In Vivo and In Vitro Gastric Emptying of Milk Replacers Containing Soybean Proteins

ABSTRACT

Gastric digestion of three milk replacers for which protein was provided either exclusively by milk powder or partially (50%) by heated soybean flour or soybean protein concentrate was studied in vivo and in vitro. In vivo gastric emptying of protein fractions of the diets was measured in six preruminant calves fitted with reentrant duodenal cannulas and used in a double 3 x 3 Latin square design. In vitro gastric emptying was studied after hydrochloric acid and rennet digestion in an artificial stomach. In vivo and in vitro flow rates of 12% TCA-insoluble N and total N were higher for the soybean diets than for the milk protein diet, indicating that the incorporation of soybean protein prevented casein from clotting. Because of this faster gastric emptying, proteolysis in the stomach was reduced. However, gastric emptying of NPN (12% TCA-soluble N) was significantly decreased only in vitro. No difference existed between the two milk replacers containing either soybean flour or soybean protein concentrate. In vivo and in vitro results were correlated suggesting that the in vitro method could be used to predict gastric digestion of protein fractions in vivo.

(Key words: artificial stomach, preruminant calf, gastric emptying, soybean)

Abbreviation key: FM = fresh matter, MP = milk powder, PN = protein N, SC = soybean protein concentrate, SF = soybean flour, TN = total N.

Received January 21, 1993.
Accepted September 3, 1993.
1Contribution Number 2149 from the Centre for Food and Animal Research.
2Contribution Number 407 from the Lennoxville Research Station, PO Box 90, Lennoxville, QC, Canada JIM 1Z3.
3Laboratoire du Jeune Ruminant, 65 rue de St Brieuc.
4Ferme expérimentale.
5Centre for Food and Animal Research.
6Département de Nutrition Humaine et de Consommation.
7Reprint requests.
8Station de Recherches Laitières.
INTRODUCTION

Soybeans, a cheaper protein source than milk, often are used in milk replacers for preruminant calves. However, BW gain and N digestibility can be decreased by 60 to 90 and 20 to 50%, respectively, when insufficiently refined soybean products are incorporated in high amounts (1, 5, 15). As with other substitute protein sources, these effects may be partially due to the lack of coagulation in the abomasum (25), faster gastric emptying of proteins (2, 9), or lower abomasal and pancreatic secretions (6, 8, 27). Moreover, antinutritional factors, such as trypsin inhibitors, could contribute to the poor digestibility of soybean protein in the preruminant calf (15). Soybean protein also could induce allergic reactions in the gastrointestinal tract of the calf (21). These undesirable effects could be reduced by treating the soybeans. Soybean concentrates (SC) prepared by extraction of fat-free soybean meal with hot aqueous ethanol showed better utilization by calves than did heated soybean flour (SF) (22). These differences between soybean preparations could be partially due to different effects on digestion and gastric emptying.

To avoid expensive and time-consuming in vivo studies on gastric digestion of substitute protein sources, Savalle et al. (18) developed an in vitro method that simulates abomasal digestion in the preruminant calf. In a previous study (2), correlations between in vivo and in vitro gastric emptying of milk replacers containing whey proteins were high \( r = 0.84 \) for protein N (PN). The present study was conducted to compare in vivo and in vitro methods of digestion for milk replacers containing soybean protein and to determine the correlations between methods.

MATERIALS AND METHODS

PROTEIN SOURCES AND DIETS

The same protein sources and diets were used for both in vivo and in vitro experiments. Milk powder (MP) was used as a control protein source. The SF (Société Industrielle des Oleagineux, Saint Laurent de Blangy, France) was prepared from dehulled seed by lipid extraction with hexane and heat treatment to denature the antitryptic factors. The SC (Aarhus Oliefabrik, Aarhus, Denmark) was obtained by hot aqueous ethanol treatment, which eliminated the oligosides and denatured most of the proteins. The SF and SC had protein contents of 48.9 and 66.5% (on a DM basis), respectively; SF still contained a fairly high residual globulin activity, as determined by ELISA with antibodies raised against the native forms (6, 3, and 15% of the activities found in raw SF for glycinin and \( \alpha- \) and \( \beta- \) conglycinins, respectively), but only \( \alpha- \) conglycinin was detectable in SC (11%) (26). Both products contained virtually no trypsin inhibitor activity.

Three milk replacers (MP, SF, and SC) containing 22% CP and 19% fat on a DM basis were prepared (Table 1). In the MP diet, milk was the only protein source. In the other two diets, 50% of the protein was provided by MP, and the rest was supplied by either SF or SC. The three milk replacers contained 1% Ca.

TABLE 1. Composition of milk replacers.

<table>
<thead>
<tr>
<th>Item</th>
<th>MP</th>
<th>SF</th>
<th>SC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat premix</td>
<td>264%</td>
<td>264%</td>
<td>264%</td>
</tr>
<tr>
<td>Skim milk powder</td>
<td>32.0</td>
<td>32.0</td>
<td>32.0</td>
</tr>
<tr>
<td>Soybean flour</td>
<td></td>
<td>24.0</td>
<td></td>
</tr>
<tr>
<td>Soybean concentrate</td>
<td></td>
<td></td>
<td>17.0</td>
</tr>
<tr>
<td>Lactose</td>
<td>12.6</td>
<td>19.0</td>
<td>26.0</td>
</tr>
<tr>
<td>Starch</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>Vitamins and minerals</td>
<td>2.4</td>
<td>4.0</td>
<td>4.0</td>
</tr>
</tbody>
</table>

1MP = Milk powder, SF = MP and soybean flour (50:50 on a CP basis), and SC = MP and soybean protein concentrate (50:50 on a CP basis).

264% Skim milk powder and 36% tallow (Groupe Lactel, PQ, Canada).

3Commercial formula to meet veal calf requirements (Groupe Lactel).
postsurgical recovery, calves were housed individually in metabolic stalls with slotted floors and kept in a room with controlled temperature and relative humidity.

The milk substitute was reconstituted with water (42°C) to contain 15.4% DM. Each calf was fed twice daily at 0830 and 1630 h. The daily total intake of milk substitute by each calf during the experiment was set at 10% of its BW on d 1 of each period. The calves were assigned to a double 3 x 3 Latin square design, and each experimental period lasted 8 d. Each period consisted of 2 d of transition to the experimental diets, 5 d of feeding the experimental diets, and 1 d of digesta collection.

Duodenal Digesta Collection. The day before duodenal digesta sampling, sodium citrate (11 g/kg of powder) was added to the milk substitute offered in the morning to prevent abomasal curd formation and to accelerate abomasal emptying (25); at the evening meal, the calves received only water with electrolytes to ensure complete emptying of the abomasum. On sampling days, calves received the experimental diets, and the digesta flowing from the abomasum during the first 6 h postfeeding were collected totally in TCA (12% final concentration) from 0 to .5, .5 to 1, 1 to 2, 2 to 3, 3 to 4, and 4 to 6 h after the morning meal. Digesta were weighed and centrifuged at 10,000 x g for 30 min at 5°C. Supernatants and sediments were stored at -20°C. Digesta collected the week before from the same calves fed their respective diets were warmed to 37°C and returned in the distal part of the cannula.

In Vitro Experiment
The in vitro method was developed to reproduce, in 3 h, 6 h of in vivo digestion in a calf. The milk substitutes were reconstituted to 15.3% DM immediately before use with deionized water at 40°C. The experimental procedure was performed as previously described by Yvon et al. (28). Briefly, 500 ml of the reconstituted milk replacer were placed in an artificial stomach (an Erlenmeyer flask shaken at 150 oscillations of 5 mm/min in a water bath at 37°C) and subjected to the action of rennet (520 mg/L of chymosin, 290 mg/L of pepsin; Boll-Hansen, Arpajon, France). The initial enzyme:substrate ratio was 1:2000 (wt/wt); diluted enzyme (2% liquid rennet) then was added at a variable flow rate following an exponential function with initial and final flow rates of 8.0 and 1.0 ml/min, respectively, and the exponential base of 1.07. A similar function was used to control the digesta emptying flow with initial and final flow rates of 10.0 and 3.0 ml/min, respectively. Acidification of the medium was controlled and adjusted so that the pH of the reaction medium followed a predetermined curve. This curve was established by setting the initial pH at 6.7, the intermediate pH (1.5 h) at 3.2, and the final pH (3 h) at 2.0 according to an exponential function.

Six effluent samples were collected separately over 3 h at 15, 30, 60, 90, 120, and 180 min after the beginning of digestion. To stop enzymatic digestion, each sample immediately was raised to pH 8.0 with 2 M NaOH using a pHstat. All assays were carried out in triplicate. Each sample was collected in TCA at a final concentration of 12% and centrifuged at 10,000 x g for 20 min. Supernatants and sediments were stored separately at -20°C until analysis.

Chemical Analysis
To study the digestion of protein fractions only, N fractions of the diets and TCA supernatants and sediments were determined by AA analysis as the sum of all AA. The AA composition was determined after acid hydrolysis (6N HCl, 110°C, 24 h under vacuum) with a Biotronik LC 5000 analyzer (Munich, Germany) for in vitro samples and with a Beckman system 6300 autoanalyzer (Palo Alto, CA) for in vivo samples. The NPN and PN represented the N content of the supernatant and the sediment, respectively. The total N (TN) content was calculated as the sum of NPN and PN.

Statistical Analysis
Data were subjected to ANOVA for a double 3 x 3 Latin square design (in vivo) or a completely randomized design (in vitro) using the general linear models procedure of SAS (17). Digesta flow composition was analyzed in a repeated measure design using the REPEATED statement of the general linear models procedure of SAS (17). When the sphericity test was significant, the multivariate method was used to test the effects of time and
time x treatment interactions; otherwise, univariate tests were employed. The closeness of the relationship between in vivo and in vitro data was evaluated by calculating Pearson's test of correlation.

RESULTS

Flow of Digesta Fresh Matter

As shown by ANOVA, the in vivo cumulative flow pattern of digesta fresh matter (FM) (i.e., the whole content passing through the duodenum, on a fresh basis) was similar for the three diets \((F = 1.58; \text{df} = 10 \text{ and } 8; \ P = .26)\) during the interval (6 h) of measurement (Figure 1). No significant global effect \((P = .18)\) occurred among the three diets. After 1 h of digestion, the cumulative recovery of digesta PM corresponded to 79, 97, and 93% of FM intake (i.e., the reconstituted milk substitute) for MP, SF, and SC diets, respectively, and was equivalent to 100% at approximately 2.5, 1.5, and 1.5 h, respectively. At 6 h, recovery reached 135, 135, and 129%, respectively.

In vitro, exponential emptying rate used was fixed using previous results (19) with preruminant calves fed milk in which endogenous secretions were measured and subtracted from the total emptying volumes. The cumulative flow of digesta FM (without secretions) leaving the artificial stomach at 3 h was set at 69% of the FM intake (Figure 1), which was slower than the gastric emptying in the in vivo experiment for the corresponding time (6 h). The same flow rate of in vitro FM was used for all three diets.

Flow of Digesta TN

The in vivo flow pattern of TN was not significantly different \((F = 1.41; \text{df} = 10 \text{ and } 8; \ P = .32)\) during the digestion (Figure 2A). However, the flow of TN was significantly higher \((P = .0003)\) with SF and SC than with MP. No significant difference \((P = .21)\) occurred between SF and SC. Cumulative flows at 6 h were 76, 111, and 107% of TN ingested for MP, SF, and SC, respectively. For SF and SC, 100% of the TN ingested was recovered between 3 and 5 h after feeding. Results were similar in vitro. The flow pattern of TN was not significantly different \((F = 2.60; \text{df} = 10 \text{ and } 4; \ P = .19)\) during the digestion (Figure 2B). However, the flow of TN was significantly higher \((P = .0013)\) with SF and SC than with MP and had a significantly higher kinetic rate during the first two periods of measurement \((P = .006 \text{ and } .002)\) respectively. At 3 h, recovery rates of TN were 45, 61, and 58% for MP, SF, and SC, respectively.

Flow of Digesta NPN

The in vivo cumulative flow of NPN was similar \((F = 2.07; \text{df} = 10 \text{ and } 8; \ P = .16)\) for the three diets (Figure 3A) and, at 6 h, represented 12.3, 9.6, and 9.9% of TN intake for MP, SF, and SC, respectively. In vitro, the flow pattern of NPN followed parallel kinetics for all three diets (Figure 3B). The flow was significantly \((P = .0003)\) lower with SF and SC than with MP and slightly lower \((P = .04)\) with SC than with SF. After 3 h, the cumulative in vitro flow reached 6.1, 4.9, and 4.2% of TN intake for MP, SF, and SC, respectively. Flow rates of NPN were about half as great in vitro as in vivo.

Flow of Digesta PN

The in vivo cumulative digesta flow pattern of PN was similar \((F = 1.57; \text{df} = 10 \text{ and } 8; \ P = .27)\) with the three diets (Figure 4A). The flow was significantly \((P = .0003)\) higher with SF and SC than with MP; no significant \((P =
difference occurred between SF and SC. The cumulative flow of PN was 35, 83, and 69% of TN ingested after 1 h and 63, 101, and 97% after 6 h for MP, SF, and SC, respectively. The in vitro cumulative flow of PN (Figure 4B) followed a pattern similar to that observed in vivo \((F = 2.93; \text{df} = 10 \text{ and } 4; P = .16).\) The flow was significantly higher for SF and SC \((P = .002 \text{ and } .004, \text{respectively})\) than for MP. After 3 h of digestion, the flow of PN, expressed as percentage of TN intake, was similar for SF (57%) and SC (54%); both of which had significantly \((P < .05)\) higher flow than that of MP (39%).

Correlation Between In Vivo and In Vitro Gastric Emptying of the Protein Fractions

Coefficients of correlation between in vivo and in vitro gastric emptying were significant \((P < .05)\) for all N fractions (Table 2). With MP, coefficients were higher \((r \geq .98; P < .001)\) than with SF and SC \((.88 \leq r \leq .93; .01 < P < .05).\) The coefficient was lowest for the PN fraction with SF \((r = .88; P < .05).\)

DISCUSSION

Gastric emptying of FM increases during the 1st h of digestion after nonmilk proteins...
are fed (10), which is related to the absence of curd formation in the abomasum (16). The presence of 50% milk protein in the SF and SC diets could have reduced this effect, which was not significant in our experiment. The apparent volumes of gastric secretions over 6 h, calculated as the difference between the volumes of FM ingested and recovered, were not significantly different (1110, 1089, and 874 ml for MP, SF, and SC, respectively). These values agree with those measured by Scanff et al. (19) for milk-fed calves and with those calculated by Guilloteau et al. (10) for calves fed a diet containing soybean protein.

The effect of incorporating soybean protein on NPN digesta flow was similar in vivo and in vitro, as reflected by their high correlation. Flow rates of NPN were about two times greater in vivo than in vitro because the in vitro emptying rate of FM was preset at a flow rate smaller than that observed in vivo. However, results were correlated despite these differences in flow rates. Differences in NPN flow rates among the three diets were not significant in vivo, although they were significant in vitro because of the better reproducibility obtained in vitro. Therefore, the in vitro system showed a decreased proteolysis for SF and SC compared with that for MP, in agreement with the results obtained previously in vitro (12) and in vivo (10) with nonmilk proteins. During the 1st h of casein digestion, the glycosylated fraction of caseinomacropeptide is released rapidly from the abomasum and is almost the only 12% TCA-soluble peptide to flow into the intestine (29). The SF and SC diets contained one-half as much casein as the MP diet, which explains the lack of significant increase in emptying of NPN with SF and SC, although emptying of TN was much faster during the 1st h. The NPN emptied after 3 h (in vivo) or 1.5 h (in vitro) probably consisted of small peptides resulting from casein hydrolysis. These peptides then were much less abundant with SF and SC than with MP because of the faster abomasal emptying of casein.

![Figure 4. Cumulative flow of protein N (PN; 12% TCA-insoluble) measured in vivo (A) and in vitro (B) with diets for which protein was provided exclusively by milk (○) or partially (50%) by either soybean flour (△) or soybean protein concentrate (★). Vertical bars are standard errors.](image)

Table 2. Correlation$^1$ between in vivo and in vitro gastric emptying of $N$ fractions.

<table>
<thead>
<tr>
<th>Fractions$^3$</th>
<th>MP</th>
<th>SF</th>
<th>SC</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPN</td>
<td>.98***</td>
<td>.93**</td>
<td>.91*</td>
</tr>
<tr>
<td>PN</td>
<td>.99***</td>
<td>.88*</td>
<td>.92**</td>
</tr>
<tr>
<td>TN</td>
<td>.99***</td>
<td>.89*</td>
<td>.93**</td>
</tr>
</tbody>
</table>

$^1$Coefficient of correlation; $n = 36$.

$^2$MP = Milk powder, SF = MP and soybean flour (50:50 on a CP basis), and SC = MP and soybean protein concentrate (50:50 on a CP basis).

$^3$PN = Protein N (12% TCA-insoluble), TN = total N.

* $p < .05$.

** $p < .01$.

*** $p < .001$.

Both TN and PN left the abomasum and the artificial stomach more rapidly with SF and SC than with the MP diet, as previously observed by several authors (7, 10). During h 1 of in vivo digestion, 2.2-fold more of the TN ingested was released as PN with SF and SC than with MP. The in vivo and in vitro gastric emptying of TN and PN of SF and SC followed that of FM, indicating that clotting phenomenon was mostly absent, although these diets contained 50% milk proteins. With a milk diet, a large part of casein is retained in the abomasal clot (3). However, with the MP diet, the in vivo cumulative flow rates of TN and PN after 6 h were about two times greater than the values obtained previously under similar conditions in vivo (2, 16) and in vitro (2). The pattern of gastric emptying of the MP diet used in our experiment was similar to that of a milk substitute containing an oxalate-NaOH buffer added to prevent coagulation in the abomasum (16) and to that of skim milk heated to 95°C for 45 s (19). This finding suggests that the MP used in our experiment was not high quality. High temperature during processing induces interactions between β-lactoglobulin and caseins, decreases coagulability of milk, and alters proteolysis (19, 24). Moreover, addition of fat to skim milk during the homogenization procedure could have an impact on protein quality and the subsequent coagulation phenomenon. Homogenization reduces the size of fat globules and consequently increases fat surface area (14). Skim milk components, especially casein, adsorb onto the surface of fat (4). Migration of casein to the fat globule surface decreases the number of sites available for coagulation and could be responsible for decreased curd firmness (14). All of these factors could be responsible for the faster gastric emptying of the MP diet in our experiment. However, because the same MP was used to formulate the three experimental milk replacers, the effects of soybean protein incorporation on gastric emptying could be compared.

Patterns of PN and TN flow in vivo and in vitro were correlated, reinforcing our previous observations with milk and whey proteins (2). Protein from the SF and SC diets was less hydrolyzed than protein from the MP diet, possibly because of the shorter time of contact with and lower susceptibility to hydrolysis by gastric enzymes (12). Furthermore, pH in the abomasum during the 1st h of digestion was not optimal for the general proteolytic activity of pepsin (pH 4 to 6). Sedgman et al. (20) observed decreased proteolytic activity with substitute proteins; Smith and Sisson (23) recorded higher pH of the abomasal content with diets containing soybean protein. Also, chymosin secretion was reduced when milk protein was replaced by soybean protein (6, 27). Heating soybeans induces denaturation of the trypsin inhibitor and lectins (13). Extraction with hot aqueous ethanol removes ethanol-soluble oligosaccharides and inactivates the allergenic factors (22), which improve the nutritional value of soybeans for the preruminant calf (21). However, in our experiment, treatment with hot ethanol did not change gastric digestion of the N fractions compared with that observed with the SF diet. Further experiments are needed to study the digestion of these diets in the other parts of the digestive tract.

**CONCLUSIONS**

Gastric emptying of PN and TN increased when soybean protein replaced 50% of milk protein in the milk substitute given to preruminant calves, possibly because of poor clotting in the abomasum. Abomasal flow of FM and NPN were similar among treatments. Proteolysis in the abomasum was reduced with soybean as a result of faster emptying of PN. No difference existed between SF and SC, which indicates that extraction with hot aqueous ethanol had no more influence than moist heating on gastric emptying of the protein fractions under our experimental conditions. In vivo and in vitro results were correlated, showing that the in vitro model could be used to study gastric digestion of different protein sources.

**ACKNOWLEDGMENTS**

The authors thank L. Neill and G. Parent for technical assistance and B. Cavanagh and S. Campbell for assistance with the daily care of the calves. This work was part of a Ph.D. program and was supported in part by Natural Sciences and Engineering Research Council of Canada University Industry Grant Number CR0044617 (Canada), a grant from France-
Québec Collaboration in Biotechnology, a grant from Ministère de la Recherche et de la Technologie (France), and a grant from Groupe Lactel (Québec) and Union Laitière Normande (France).

REFERENCES


