of so called "urea toxicity" are misleading because of difficulty of diagnosis. At the Oklahoma Station chronic toxicity could not be produced by feeding 500-pound steers as much as 1 pound of urea per day. The urea was fed in increasing amounts in a ration of 4 pounds of prairie hay, 3 pounds of corn, 6 pounds of molasses, and minerals (bone meal and salt) over a period of 125 days. Acute toxicity was produced by withholding feed for 48 hours and then offering a mixture of corn, molasses, and urea in such amounts (2 to 3 pounds) that the animals consumed about 20 grams of urea per 100 pounds of body weight. Staggering, prostration, convulsions, and bloating were common symptoms. In these cases, as in previous ones (*5*), blood ammonia values were elevated and the animals either died within a few hours or completely recovered.

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RUMINANT NUTRITION

In Vivo and in Vitro Nutritional Requirements of Rumen Microorganisms

RONALD R. JOHNSON, ORVILLE G. BENTLEY, J. W. HIBBS, and H. R. CONRAD

Departments of Animal Science and Dairy Science, Ohio Agricultural Experiment Station, Wooster, Ohio

The composition of the ration has an important influence on the digestive and synthetic processes of rumen microorganisms. Both rumen inoculations and changes in the ratio of roughage to grain fed to dairy calves altered the establishment and proportion of microorganisms typically found in the rumens of adult dairy cattle. Ruminal synthesis of vitamin B_{12} can be affected by lack of cobalt. In vitro studies indicate that rumen microorganisms require nutritional factors present in natural feedstuffs, in addition to energy, nitrogen, and minerals. Addition of rumen liquor, extracts of roughage and protein concentrate, or fermentation by-products to the in vitro fermentation medium increases the rate of cellulose digestion. Several laboratories have obtained a cellulolytic response with B vitamins, C_5 to C_6 fatty acids, amino acids, and partial hydrolyzates of proteins.

STUDIES ON RUMINANT NUTRITION conventionally involve evaluating and investigating the effect of various feeds and levels of nutrition on performance—i.e., rate of gain, maintenance, reproduction, and carcass quality of animals. Some early investigators realized the possible importance of the contribution of the bacteria within the rumen to the utilization of feeds by the animal. Tappenheimer in 1884 [cited by Phillipson (*33*)] used ox rumen bacteria to study the fermentation of cellulose and found that large amounts of volatile fatty acids were produced. Zuntz and Hagemann in 1891 [cited by McNaught and Smith (*29*)] postulated that nonprotein nitrogen might be converted to protein by the microbes in the rumen and that subsequent digestion of the microbes would contribute to the protein requirement of the host animal. However, it was not until 1922 that Henneberg [cited by Baker and Harriss (*2*)] first used microscopy to study microorganisms in relation to cellulose digestion.

In recent years, the importance of the rumen microflora population in the nutrition of the ruminant animal has been stressed. Consequently, many studies on ruminant nutrition now emphasize the nutrition of the rumen bacteria themselves. The development of in vitro techniques for studying their nutrition has assisted these investigations greatly.

Basically, the ruminant is a roughage consumer—i.e., it utilizes high fiber feeds, and it finds its greatest economic utility in that role. However, with the increased desire for rapid production of not only more quantity but better quality of meat, many cattle and sheep are now given rations high in starch and low in fiber. Changes in the proportion of fiber to starch affect not only the energy content of the ration but many processes in the rumen as well.

Burroughs and coworkers (*8*) showed that cellulose digestion was depressed by feeding starch to steers, while the presence of starch often improves synthesis of B vitamins (*25, 28*) and urea utilization (*30*). Using in vitro rumen fermentations, Arias and coworkers (*1*) found that small amounts of readily available sources of energy promoted cellulose digestion but larger amounts inhibited it. Again, these energy sources increased urea utili-

Table I. Influence of Type of Hay Fed and Hay-Grain Ratio on Digestibility of Cellulose, Dry Matter, Protein, and Daily Nitrogen Retention in 12-Week-Old Calves[a]

No. of Calves	Kind of Hay Fed	Hay-Grain Ratio	Dry Matter, %	Cellulose, %	Protein, %	Daily Nitrogen Retention, G./100 Lb.
3	Alfalfa	4:1	66.0	65.6	66.8	7.13
3	Alfalfa	3:2	68.9	70.5	71.6	12.90
3	Alfalfa	2:3	72.5	60.3	72.3	13.02
3	Clover-timothy	4:1	66.6	69.2	57.7	7.77
3	Clover-timothy	3:2	69.0	68.9	60.2	10.84
3	Clover-timothy	2:3	70.1	60.1	67.1	12.72

[a] Data from (*10*).

zation. Hunt, Bentley, and Hershberger (*23*) also noted that the addition of starch increased B vitamin synthesis by rumen microorganisms in vitro.

Moir and Williams (*32*) showed that the total numbers of bacteria in the rumen increased with increased protein intake of the animal. Moir (*31*) then found that the total number of bacteria varied with the season of the year—i.e., the numbers increased as the animals were changed from "dry" to "green" grazing conditions. This was explained on the basis of protein content of the grass. Similar findings were reported by Gall and others (*14*).

Loosli and coworkers (*27*) and Thomas and coworkers (*38*) reported that the addition of elemental sulfur or sodium sulfate to a sheep ration increased the retention of urea nitrogen. The importance of sulfate and cobalt in maintaining high numbers of rumen bacteria was also reported by Gall and associates (*15*, *16*). Hunt and associates (*24*) noted by in vitro studies that sulfur as sodium sulfate or methionine was necessary for B vitamin synthesis, urea utilization, and cellulose digestion by the rumen bacteria.

It is apparent that the composition of the rations and the nutrients contained can affect the activity and performance of the rumen microflora. Some of these relationships are discussed as they represent examples of the progress in the field of animal nutrition. The major topics considered are the effect of energy sources on the microflora and their relationship to the early establishment of rumen function in the young calf, dietary cobalt and vitamin B_{12} synthesis, and some recent work on growth factors for the microflora as grown in vitro.

No attempt is made to discuss the bacteriology of the rumen flora. Hungate (*22*) and Doetsch and Robinson (*12*) discuss this subject and have assembled an extensive bibliography. Toxic or adverse factors in feed as they affect the rumen flora are not discussed because of limited knowledge concerning this subject.

Energy Sources

Hibbs, Pounden, and Conrad, at the Ohio Station, have carried out an extensive series of experiments dealing with the effect of varying hay-grain ratios on the establishment of an adult type of rumen flora in young dairy calves (*10*, *11*, *20*, *21*, *34*, *35*). To establish a measure of rumen development, observations were made on the rumen microflora and microfauna from adult dairy animals fed normal dairy rations consisting of large amounts of roughages. Certain "indicator" bacteria were selected and classified as hay I and hay II and grain I and grain II, based on the type of feed that best supported their establishment in the rumen. On high hay rations, the hay I and II bacteria were numerous, while the grain I and II bacteria were absent. As the ratio of hay to grain in the ration became equal to or less than 1.0, the hay types became less numerous and the grain I and II bacteria increased in numbers. On all grain rations, there were very few hay-type organisms.

The effects of varying hay-grain ratios on the degradative and synthetic abilities of the rumen microflora were also studied. Conrad and Hibbs had shown that cud inoculations at an early age increased the digestibility of the protein and cellulose by calves fed rations relatively high in roughage (*10*). This illustrates that the kinds and numbers of microorganisms present in the rumen have an effect on digestion. Quin (*36*) had previously shown that utilization of sugar and cellulose was markedly depressed following a 48-hour period of starvation, again illustrating the effect of depleted numbers of bacteria in the rumen on digestion therein.

Table I illustrates digestion of cellulose and protein in inoculated calves fed rations containing different hay-grain ratios (*10*). Cellulose digestibility was significantly lower on the 2 to 3 ratio than on the 3 to 2 ratio. These data agree with the results cited earlier (*1*, *8*), which showed in both in vivo and in vitro tests that a small amount of starch or grain in the ration may increase cellulose digestion but a larger amount decreases its digestion. Protein digestibility and nitrogen retention increased as the proportion of grain in the ration increased, which agrees with the work of Lofgreen, Loosli, and Maynard (*26*) concerning the effect of energy on nitrogen retention.

Data obtained by Conrad and Hibbs (*11*) on the thiamine and riboflavin synthesis in calves fed varying ratios of hay to grain are summarized in Table II. Thiamine synthesis showed no consistent relationship to the types of ration fed. Riboflavin synthesis, however, showed a consistent increase as the percentage of grain in the ration was increased, especially when mature timothy hay was used as the roughage. When legume hay was used in the ration, no significant differences were observed among various hay-grain ratios.

Table III gives the fatty acid content of rumen liquor from calves fed various hay-grain ratios (*20*). The total steam-volatile fatty acid concentration did not vary with the different ratios. However, butyric acid was considerably higher at the higher grain levels than at the 4 to 1 hay-grain ratio. With this rise in

Table II. Influence of Hay-Grain Ratio on Synthesis of Thiamine and Riboflavin in Rumee of Calves Fed Mature Timothy Hay and Clover-Timothy Hay as Only Roughage[a]

Hay-Grain Ratio	Type of Hay	Thiamine, γ/G. Dry Matter		Riboflavin, γ/G. Dry Matter	
		Rumen liquor	Ration fed	Rumen liquor	Ration fed
All hay	Timothy	2.90	1.63	15.8	4.42
12:1	Timothy	2.18	1.76	17.1	4.36
6:1	Timothy	4.73	1.95	21.0	4.29
4:1	Timothy	10.11	2.19	30.6	4.22
2:1	Timothy	6.64	2.54	49.2	4.08
1:1	Timothy	5.20	2.93	46.3	4.00
0.6:1	Timothy	2.60	3.07	45.4	3.90
All grain		4.06	3.94	47.4	3.58
4:1	Clover-timothy	8.5	2.14	31.7	9.80
3:2	Clover-timothy	11.2	2.54	38.0	7.80
2:3	Clover-timothy	8.9	2.88	44.2	5.16

[a] Data from (*11*).

butyric acid there was a concomitant fall in acetic and propionic acids. This indicates that the type of fermentation is different at the various hay-grain ratios and is attributed to changes observed in the various types of rumen bacteria.

Studies with Cobalt

It has been demonstrated by many workers that ruminants require cobalt in their ration. Workers in Australia and New Zealand found that certain severe wasting diseases of sheep, classified as "enzootic marasmus," were associated with forages grown on soils with low amounts of available cobalt. Top dressing the soil where roughage was grown or supplementing the animal ration directly with cobalt brought about miraculous recoveries in affected animals. In more recent years, similar cobalt deficiencies have been shown to exist in the United States, Canada, and other parts of the world. A cobalt deficiency is usually characterized by a severe loss of appetite, drop in numbers of bacteria in the rumen, and subsequent anemia and general emaciation, leading to the death of the animal.

In studies at the Ohio Station, Bentley and coworkers (*6*) found that when the ash from good quality alfalfa hay was used to supplement a fattening ration containing poor quality timothy hay as the roughage, the rate of gain of steers on this ration was markedly increased (Table IV). Further investigation showed that a synthetic trace mineral mix brought about similar performance. Subsequently, it was shown that cobalt was the element primarily responsible for the effect. That vitamin B_{12} was important in this response was suggested by the increased vitamin B_{12} content in the feces and soft tissues of the steers receiving the trace minerals or cobalt. Digestibility of the ration nutrients was not increased by cobalt supplementation.

Since the discovery of vitamin B_{12} and the presence of cobalt in the vitamin B_{12} molecule, investigators have conclusively shown that the chief function of cobalt in ruminant rations is in promoting vitamin B_{12} synthesis by the rumen microorganisms. Cobalt deficiencies have been cured by either intravenous or oral administration of vitamin B_{12}, providing sufficient quantities are given. Yet it has not been fully determined whether the vitamin B_{12} is more important for the host animal or for the rumen microflora.

Table III. Effect of Hay-Grain Ratio on Volatile Fatty Acid Content of Rumen Juice of 12-Week-Old Jersey Calves[a]

	Hay-Grain Ratio		
	4:1	3:2	2:3
No. of calves	4	4	2
Total steam-volatile fatty acids, meq./l	88.0	84.8	84.5
Av. acetic, mole %	72.5	66.5	63.6
Av. propionic, mole %	20.6	21.1	18.3
Av. butyric, mole %	6.9	12.4	18.1

[a] Data from (*20*).

Table V. Effect of Starch on Synthesis of Riboflavin and Vitamin B_{12}, Cellulose Digestion, and Nitrogen Utilization by Rumen Microorganisms Grown in Vitro[a]

Test of Activity	Good Hay[b]		Poor Hay[b]	
	Starch[c]	No starch	Starch	No starch
Riboflavin synthesized, mg.	98	39	105	35
Vitamin B_{12} synthesized, mg.	12.7	8.0	5.1	1.4
Cellulose digested, %	57.5	65.1	11.8	48.0
Ammonia nitrogen utilized, mg.	588	424	468	424

[a] Data from (*24*).
[b] "Good hay" and "poor hay" refer to in vitro flasks inoculated with rumen liquor taken from steers fed good quality alfalfa hay and poor quality timothy hay, respectively.
[c] 1 g. starch per 100 ml. in vitro rumen medium.

In Vitro Nutritional Studies with Rumen Microorganisms

In order to study more advantageously the nutrition of the rumen organisms, many laboratories have developed methods for growing these organisms in vitro. By growing the rumen bacteria in laboratory flasks, one is able to control growing conditions and the nutrient medium. Bacterial activity can then be measured by such standards as cellulose digestion, nitrogen utilization, vitamin synthesis, and volatile fatty acid production.

Burroughs, Headley, Bethke, and Gerlaugh (*9*) at the Ohio Station were among the earliest to use the in vitro fermentation technique to study rumen microorganisms. In the early experiments, rumen contents were removed from fistulated steers and used to inoculate flasks containing the purified basal medium of cellulose, glucose, minerals, and urea.

With this procedure, Hunt, Bentley, Hershberger, and Cline (*24*) showed that bacteria taken from an alfalfa-fed steer performed differently from bacteria taken from a steer fed timothy hay when the bacteria were grown in vitro (Table V). The bacteria from the animal fed poor quality timothy synthesized less vitamin B_{12}, utilized less ammonia nitrogen, and digested less cellulose than those taken from the alfalfa-fed animal. The addition of starch to either type of inoculum increased vitamin synthesis and ammonia nitrogen utilization but decreased cellulose digestion.

Recently, several laboratories have refined the in vitro rumen fermentation technique by using centrifuged cells and washed cell suspensions for inoculating the fermentations. By this means, nutrients contributed by the rumen liquor can be eliminated. When this was done, the activity of rumen microorganisms in vitro has been shown to be stimulated by certain unidentified

Table IV. Effect of Iron, Trace Mineral Mixture, Alfalfa Ash, and Cobalt on Steer Performance[a]

	1952 Rations				1953 Rations		
	Basal	Basal + Fe	Basal + trace minerals[b]	Basal + alfalfa ash[c]	Basal	Basal + cobalt[d]	Basal + trace minerals
No. of steers	3	3	3	3	4	4	4
Av. daily gain (140 days)	1.34	1.21	1.96	1.89	1.26	1.52	1.75
Total feed per 100-lb. gain, lb.	878.0	884.0	720.0	725.0	829.0	815.0	764.0
Vitamin B_{12} content							
Of feces, γ/g. dry weight	0.13	0.16	1.23	...	0.24	1.20	1.53
Of liver, γ/g. wet weight	0.05	...	0.38	...	0.12	0.68	0.69
Of kidney, γ/g. wet weight					0.05	0.21	0.22

[a] Data taken from (*6*).
[b] Mixture of manganese, copper, zinc, cobalt, and iron.
[c] 75 g. alfalfa ash per steer per day.
[d] 0.5 mg. cobalt per steer per day.

factors. Doetsch, Robinson, and Shaw (*13*) found that rumen juice itself stimulated the activity of washed suspensions of rumen bacteria. Ruf, Hale, and Burroughs (*37*) showed that extracts of cow manure and several natural feedstuffs stimulated cellulose digestion by rumen bacteria. Hall, Cheng, and Burroughs (*18*) found that biotin and vitamin B_{12} similarly stimulated these organisms.

Bentley and coworkers (*3–5*) at the Ohio Station have reported a series of in vitro studies in which rumen bacteria separated from rumen liquor with a Sharples supercentrifuge were used to inoculate flasks containing purified ingredients. It was found that autoclaved rumen liquor or water extracts of good quality roughages, protein concentrate feeds, and fermentation byproducts contained factors which stimulated the cellulolytic activities of a mixed rumen population. These stimulatory factors were stable to autoclaving. When rumen liquor was acidified and distilled, the factor activity was recovered in the distillate, whereas distillation from alkaline solutions did not volatilize the activity. This suggested that the factor(s) was a volatile acid. Table VI illustrates the cellulolytic factor activity of several volatile fatty acids. Valeric, caproic, and to a lesser extent, isobutyric and isovaleric acids exhibited marked stimulatory activity. Table VII shows that in addition to valeric (or caproic) acid, the B vitamins biotin and aminobenzoic acid were necessary for maximum activity. Analysis of rumen liquor showed that valeric acid was the major stimulatory fatty acid present in that natural material.

Table VI. Cellulolytic Factor Activity of Fatty Acids for Rumen Microorganisms in Vitro[a]

Additions to Basal Medium	Cellulose Digestion, %
None	19.0
Rumen juice supernatant	60.0
Rumen juice supernatant, acid distillate	56.0
Formic acid	5.0
Acetic acid	18.0
Propionic acid	20.0
Butyric acid	23.0
Isobutyric acid	38.0
Valeric acid	55.0
Isovaleric acid	44.0
Caproic acid	49.0
Heptanoic acid	29.0
C_8, C_9, or C_{10} acids	<20.0

[a] Data from (*3*).

Unlike the factor in rumen liquor, the "cellulolytic" factor activity in materials such as alfalfa extract, yeast extract, and distiller's solubles was not volatile by steam distillation from either acidic or alkaline solutions. When passed through a Dowex 50 column, however, the activity remained with the ninhydrin-positive fraction, suggesting amino acids or peptides as the active substances. This possible relationship between amino acids and cellulolytic factors was investigated and several amino acids were found to possess some stimulatory activity. Valine and proline, in combination, were the most active with less but noticeable activity from arginine, aspartic acid, lysine, and methionine. Biotin and *p*-aminobenzoic acid were also necessary for amino acid activity.

Similar findings have been reported by other laboratories. Hall and others (*19*) found that partial hydrolyzates of certain proteins stimulated cellulose digestion by washed rumen bacteria, suggesting that certain peptides or amino acids were active. Bryant and Doetsch (*7*) found that a combination of a branched-chain and a straight-chain fatty acid (C_5 to C_6) was necessary for maximum growth of *Bacteroides succinogenes*, a cellulolytic bacteria isolated from the rumen. Stimulation of rumen bacteria by natural materials was also reported by Garner, Muhrer, and Pfander (*17*).

The exact nature of the factors in natural materials which stimulate growth of rumen microorganisms has not been fully determined, and the mechanism of this stimulation is not known. These phases are at present under investigation.

Summary and Conclusions

Feedstuffs contain many substances and factors which may change or stimulate the type of bacterial population existing within the rumen. The form of energy available to the bacteria may drastically affect the types of bacteria and protozoa present. High starch rations promote the predominance of groups of bacteria different from those promoted by high fiber rations. The amount and nature of the protein in the ration may affect both the types and numbers of bacteria existing in the rumen. Many of the microflora are dependent on adequate amounts of trace minerals, such as sulfur and cobalt.

Table VII. Effect of Biotin, *p*-Aminobenzoic Acid, and Valeric Acid on Cellulose Digestion by Rumen Microorganisms Grown in Vitro[a]

Additions to Medium	% Cellulose Digested	
	Expt. A	Expt. B
Biotin and PABA	21.6	22.8
Valeric acid, 10 mg./flask (VA)	19.3	31.9
VA + biotin	58.0	44.3
VA + PABA	27.3	36.4
VA + biotin + PABA	61.3	56.9
VA + biotin + PABA + B_{12}	54.6	..
Centrifuged rumen juice	62.5	61.4

[a] Data from (*3*).

Recently, in vitro studies have revealed that certain of the rumen bacteria are stimulated by B vitamins (biotin, *p*-aminobenzoic acid, and vitamin B_{12}) and by 5- and 6-carbon fatty acids. The present data indicate that other factors in natural feeds stimulate these bacteria. The nature of these factors is unknown.

The significance of these findings is realized only when one is aware that the well being and performance of the ruminant animal are directly related to the proper performance of the rumen bacteria themselves. Hence, studies on the nutrition of these microorganisms can have a direct bearing on animal production.

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NUTRIENTS IN FERMENTATION PRODUCTS

Determination and Comparison of Amino Acid Composition of Yeast and Distiller's Solubles

J. W. SPANYER, Jr., and ALAN T. THOMAS

Brown-Forman Distillers Corp., Louisville, Ky.

By monodimensional paper chromatography 15 amino acids were estimated in a strain of *Saccharomyces cerevisiae* and 13 amino acids in distiller's solubles. The materials and procedures used to procure the data are described.

PAPER CHROMATOGRAPHY occupies an important position in the separation and identification of small amounts of similar compounds in a complex mixture. The technique is well suited for the determination of amino acids in microgram quantities. Several methods for quantitative estimation of the individual amino acids have been proposed (*2, 5, 7*).

The separation and estimation of 15 amino acids in a strain of *Saccharomyces cerevisiae* and 13 in distiller's solubles are reported in this paper. The same procedure was used in the chromatographic examination of both the yeast and the solubles.

Materials and Reagents

Chromatographic Chambers. Two chambers were built from $^5/_8$-inch plywood, one to hold three trough and tray assemblies (Schaar and Co.) and the other to hold six. The chambers and lids were paraffined inside and the lids were held in place by wing nuts and bolts hinged to the sides of the box. Rubber tubing was glued around the top edge of the boxes, forming an effective gasket when the lids were tightened. Holes were drilled in the lid directly over each trough for the addition of solvent, after equilibration. These holes were stoppered with rubber stoppers. Atmospheric equilibration was accomplished by lining the walls of the chambers with filter paper, held in place by thumb tacks. The ends of the filter paper dipped into a photographic tray filled with solvent.

Solvent Systems. Solvent 1, 4 parts of phenol (Mallinckrodt, Gilt Label) to 1 part of water plus 20 mg. of 8-quinolinol per 500 ml. of solvent. A beaker containing 0.3% ammonia was placed in the chamber when this solvent was used.

Solvent 2, 2-butanol and 3% ammonia in the ratio of 3 to 1.

Solvent 3, 2-butanol, water, and formic acid in the ratio of 120 to 40 to 1.

Whatman No. 1, $18^1/_4 \times 22^1/_2$ inch chromatographic paper, and a Macbeth Ansco Model 12 color densitometer were also used.

Procedure and Results

Two liters of media from each of 24 pilot plant fermentations were centrifuged to provide the yeast samples used in the work described in this paper. The solids were washed twice and made up to a volume of 250 ml. with demineralized water. Five-milliliter samples of the resuspended yeast (containing about 32 grams of solids per liter) were hydrolyzed under total reflux for 18 to 20 hours with 10 ml. of 6*N* hydrochloric acid. Excess hydrochloric acid was removed by evaporation on a water bath, and the samples were placed in a vacuum desiccator. They were then taken up in exactly 1 ml. of isopropyl alcohol (the isopropyl alcohol inhibits bacterial action on the hydrolyzate). Further dilutions were made when necessary.

Samples of 2.5 grams of distiller's solubles from four different production dates were made up to 250 ml. with demineralized water. Five-milliliter aliquots of these samples were hydrolyzed under the same conditions as the yeast and taken up in 10% isopropyl alcohol. For the determination of tryptophan, the samples were also hydrolyzed with 5*N* barium hydroxide for 18 to 20 hours under total reflux. The barium was precipitated, after hydrolysis, by neutralization with sulfuric acid. The neu-