Comparisons among species indicated that the *p*H optima for most of the enzymes varied. The *p*H optima for the assay of glutamic oxaloacetic transaminase and phosphotransacetylase varied the most among species. The highest optimal *p*H (8.0) for glutamic oxaloacetic transaminase was in *R. albus*, and the lowest (*p*H 6.8) was in *B. amylophilus*. The lowest *p*H optimum (6.0) for phosphotransacetylase was in *P. elsdenii*, and the highest (*p*H 8.0) was in *S. bovis* and *B. fibrisolvens*.

In the present work, only one strain per species was studied. Since strain differences in both nutritional requirements and end products have been observed, differences in specific activities of various enzymes might be expected. The in vitro activities of the species used in the present study

TABLE 7. *Pyridine nucleotide specificity of several enzymes*

Microorganism	Enzymes							
	Lactate dehydrogenase		Glutamate dehydrogenase		Malate dehydrogenase,[a] NAD	Malic enzyme[a] NADP	Isocitrate dehydrogenase	
	NAD	NADP	NAD	NADP			NAD	NADP
Ruminococcus flavefaciens	+	−	−	+	−	+	−	+
R. albus	−	−	+	−	−	+	−	+
Bacteroides succinogenes	+	−	−	+	−	+	−	+
B. ruminicola	+	−	−	+	+	−	−	−
B. amylophilus	+	−	−	+	+	−	−	+
Peptostreptococcus elsdenii	−	−	+	−	+	−	+	−
Streptococcus bovis	+	−	−	+	−	−	−	−
Selenomonas ruminantium	+	−	−	+	−	+	−	−
Butyrivibrio fibrisolvens	+	−	−	+	+	+	−	+

[a] See text for discussion of malate dehydrogenase and malic enzyme.

TABLE 8. *Optimal pH values of several enzymes determined at the conclusion of enzymatic assay*

Assay	Microorganism								
	Ruminococcus flavefaciens	*R. albus*	*Bacteroides succinogenes*	*B. ruminicola*	*B. amylophilus*	*Peptostreptococcus elsdenii*	*Streptococcus bovis*	*Selenomonas ruminantium*	*Butyrivibrio fibrisolvens*
Aldolase	6.8	7.0	6.8	7.0	7.0	6.4	7.0	6.8	6.8
Ketolase	6.4	7.0	6.4	6.8	7.0	6.4	6.8	6.8	6.4
Phosphotransacetylase	7.0	7.6	7.2		6.8	6.0	8.0		8.0
Lactate dehydrogenase[a]	7.0		7.2	6.8	7.0		6.8	7.0	6.8
Lactate dehydrogenase[b]				7.2		7.2	7.2	7.2	7.2
Propionyl-CoA dehydrogenase						7.6		7.2	
Butyryl-CoA dehydrogenase						7.6		7.2	7.0
Crotonase-β-hydroxybutyryl-CoA dehydrogenase				7.0		7.0	7.6	7.6	7.0
Glutamate dehydrogenase	7.2	8.0	7.6	7.2	7.6	8.0	7.2	7.6	7.0
Glutamic-oxaloacetic transaminase	7.0	8.0		7.0	6.8	7.6	7.2	7.0	
Succinate dehydrogenase	7.0		7.6	7.2	7.2		7.6	7.2	7.2
Lactyl-CoA dehydrase				7.6		8.0			
Diaphorase	7.2	7.2	7.0	7.0	7.2	7.0	7.6	6.8	6.8

[a] Pyridine-linked.
[b] Not pyridine-linked.

probably do not reflect their activities in vivo, but species differences in several enzymatic parameters suggest that enzymatic measurements on cell-free extracts of the mixed rumen microbial population might be used to estimate contributions of various microbial species to the end products found in the rumen. Differences in pyridine nucleotide requirements and rates of forward and reverse reactions, and enzymes unique to only a few species, appear to offer the greatest opportunity for estimating contributions of different species. Utilization of enzymatic techniques in conjunction with other types of measurements used previously in studying rumen metabolism should permit attainment of a better understanding of the roles of the various microbial species in the rumen.

LITERATURE CITED

1. ALLISON, M. J., M. P. BRYANT, AND R. N. DOETSCH. 1962. Studies on the metabolic function of branched-chain volatile fatty acids, growth factors for ruminococci. I. Incorporation of isovolerate into leucine. J. Bacteriol. 83:523–532.
2. AYERS, W. A. 1958. Phosphorylation of cellobiose and glucose by *Ruminococcus flavefaciens*. J. Bacteriol. 76:515–517.
3. BALDWIN, R. L. 1965. Pathways of carbohydrate metabolism in the rumen, p. 379–389. *In* R. W.

Dougherty [ed.], Physiology of digestion in the ruminant. Butterworths, Washington, D.C.
4. BALDWIN, R. L., AND D. L. PALMQUIST. 1965. Effect of diet on the activity of several enzymes in extracts of rumen microorganisms. Appl. Microbiol. **13**:194–200.
5. BALDWIN, R. L., W. A. WOOD, AND R. S. EMERY. 1963. Conversion of glucose-C^{14} to propionate by the rumen microbiota. J. Bacteriol. **85**: 1346–1349.
6. BALDWIN, R. L., W. A. WOOD, AND R. S. EMERY. 1965. Lactate metabolism by *Peptostreptococcus elsdenii*: evidence for lactyl-coenzyme A dehydrase. Biochim. Biophys. Acta **97**:202–213.
7. BENZIMAN, M., AND Y. GALANTER. 1964. Flavine adenine dinucleotide-linked dehydrogenase from *Acetobacter xylinum*. J. Bacteriol. **88**: 1010–1018.
8. BERNATH, P., AND T. P. SINGER. 1962. Succinic dehydrogenase, p. 597–614. *In* S. P. Colowick and N. O. Kaplan [ed.], Methods in enzymology, vol. 5. Academic Press, Inc., New York.
9. BRYANT, M. P. 1956. The characteristics of strains of *Selenomonas* isolated from bovine rumen contents. J. Bacteriol. **72**:162–167.
10. BRYANT, M. P. 1959. Bacterial species of the rumen. Bacteriol. Rev. **23**:125–153.
11. BRYANT, M. P., AND L. A. BURKEY. 1953. Cultural methods and some characteristics of the more numerous groups of bacteria in the bovine rumen. J. Dairy Sci. **36**:205–217.
12. BRYANT, M. P., AND L. A. BURKEY. 1953. Numbers and some predominant groups of bacteria in the rumen of cows fed different rations. J. Dairy Sci. **36**:218–224.
13. BRYANT, M. P., I. H. ROBINSON, AND H. CHU. 1959. Observations on the nutrition of *Bacteroides succinogenes*—a ruminal cellulolytic bacterium. J. Dairy Sci. **42**:1831–1847.
14. BRYANT, M. P., AND N. SMALL. 1956. The anaerobic monotricious butyric acid-producing curved rod-shaped bacteria of the rumen. J. Bacteriol. **72**:16–21.
15. BRYANT, M. P., N. SMALL, C. BOUMA, AND H. CHU. 1958. *Bacteroides ruminicola* n. sp. and *Succinomonas amylolitica*, the new genus and species. Species of succinic acid-producing anaerobic bacteria of the bovine rumen. J. Bacteriol. **76**:15–23.
16. BURCHALL, J. J., R. A. NIEDERMAN, AND M. J. WOLIN. 1964. Amino group formation and glutamate synthesis in *Streptococcus bovis*. J. Bacteriol. **88**:1038–1044.
17. DEHORITY, B. A. 1966. Characterization of several bovine rumen bacteria isolated with a xylan medium. J. Bacteriol. **91**:1724–1729.
18. DE LA HABA, G., AND E. RACKER. 1955. Crystalline transketolase from baker's yeast, p. 375–380. *In* S. P. Colowick and N. O. Kaplan [ed.], Methods in enzymology, vol. 1. Academic Press, Inc., New York.
19. ELSDEN, S. R., B. E. VOLCANI, F. M. C. GILCHRIST, AND D. LEWIS. 1956. Properties of a fatty acid forming organism isolated from the rumen of sheep. J. Bacteriol. **72**:681–693.
20. GUTIERREZ, J. 1953. Numbers and characteristics of lactate utilizing organisms in the rumen of cattle. J. Bacteriol. **66**:123–128.
21. HAMLIN, L. J., AND R. E. HUNGATE. 1956. Culture and physiology of a starch-digesting bacterium (*Bacteroides amylophilus* n. sp.) from the bovine rumen. J. Bacteriol. **72**:548–554.
22. HUNGATE, R. E. 1957. Microorganisms in the rumen of cattle fed a constant diet. Can. J. Microbiol. **3**:289–311.
23. HUNGATE, R. E., M. P. BRYANT, AND R. A. MAH. 1964. The rumen bacteria and protozoa. Ann. Rev. Microbiol. **18**:131–166.
24. KORNBERG, A. 1955. Lactic dehydrogenase of muscle, p. 441–443. *In* S. P. Colowick and N. O. Kaplan [ed.], Methods in enzymology, vol. 1. Academic Press, Inc., New York.
25. KUN, E. 1963. Malate dehydrogenases, p. 149–160. *In* P. D. Boyer, H. Lardy, and K. Myrback [ed.], The enzymes, vol. 7. Academic Press, Inc., New York.
26. LADD, J. N. 1959. The fermentation of lactic acid by a gram-negative coccus. Biochem. J. **71**: 16–22.
27. LADD, J. N., AND D. J. WALKER. 1959. The fermentation of lactate and acrylate by the rumen microorganism LC. Biochem. J. **71**:364–373.
28. LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL. 1951. Protein measurement with Folin phenol reagent. J. Biol. Chem. **193**:265–275.
29. MCCORMICK, N. G., E. J. ORDAL, AND H. R. WHITELEY. 1962. Degradation of pyruvate by *Micrococcus lactilyticus*. I. General properties of the formate exchange reaction. J. Bacteriol. **83**:887–898.
30. NYGAARD, A. P. 1961. Kinetic properties of yeast D(−)-lactate cytochrome c reductase. J. Biol. Chem. **236**:2128–2132.
31. PALMQUIST, D. L., AND R. L. BALDWIN. 1966. Enzymatic techniques for the study of carbohydrate utilization in the rumen. Appl. Microbiol. **14**:60–69.
32. PAZUR, J. H., E. W. SHUEY, AND C. E. GEORGI. 1958. The conversion of D-xylose into volatile organic acids by rumen bacteria. Arch. Biochem. Biophys. **77**:387–394.
33. PITTMAN, K. A., AND M. P. BRYANT. 1964. Peptides and other nitrogen sources for growth of *Bacteroides ruminicola*. J. Bacteriol. **88**:401–410.
34. ROSE, J. H. 1955. Acetate kinase of bacteria, p. 591–595. *In* S. P. Colowick and N. O. Kaplan [ed.], Methods in enzymology, vol. 1. Academic Press, Inc., New York.
35. SCHWERT, G. W., AND A. D. WINER. 1963. Lactate dehydrogenase, p. 127–148. *In* P. D. Boyer, H. Lardy, and K. Myrback [ed.], The enzymes, vol. 7. Academic Press, Inc., New York.

36. SINGER, T. P. 1965. Comparative biochemistry of succinate dehydrogenase: forms and functions, p. 448–481. *In* T. E. King, H. S. Mason, and M. Morrison [ed.], Oxidases and related redox systems, vol. 1. John Wiley & Sons, Inc., New York.
37. SMITH, P. H., AND R. E. HUNGATE. 1958. Isolation and characterization of *Methanobacterium ruminantium* n. sp. J. Bacteriol. **75**:713–718.
38. STADTMAN, E. R. 1955. Phosphotransacetylase from *Clostridium kluyveri*, p. 516–599. *In* S. P. Colowick and N. O. Kaplan [ed.], Methods in enzymology, vol. 1. Academic Press, Inc., New York.
39. STADTMAN, E. R. 1957. Preparation and assay of acrylyl-coenzyme A and other thiol esters; use of hydroxylamine, p. 931–941. *In* S. P. Colowick and N. O. Kaplan [ed.], Methods in enzymology, vol. 3. Academic Press, Inc., New York.
40. TAYLOR, J. R. 1955. Aldolase from muscle, p. 310–315. *In* S. P. Colowick and N. O. Kaplan [ed.], Methods in enzymology, vol. 1. Academic Press, Inc., New York.
41. WALLNÖFER, P., R. L. BALDWIN, AND E. STAGNO. 1966. Conversion of ^{14}C labelled substrates to volatile fatty acids by the rumen microbiota, *in press*.
42. WOLIN, M. J., AND G. WEINBERG. 1960. Some factors affecting the growth of *Streptococcus bovis* on chemically defined media. J. Dairy Sci. **43**:825–830.