Simple Determination of Microbial Protein in Rumen Liquor

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ABSTRACT
A method for microbial protein determination based on Lowry's assay was devised. Differential centrifugation was used to separate bacterial cells from rumen liquor. Proteins from cells were released by suspending in .25 N sodium hydroxide and heating in boiling water bath for 10 min. Protein was determined by the Folin phenol method. The method is simple, accurate, reproducible, sensitive, specific, brief, and can be performed with small amount of rumen liquor (5 ml). A large number of samples can be handled conveniently.

INTRODUCTION
Determination of microbial protein synthesis is significant in evaluation of different nitrogenous compounds in ruminant rations. The most widely used methods for determination of microbial protein employ precipitating agents such as trichloroacetic acid (TCA), perchloric acid (HClO₄), tungstic acid, and picric acid (1) in addition to other methods (2). However, all these methods have limitations (2). We report a method for determination of microbial protein based on the method of Lowry et al. (6).

MATERIALS AND METHODS
Rumen fluid was obtained from a fistulated male calf weighing 150 kg and fed 1.5 kg concentrate mixture daily containing maize (35 parts), linseed cake (32 parts), wheat bran (30 parts), salt (2 parts), and mineral mixture (1 part). Wheat straw was fed ad libitum. The rumen liquor was strained through four layers of cheesecloth to remove large feed particles and debris. The strained rumen liquor was shaken in a magnetic stirrer (400 rpm) for 45 s to remove the microbes adsorbed on the feed particles. It then was centrifuged at 408 × g for 5 min for removing protozoa and remaining feed particles. Aliquots of 5 ml each were centrifuged at 25,000 × g for 20 min. Supernatants were discarded, and cells obtained were washed with distilled water (McDougall's buffer (7) may be preferred to avoid cell lysis) and centrifuged as before. The supernatants again were discarded, and the "washed cells" were suspended in 15 ml of .25 N NaOH and heated in boiling water bath for 10 min. Contents then were centrifuged at 25,000 × g for 30 min, and supernatant was collected. Protein in an aliquot of .5 ml was determined by the method of Lowry et al. (6) with bovine serum albumin as standard. Protein in 5 ml of rumen liquor was calculated by multiplication of a factor of 30. In a separate experiment, aliquots of 20 ml each obtained after removing the protozoa and feed particles were subjected to treatments as mentioned above, and "washed cells" obtained were transferred to Kjeldahl flasks for micro-Kjeldahl nitrogen estimation (5).

For comparison, microbial protein also was determined by protein precipitating agents as described by Barr et al. (1). Aliquots of 10 ml of rumen liquor, obtained after removing the feed particles and protozoa, were taken, and 2.5 ml of 64.5% TCA, or 2.5 ml of 2 M HClO₄, or 10 ml of 1% picric acid, or 3.8 ml of 10% sodium tungstate and 3.8 ml of 2/3 N sulfuric acid were added to each sample. The precipitates obtained after washing with distilled water were not taken for micro-Kjeldahl determination (5) as mentioned in (1) but were suspended in 30 ml of .25 N NaOH, heated in boiling water bath for 10 min, and protein was estimated by the method of Lowry et al. (6).

For optimizing extraction conditions, the "washed cells" were digested in .25 N NaOH for 10, 15, 20, 30, 45, and 60 min in a boiling water bath. For the optimum concentration of NaOH for the extraction of protein, the "washed cells" were digested in .25 N, .50 N, and .75 N NaOH in a boiling water bath for 10 min. The

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contents were centrifuged at 25,000 x g for 30 min, and protein in the supernatant was determined as previously described.

In another experiment the "washed cells" were suspended in 7.5 ml distilled water and sonicated at 70 mA for 10 min. The sonicated material was centrifuged at 25,000 x g for 30 min, and the supernatant (Sup-I) was collected. The debris was suspended in 7.5 ml of .5 N NaOH and heated in boiling water bath for 10 min, and supernatant was collected after centrifugation at 25,000 x g for 30 min. This supernatant and Sup-I were mixed, and protein was determined.

The contribution of protein by microbes was determined by our taking 10 ml samples of liquor free of protozoa and feed particles and centrifuging at 25,000 x g for 20 min. Supernatants collected were treated with TCA, HClO₄, tungstic acid, or picric acid, and protein was determined on the precipitate from each sample. These amounts were subtracted from amounts obtained for whole rumen liquor by the corresponding precipitating agents to arrive at the contribution of microbes, which should be true microbial protein.

The term microbial protein is a misnomer for bacterial protein estimation in this procedure but has been retained for clarity of results with reference to the published literature wherein workers (2) have estimated only bacterial protein but termed it microbial protein.

RESULTS AND DISCUSSION

Recovery of protein was not increased when time of heating in boiling water bath was increased from 10 min to 60 min. Similarly, protein did not differ when cells were digested in .25, .50, or .75 N NaOH. This suggested that .25 N NaOH is sufficient to dissolve all protein of bacterial cells when heated for 10 min. The sonication and treatment of debris with NaOH was expected to release more protein, if present. However, no difference in microbial protein was observed with an additional step of sonication. This suggested that suspension of cells in .25 N NaOH and keeping in boiling water bath for 10 min extracts the protein of cells completely.

In comparisons of protein precipitating agents, HClO₄ was the best precipitating agent followed by TCA, tungstic acid, and picric acid (Table 1). Such have been compared by other workers (1, 8, 10, 14), and the difference is attributed to a number of factors (1). The discrepancy between microbial protein determinations make these methods undesirable.

<table>
<thead>
<tr>
<th>Agents</th>
<th>Bacteria plus soluble protein (mg/10 ml rumen liquor)</th>
<th>Error (%)</th>
<th>Soluble protein (mg/10 ml supernatant)</th>
<th>Bacterial protein (mg/10 ml rumen liquor)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>X</td>
<td>SE</td>
<td>X</td>
<td>SE</td>
</tr>
<tr>
<td>Perchloric acid</td>
<td>26.40*</td>
<td>.73</td>
<td>+260</td>
<td>16.19*</td>
</tr>
<tr>
<td>Trichloroacetic acid</td>
<td>14.70*</td>
<td>.21</td>
<td>+145</td>
<td>4.60*</td>
</tr>
<tr>
<td>Tungstic acid</td>
<td>1.087**</td>
<td>.20</td>
<td>-89</td>
<td>0</td>
</tr>
<tr>
<td>Picric acid</td>
<td>.367**</td>
<td>.01</td>
<td>-96</td>
<td>0</td>
</tr>
<tr>
<td>Present method</td>
<td>...</td>
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*Precipitated protein from rumen liquor after removing protozoa and feed particles.
<table>
<thead>
<tr>
<th></th>
<th>Error (mg/10 ml supernatant)</th>
<th>Bacterial protein (mg/10 ml rumen liquor)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>X</td>
<td>SE</td>
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</table>

*Errors in the microbial protein obtained by precipitating agents (column 2) as compared with the average of the present method (column 5).

**Precipitated protein in supernatant obtained after high speed centrifugation of supernatant remaining after removing feed particles and protozoa.

*Average of 8 determinations in duplicate on 8 days.

**Average of 3 determinations in duplicate on 3 days.

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Moreover, the amounts obtained by HClO₄ and TCA precipitating agents are much higher than those by the method reported here (Table 1), because the precipitating agents have been used on the whole rumen liquor, which includes protein content of the nonmicrobial soluble fraction also, the origin of which could be feed and saliva. Hence, the protein obtained by TCA or HClO₄ precipitation methods could not be called true microbial protein. Table 1 also shows that true microbial protein obtained as mentioned in materials and methods by TCA and HClO₄ methods is the same as obtained by the method reported here. The same was not true for the other precipitating agents, tungstic and picric acids. However, there was a wide difference in protein obtained by precipitating the rumen liquor (14.70 and 26.4 mg/10 ml) or supernatant (4.60 and 16.19 mg/10 ml) by TCA and HClO₄. But the difference in protein precipitated by the two reagents in the whole rumen liquor is almost the same as the difference in the supernatant (free of cells). It appears that HClO₄ completely precipitated proteins both from whole rumen liquor and supernatant (free of cells). However, TCA precipitated completely the protein of cells but could not precipitate completely the soluble proteins in rumen fluid. We confirmed this by suspending the "washed cells" (obtained from 10 ml of rumen liquor) in 10 ml of distilled water and then precipitating protein by TCA or HClO₄. The amounts were the same as by the method reported here. Evidently the digestion of microbial cells in NaOH and the amounts obtained by the method of Lowry et al. (6) represented the true microbial protein unaffected by different interfering compounds (6, 9). Moreover, concordance in the amounts by TCA or HClO₄ precipitation of "washed cells" and direct extraction with NaOH further confirms that there was complete extraction of microbial protein by our method.

Barr et al. (1) developed a method to determine bacterial and protozoal proteins in in vitro fermentation mixtures by elimination of low speed centrifugation when highly cooked substrates are fed to the animals, because the highly cooked substrates lead to the formation of a polysaccharide layer around bacteria and, hence, are lost along with protozoa and feed particles by low speed centrifugation. Under such conditions, in the present method, too, we can eliminate the low centrifugation step and estimate the microbial protein by suspending the pellet obtained by direct high speed centrifugation in .25 N NaOH, heating for 10 min in boiling water bath, and using the method of Lowry et al. (6) instead of micro-Kjeldahl method (5). This is because the means when cells were subjected to micro-Kjeldahl method (6.25 × N = 11.91 ± .90 mg/10 ml rumen liquor) were higher when than the cells were digested in .25 N NaOH and protein estimated by Lowry's method (10.14 ± .89 mg/10 rumen liquor). The higher means with micro-Kjeldahl method could be from estimation of nitrogen compounds other than protein of the cell as well (13). In spite of instability of reagents and nonlinearity of the standard curve, the Lowry's method (6) is more specific, simple, sensitive, and accurate as compared to micro-Kjeldahl method (5, 9, 13).

Bacteria in the rumen adhere to feed particles (3). Shaking of strained rumen liquor has been used to detach bacteria from the feed particles. With and without shaking, the microbial protein was 10.14 ± .89 and 8.09 ± .74 mg/10 ml rumen liquor. The greater microbial protein after shaking of the strained rumen liquor showed that shaking is removing bacteria attached to feed particles, but it is likely the shaking may not be detaching all bacteria from feed particles. Therefore, the method reported here and the methods using precipitating agents (2) may not give the protein contributed by all the bacteria of the rumen liquor. There is a need to develop a method for complete extraction of bacteria adsorbed on feed particles. The use of the method for complete detachment of bacteria from feed particles with the method of bacterial protein determination reported here would quantitate the bacterial content of rumen digesta.

In conclusion, a simple, sensitive, reproducible, and accurate method based on Lowry's assay was developed for microbial protein determination. It has a number of advantages over other methods; it takes little time, can be performed with small amount of rumen liquor (5 ml), and a large number of samples can be handled conveniently. Evidence is sufficient that there is complete extraction of proteins by the method reported here unlike other methods employing different precipitating agents. Besides, the microbial protein determined by
precipitating agents (1) does not represent true microbial protein.

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REFERENCES


