The Use of Nitrogen-15 Labeling for the Assessment of Leguminous Protein Digestibility

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Summary This study evaluated the digestibility of leguminous protein labeled with $^{15}$N, by using nitrogen balance and quantitation of fecal endogenous nitrogen (FEN), determined by isotopic dilution, in order to correct apparent values. Seeds of common beans, cowpea and common pea labeled with 1,000 atoms% of $^{15}$N-excess were used as protein sources in diets for 60 male Wistar rats, during a 6-day assay. The real digestibility values obtained with FEN were 77.6, 84.4, and 86.3% for common beans, cowpea and common pea, respectively. They were higher and statistically different ($p<0.05$) than true digestibility values, corrected by non-protein diet. FEN showed a direct, moderate and positive relation with weight of dry matter eaten, initial body weight, weight gain and weight of dry matter of feces, the latter showing the highest correlation, with a coefficient $r=0.8930$ at 1% level.

Key Words leguminous, protein, digestibility, nitrogen-15, rat

The nutritional value of legumes and particularly beans, has been studied mainly concerning their quality as protein source, due to their lower costs compared to animal sources and high consumption, especially in developing countries (1). The low digestibility of these proteins when raw, is due mainly to the presence of antinutritional factors, like proteases inhibitors and lectins. Thermal inactivation of these factors by cooking results in higher digestibility values than those obtained with raw leguminous proteins; however, the values are still lower when compared with animal proteins like casein (2).

The influence of these seeds on rat fecal endogenous nitrogen excretion as on digestive secretions and epithelial desquamation, was investigated (2-4) and, in all cases, authors found increased secretion and excretion by animals fed cooked legumes, which could indirectly contribute to decreased protein digestibility values. By using $^{15}$N isotopic labeling of bean protein, it was possible to identify the contribution of endogenous nitrogen to total fecal nitrogen excreted by animals fed
these labeled seeds and to determine the digestibility values, which were called real (5). In this study, the real protein digestibilities of cooked common bean (*Phaseolus vulgaris*, L.), cowpea (*Vigna unguiculata*, L.) and common pea (*Pisum sativum*, L.), labeled with $^{15}$N were determined with the correlations between FEN and the animal weight, weight gain, dry matter eaten and dry weight of feces.

**EXPERIMENTAL**

*Labeled protein sources.* Legume seeds from pure plant cultivars of common bean (*Phaseolus vulgaris*, L., cv. Aroana-80), cowpea (*Vigna unguiculata*, L., cv. Pitiúba) and common pea (*Pisum sativum*, L., cv. Torta-de-Flor-Roxa) obtained from the Centro Nacional de Pesquisa de Arroz e Feijão, Empresa Brasileira de Pesquisa Agropecuária, Goiânia, Goiás, and the Instituto Agronomico de Campinas, Campinas, São Paulo, Brasil were utilized in this study. The seeds were planted in experimental plots at the Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Piracicaba, São Paulo, Brasil, cultivated with $(^{15}\text{N})\text{H}_4\text{SO}_4$ enriched with 10 atoms% of $^{15}$N, and received common agricultural procedures. At harvest, the leguminous seeds had $1.000\pm0.142$ atoms% of $^{15}$N-excess. After selection, washing and soaking in tap water by 12h, the seeds of common bean and cowpea were cooked under pressure (14.7psi) for 20 and 15 min, respectively, and the common pea was cooked in boiling water for 20 min at atmospheric pressure. The cooked seeds were freeze-dried and ground to 40-mesh size, in order to prepare the experimental diets.

*Diets.* The experimental diets were isoprotein, containing $10.7\pm0.4$% crude protein ($N \times 6.25$) and $3.2\pm0.7$% original crude fiber. They were prepared to contain 8% lipids, taking into account the original level from seeds and added soy oil (6), 4% salt mixture (7), and 2% vitamin diet fortification mixture (8). All diets were completed to 100% with a mixture of 1:3 sucrose and cornstarch and were isocaloric ($362.2\pm19.3$ kcal/100 g). A non-protein diet and a casein diet (control) were also utilized. In the former, the protein was replaced by the same sucrose and cornstarch mixture; in the later, casein (10.6% crude protein, $N \times 6.38$) was used as the only protein source for the nitrogen balance assay; both received 3% cellulose fiber (Microcel, Blanver, pharmaceutical grade) for comparative purposes.

*Biological assay.* Sixty weanling male Wistar rats weighing $88.30\pm6.09$ g when assigned to treatments were used. The rats with the same weight mean were randomly distributed in groups of twelve per treatment. The rats were maintained in individual metabolic cages, with temperature control ($22\pm2^\circ C$) and alternate cycles of light and dark (12h) and received the diets and water *ad libitum*. Prior to the 6-day feeding experimental period, the rats were fasted for 24 h to empty the stomach and reduce the content of the intestine, in order to avoid contamination with unlabeled feed residues; then, they were maintained for 2 days on the experimental diets in an adaptation period. From the third to the sixth day, feces were collected and analyzed for total nitrogen and nitrogen-15. The nitrogen of endogenous origin

was determined by the isotopic dilution method, taking into account the content of \(^{15}\)N in the feces, in the diets and natural \(^{15}\)N (0.375\(\pm\)0.001\%, also determined in this investigation), as described by Oliveira and Sgarbieri (5). The weight gain and the digestibilities of proteins—apparent, true (i.e. corrected by non-protein diet) and real, corrected by isotopic dilution of \(^{15}\)N (5)—were also determined.

**Analytical methods.** Total nitrogen was determined by the Kjeldahl (semi-micro) procedure according to Williams (9).

**Nitrogen-15:** Nitrogen-15 in the protein sources, diets and feces of rats was determined by the procedure recommended by Bremner (10), using a Varian Matt-230 mass spectrometer.

**Total lipids:** Determined in the protein sources by the method of Bligh and Dyer (11).

**Crude fiber:** Determined in the protein sources by “Weende” procedure according to Pearson (12).

**Statistical treatment.** The experimental design utilized was the “Completely Random Design” (13). Comparison between means was done by analysis of variance (F-test) and Duncan’s test (14) at 5\% level. Correlations among the experimental data were calculated by linear regression analysis and tested by the Pearson’s linear correlation method (15) and their strengths were classified according to Levin (16).

### RESULTS

Table 1 shows the intake, fecal excretion and absorption of total nitrogen, FEN excretion and apparent, true and real digestibilities obtained. The nitrogen intake by rats fed common pea diet did not differ from those fed casein diet. In the case of common bean and cowpea diets, the intakes were similar but lower than those of rats fed the casein diet \((p<0.05)\). The total fecal nitrogen excretion did not show statistical difference among leguminous diets in the 4-day balance, with a mean value of 202\(\pm\)22 mg, which was 3.7-fold higher than that of the casein-fed rats \((55\pm17\text{ mg})\). Likewise, there was no difference in FEN excretion among leguminous diets, with a mean value of 49\(\pm\)4 mg, corresponding to 2.2-fold of that excreted by rats fed non-protein diet, which was 22\(\pm\)7 mg.

For weight gain, the leguminous diets also resulted in similar values, with a mean of 8.4\(\pm\)2.8 g at the end of the experiment, whereas the non-protein group showed \(-7.2\pm1.0\text{ g}\).

All rats fed leguminous diets showed lower nitrogen absorption than those fed the casein diet. Among the legumes the highest absorption was shown by rats fed common pea diet, followed by those fed cowpea and common bean diets, respectively. The apparent, true and real digestibilities obtained from cowpea and common pea diets were higher than those obtained from common bean diet. The casein diet showed 15 to 25\% better digestibility values than the leguminous proteins under study. The horizontal comparison of data of Table 1 made possible the observation that true digestibility values did not differ significantly from apparent
Table 1. Nitrogen intake (NI), fecal nitrogen (FN), nitrogen absorption (NA), fecal endogenous nitrogen (FEN) and apparent, true, and real digestibilities obtained with Wistar rats fed common pea, common bean and cowpea labeled with $^{15}$N, in a 4-day balance period (mean value with standard deviation for groups of 12 rats).

<table>
<thead>
<tr>
<th>Protein source</th>
<th>NI</th>
<th>FN</th>
<th>NA</th>
<th>FEN</th>
<th>Digestibility %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(mg)</td>
<td></td>
<td></td>
<td></td>
<td>App. True Real</td>
</tr>
<tr>
<td>Common pea</td>
<td>1,009&lt;sup&gt;a&lt;/sup&gt;± 144</td>
<td>195&lt;sup&gt;a&lt;/sup&gt;± 52</td>
<td>814&lt;sup&gt;a&lt;/sup&gt;± 119</td>
<td>53&lt;sup&gt;a&lt;/sup&gt;± 10</td>
<td>80.7&lt;sup&gt;c&lt;/sup&gt;± 3.8</td>
</tr>
<tr>
<td>Common bean</td>
<td>777&lt;sup&gt;b&lt;/sup&gt;± 152</td>
<td>226&lt;sup&gt;a&lt;/sup&gt;± 57</td>
<td>551&lt;sup&gt;b&lt;/sup&gt;± 113</td>
<td>51&lt;sup&gt;a&lt;/sup&gt;± 10</td>
<td>70.9&lt;sup&gt;b&lt;/sup&gt;± 4.5</td>
</tr>
<tr>
<td>Cowpea</td>
<td>854&lt;sup&gt;b&lt;/sup&gt;± 135</td>
<td>184&lt;sup&gt;a&lt;/sup&gt;± 48</td>
<td>670&lt;sup&gt;c&lt;/sup&gt;± 97</td>
<td>44&lt;sup&gt;a&lt;/sup&gt;± 7</td>
<td>78.8&lt;sup&gt;c&lt;/sup&gt;± 3.7</td>
</tr>
<tr>
<td>Casein</td>
<td>1,074&lt;sup&gt;a&lt;/sup&gt;± 200</td>
<td>55&lt;sup&gt;b&lt;/sup&gt;± 17</td>
<td>1,019&lt;sup&gt;d&lt;/sup&gt;± 191</td>
<td>1,019&lt;sup&gt;d&lt;/sup&gt;± 7</td>
<td>94.9&lt;sup&gt;d&lt;/sup&gt;± 1.2</td>
</tr>
<tr>
<td>Non-protein</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>22&lt;sup&gt;b&lt;/sup&gt;± 7</td>
</tr>
</tbody>
</table>

<sup>a, b, c, d</sup> Values in the same vertical column not sharing a common superscript letter, or not in the same line underlined, differ significantly ($p<0.05$).

Fig. 1. Scatter diagram and linear regression showing the correlation between fecal endogenous nitrogen (FEN) and dry matter of feces excreted by rats fed common pea (△), common bean (▲), cowpea (○), and non-protein diet (●). $y = 12.3 + 8.7x$, $r = 0.89**$. 

ones. Nevertheless, the real digestibilities were significantly higher ($p < 0.05$) than the true digestibilities for all experimental diets.

Figure 1 shows the scatter diagram and the linear regression of the correlation between FEN and dry matter of feces, with a coefficient $r = 0.8930$ at 1% level. For the other variables studied, the correlation coefficients were: $r = 0.7094$ for dry matter eaten, $r = 0.8144$ for body weight at the beginning of the experiment and $r = 0.8384$, for weight gain.

**DISCUSSION**

In spite of differences between nitrogen absorption values, the rats fed leguminous diets showed similar excretion of FEN, with no statistical difference and the FEN was higher than those fed the non-protein diet. Thus, the determination of leguminous protein digestibility using the endogenous fecal nitrogen excreted by a group of animals fed non-protein diet, in order to correct the apparent values, underestimatated the results. These data are in accord with the reports of Oliveira and Sgarbieri (17) who worked with cooked bean diets and determined the endogenous excretion by rats labeled with intraperitoneal injection of $[^{14}\text{C}]$glycine. These authors found a radioactivity content of 2.0 to 2.5-fold higher in feces of rats fed bean diets than those fed casein diet. The influence of leguminous diet on the stimulation of endogenous excretion was also referred to by Bender and Mohammadiha (3). These authors suggested that low digestibility values normally assigned to these legumes are not due to limited absorption of their protein. This observation was also made by Fairweather-Tait and co-workers (18), who determined the influence of bean diets in the dry weight of feces, which showed values 2.5-fold higher than those obtained with a casein diet, and a increase of 2.9-fold in total fecal nitrogen. Although true digestibility values were higher than apparent ones, there was no significant difference among them. However, by correcting the digestibility with FEN determined by $^{15}\text{N}$ isotope technique, the obtained values were higher and statistically different from true protein digestibility of the legumes studied.

These facts confirm that endogenous excretion stimulated by cooked legume intake, results in false decreased digestibility values, when calculated by correction with fecal nitrogen excreted by rats fed non-protein diet.

In spite of the fact that the protein sources under study were from three different legumes, there are similarities between the FEN values and, for all three, the real digestibilities were higher than the true digestibilities, although different from each other. These facts show the adequacy of isotopic labeling technique with $^{15}\text{N}$ to evaluate protein digestibility.

Many investigators had already studied the variables involved in endogenous excretion in feces of animals and men. Blaxter and Wood (19) tried to correlate this excretion with total quantity of feces. Mitchell and Bert (20) studied the possible relation with dry matter eaten. Causeret and co-workers (21) found, in Wistar rats,
positive correlations between FEN and body weight, diet intake and dry matter of feces, the latter expressed by a higher coefficient than the former. However, in all these studies, the determination of nitrogen of endogenous origin was based on the use of rats fed non-protein diet. In the present investigation, by using protein and non-protein diets, it was verified that FEN, determined by isotopic dilution of $^{15}$N, showed correlations with the variables dry matter eaten, body weight, body weight gain and dry weight of feces. For all variables, it was possible to establish a positive correlation, with moderate coefficients and the highest value was for dry weight of feces. Therefore, the data reported by those authors op. cit. were confirmed since all variables had influenced the endogenous excretion of nitrogen by rats fed protein diets.

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