RUMEN MICROORGANISMS

Factors Influencing Protein Synthesis By Microorganisms in Vitro

DONALD B. HUDMAN and H. O. KUNKEL, with the technical assistance of BETTY J. HOOD

Departments of Biochemistry and Nutrition and Animal Husbandry, Texas A. & M. College System, College Station, Tex.

Studies were set up in order to evaluate a number of factors that might influence the synthesis of protein from nonprotein nitrogen by rumen bacteria using the in vitro or "artificial rumen" technique. Protein synthesis was followed by determining the tungstic acid–precipitable nitrogen in fermentation flasks containing a total volume of 0.3 liter of inoculum and media. Using a level of 0.6% or more urea or the equivalent in other nitrogen sources, maximum synthesis was often obtained within 3 to 6 hours of incubation at 39° C. With low levels of urea, an atmosphere of carbon dioxide was more effective than one of nitrogen, but at the optimum urea concentrations, only anaerobiosis seemed to be necessary. Urea and ammonium acetate were the best nitrogen sources used. The greatest variation in results was due to the level and type of rumen inoculum used. There appeared to be a distinct optimum level of rumen inoculum, below and above which net protein synthesis was decreased. Three grams of glucose plus 9 to 15 grams of cellulose per liter of medium supply the energy required for maximum protein synthesis.

THE IN VITRO OR "ARTIFICIAL RUMEN" METHOD of studying rumen microorganisms provides a controlled means of evaluating factors affecting the growth and metabolism of these organisms. Pearson and Smith (*5*) using this technique demonstrated that the synthesis of protein from urea was due to microbial activities. They showed that the utilization of urea involved first a conversion to ammonia and then the synthesis of protein (*4*). Ammonium bicarbonate was also reported (*5*) to be more efficient than ammonium sulfate and equal to urea as a nitrogen source. Burroughs *et al.* (*2*) concluded that the nitrogen requirements of rumen bacteria are relatively simple.

The polysaccharides, starches and inulin, the trisaccharide, raffinose, and several disaccharides, hexoses, and pentoses were readily utilized by bacteria in protein synthesis (*3*). Arias *et al.* (*1*) used the in vitro technique to show that a variety of carbohydrate sources would aid synthesis of bacterial protein from urea.

Studies in this laboratory were set up in an attempt to use the techniques of Burroughs *et al.* (*1, 2*) to evaluate the synthetic capabilities of samples of rumen contents taken from sheep fed various diets. It seemed desirable to determine first the effects of factors that might influence the bacterial synthesis of protein from nonprotein nitrogen, and thereby provide information concerning optimum conditions. Therefore, the effects of nitrogen source, gas phase, amount of rumen inoculum, and amount of added energy were investigated.

Methods and Materials

The animals used in these experiments were adult sheep maintained on an alfalfa hay diet of good quality. Rumen inoculum for each experiment was obtained from sheep by the use of a water aspirator fitted with plastic Tygon tubing and was collected in a suction flask insulated by ground cellulose. The inoculum was filtered through a fine-mesh nylon cloth.

A measured amount of inoculum was placed in a series of 500-ml. fermentation flasks (Erlenmeyer flasks) that contained a source of carbohydrate and salts (Table I) to supply the nutritional requirements of the rumen microorganisms. A source of nitrogen was added to the fermentation flasks and the flask contents were made up to a volume of 333 ml. with distilled water. The contents were adjusted to a pH of 6.5 to 6.6 and the flasks were fitted with a two-holed rubber stopper which held an aeration tube for the addition of a gas and an outlet for the removal of gases. The flasks were placed in a water bath at 39° C. and a gas was bubbled through the flask contents to stir the media constantly and to provide a controlled atmosphere by rapidly removing fermentation gases. The flask contents were adjusted to a pH of 6.5 to 6.6 every 3 hours.

A sample of the flask contents was removed at various time intervals and placed under refrigeration. Twenty-five-milliliter portions were treated with

Table I. Mineral Additions to Media

	Flask Contents, Mg./Ml.
$MgSO_4.7H_2O$	0.18
$FeSO_4.7H_2O$	0.10
Na_2SO_4	0.23
$CaCl_2$	0.06
KCl	0.59
NaCl	0.59
$CoCl_2$	0.001
NaH_2PO_4	4.93
$NaHCO_3$	4.14

Table II. Effect of Level of Nitrogen Source Under Carbon Dioxide and Nitrogen on Protein Synthesis

Gas Phase	Nitrogen Source	Level, %	Protein Synthesized, Mg. per Flask[a] 3 hours	6 hours	9 hours
CO_2	Urea	0.1	−5	192	155
		0.3	232	282	508
		0.6	579	825	788
		0.9	835	909	976
	Ammonium citrate[b]	0.33	−23	62	450
		2.3	352	412	960
		3.4	544	464	1233
N_2	Urea	0.2	−78	−115	−37
		0.3	98	80	148
		0.6	474	631	389
		0.9	751	485	938
	Ammonium citrate[b]	0.33	0	12	315
		1.1	152	209	612
		2.3	331		1064
		3.4	597	719	1014

[a] 3 grams of glucose, 3 grams of cellulose per flask.
[b] Calculated to provide quantity of nitrogen equal to urea levels used.

15 ml. of 10% sodium tungstate and 60 ml. 0.33 *N* sulfuric acid, allowed to stand 24 hours, and filtered through Reeve Angel No. 812 filter paper. The precipitate was washed with small amounts of 0.33 *N* sulfuric acid and analyzed for its nitrogen content by the Kjeldahl method. The increase in tungstic acid–precipitable nitrogen, calculated as protein (N × 6.25), was used as the criterion for protein synthesis.

Results of Experiments

Nonprotein Nitrogen Level Typical effects of various concentrations of nonprotein nitrogen source on the synthesis of protein by the rumen microorganisms under atmospheres of carbon dioxide and nitrogen are given in Table II. Under both gas phases, the concentration of nitrogen source, urea or ammonium citrate, has a marked effect on the total amount of protein synthesized and the time course of the synthesis. Maximum protein synthesis was not obtained in the 9-hour run until the concentration of nitrogen source was at least 0.6% urea or 2.3% ammonium citrate. Maximum protein synthesis was not obtained before 9 hours at the low levels (0.1% urea or 0.33% ammonium citrate) of nitrogen source, but with the higher levels (0.6% or more of urea or the equivalent in other nitrogen sources) it was often obtained within 3 hours of incubation.

These results differ from those of Pearson and Smith (*5*), who reported that 75 to 100 mg. of urea per 100 grams of rumen contents appear to be the optimum concentrations. The 100-mg. level is equivalent to the 0.1% urea level used in this study. The reasons for these differences in results are not clear at this time.

Gas Phase The effects of the gas phase are shown in Table II. At low levels of urea somewhat more protein was synthesized when a high carbon dioxide tension was maintained. However, from the results of this and several other experiments, it appeared that at the higher concentrations of urea, the gas phase made little difference as long as anaerobic conditions were maintained.

The effect of oxygen was demonstrated in an experiment in which carbon dioxide, nitrogen, and a 94% air–6% carbon dioxide mixture were bubbled through the flask contents. The net protein synthesis in 15 hours under carbon dioxide was 730 mg. of protein per flask, under nitrogen 673 mg., and under the air–carbon dioxide mixture 332 mg. These results indicate that oxygen inhibits the protein synthesis by rumen microorganisms.

Nitrogen Source A series of experiments was set up to determine further the effects of various nitrogen sources on protein synthesis. Urea has been the substrate used by most investigators (*1–5*), but there appear to be relatively few data concerning the effect of the nitrogen form in the in vitro technique of measuring microbial protein synthesis. Burroughs *et al.* (*2*) have concluded that the major nitrogen requirements of rumen microorganisms are relatively simple. Pearson and Smith (*5*) demonstrated a lowered efficiency when ammonium nitrogen was supplied as the sulfate, as compared to a supply of nitrogen as ammonium bicarbonate or urea.

The results of two experiments of special interest are presented in Table III. Nitrogen was supplied as urea and ammonium acetate, bicarbonate, or citrate. In these experiments ammonium acetate was as effective as urea, while less synthesis was noted when ammonium citrate or bicarbonate supplied the nitrogen. Thus, the results with citrate were highly variable. In the results presented in Table II, the protein synthesis was slightly greater with the citrate than with urea. A possible combination of the citrate with the calcium and perhaps other metallic ions to form undissociated salts may be responsible for a decreased activity. The citrate, therefore, under certain conditions may produce a mineral deficiency. The lowered activity with the bicarbonate is probably not due to anything inherent in the form in which the nitrogen is presented, but it is a likely result of the additional manipulations required. In order to maintain a constant pH, the addition of a considerable amount of hydrochloric acid was required before incubation. Perhaps for this reason, ammonium bicarbonate is not a desirable nitrogen source for in vitro studies.

Inoculum Level The results of three experiments in which the inoculum level was varied are reported in Table IV. Low levels of inoculum (20 and 40 ml. of rumen fluid) resulted in a lowered net synthesis of protein, especially when synthetic activity of the inoculum was relatively low, as in experiment 9. In most cases throughout these studies, an inoculum level of 60 ml. of rumen fluid appeared to give maximum synthesis; lower and greater amounts of inoculum resulted in less net protein increases. An example of the effect of too much inoculum is seen in experiment 11, where an increase in the amount of rumen fluid used from 75 ml. to 200 ml. actually resulted in a decrease in protein at 3 hours of incubation and a lowered net protein synthesis at 9 hours.

The effects of inoculum level may be the results of several factors. The low-

Table III. Effects of Nitrogen Source on Protein Synthesis

No.	Nitrogen Source	Level, %	Protein Synthesized, Mg. per Flask[a, b] 1.5 hours	3 hours	6 hours	9 hours
11	Gas phase, N_2					
	Ammonium acetate	2.3		793	676	957
	Ammonium bicarbonate	2.4		180	170	329
	Ammonium citrate	3.4		−2	42	59
	Urea	0.9		526	606	614
14	Gas phase, CO_2					
	Ammonium acetate	2.3	800	890	979	1006
	Ammonium bicarbonate	2.4	569	879	724	355
	Ammonium citrate	3.4	600	969	687	321
	Urea	0.9	695	1140	1072	782

[a] 3 grams of glucose, 3 grams of cellulose per flask.
[b] Average of duplicate flasks.

Table IV. Effect of Varying Levels of Inoculum on Extent of Protein Synthesis from Urea

Expt. No.	Inoculum Level, Ml.	Protein Synthesized, Mg. per Flask[a] 3 hours	6 hours	9 hours
6[b]	20	484	466	...
	40	558	635	...
	60	669	760	...
	80	585	656	...
9[b]	20	247	...	328
	40	196	...	363
	60	296	...	462
	80	264	...	605
11[c]	75	611	1142	1336
	200	−120	561	772

[a] Each value is average from duplicate flasks.
[b] Urea 0.6%; gas phase, CO_2.
[c] Urea 0.9%; gas phase, CO_2.

ered synthesis due to a low level of inoculum may indicate that a certain concentration of bacteria is necessary for maximum activity—that is, the bacterial synthesis of protein depends upon a synergism between the various bacterial species in the rumen. It may also indicate that the rumen inoculum supplies certain nutrients which stimulate bacterial growth. The lag in synthesis with the very high levels of inoculum may be the result of a rapid breakdown of preformed protein when the microbial population is high, or of an insufficient supply of energy.

In 10 experiments using 75-ml. level of rumen fluid, the protein content of inoculum varied from 240 to 890 mg. The maximum total protein determined after incubation varied from 1420 to 2000 mg. per flask when a level of 0.9% urea was used. The correlation between the protein content of the inoculum and the maximum protein content of the flasks after incubation was 0.78, a correlation that is highly significant statistically ($p < 0.01$).

Energy Source

The effects of various carbohydrate additions have received considerable attention (*1*, *3*). Therefore, in the present study, only variations in the level of a soluble source of energy (glucose) and an insoluble source (cellulose) were investigated. The results of three selected experiments in which various effects of carbohydrate additions were observed are presented in Table V. In experiment 25, the need for a small amount of soluble carbohydrate was indicated, while in experiment 32, in which a different inoculum was used, the addition of glucose and cellulose alone or together did not result in a very great increase in protein synthesis. Thus, variation in inoculum may affect the results obtained when various energy sources are studied in the in vitro system.

In the present studies 3 grams of glucose with 3 grams of cellulose per flask were the levels used routinely, although it appeared that 1 gram of glucose with 4.8 grams of cellulose would provide for an equal amount of protein synthesis. In cases where the maximum increase in protein was obtained by 3 or 4 hours of incubation, the cessation of protein synthesis does not appear to be due to a lack of energy. As may be seen from the results of experiment 28 (Table V), the addition of 3 grams of glucose every 3 hours did not result in increased or continued protein synthesis.

Discussion

Maximum protein synthesis appeared to be evident within 9 hours of incubation, often within 3 to 6 hours. It is suggested from these results that in using the in vitro technique to evaluate the synthetic ability of a sample of rumen contents an incubation time of 4 to 6 hours be used. The flask contents should be sampled at 1- or 2-hour periods during the incubation.

Other conditions which are suggested to be optimum for the in vitro synthesis of protein by rumen bacteria under the conditions described in the present work are:

1. An inoculum level of about 200 ml. of rumen fluid per liter of incubation media.
2. A nonprotein nitrogen level of 0.3% (equivalent to 0.64% urea).
3. Anaerobic conditions during incubation, with carbon dioxide probably the preferred gas phase when urea or ammonium bicarbonate is the nitrogen source.
4. Three grams of glucose plus 9 to 15 grams of cellulose per liter of media.

A number of factors carried by the inoculum affect the results obtained. If the in vitro technique is used, an effort must be made to control these factors. This control is often difficult to accomplish even when a single animal on a given diet is used as a source of inoculum. At the same time, because the variation in samples of rumen contents appears to affect the results so markedly, the in vitro technique may prove to be an excellent means of evaluating periodical changes in the rumen microbial population. There is no assurance that the synthesis of protein under conditions reported here is accomplished by the same population of bacteria responsible for nonprotein nitrogen utilization in the ruminant.

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Table V. Effects of Level of Glucose and Cellulose on Protein Synthesis from Urea

Expt. No.	Carbohydrate Additions, Grams Glucose	Cellulose	Protein Synthesized, Mg. per Flask[a, b] 3 hours	6 hours	9 hours
25	0	3	276	385	420
	1	4.8	516	1058	1247
	3	3	611	1142	1336
32	0	0	186	619	656
	0	5.7	223	569	751
	1	4.8	306	653	855
	1	0	198	662	920
	2	0	256	633	863
	3	0	351	647	906
	3	3	252	669	888
28	3	3	1140	1070	780
	9[c]	3	1354	1190	990

[a] 75 ml. inoculum; 0.6% urea; gas phase, CO_2.
[b] Each value average of duplicate flasks.
[c] 3 grams of glucose added every 3 hours.