In vitro digestion studies using $^{14}$C-labelled polyethylene glycol (PEG) 4000: comparison of six tanniniferous shrub legumes and the grass *Panicum maximum*

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Abstract

The in vitro digestibility of dry matter (IVDMD) and of nitrogen (IVND) was measured on six leguminous tropical shrubs and the grass *Panicum maximum*, in the presence and absence of polyethylene glycol (PEG) 4000. The PEG was spiked with $^{14}$C-labelled PEG 4000 so that PEG remaining in the residues after digestion could be calculated. This was subtracted from the residues to enable a corrected IVDMD (CIVDMD) to be calculated. The plant samples were also mixed with labelled PEG in a buffer medium for 24 h, and the remaining PEG in the supernatant measured to estimate the PEG bound to the sample. PEG binding was then related to IVND and to the difference between the IVND of the samples measured with and without PEG. In the absence of PEG, the ranking of the species for IVDMD was: *Gliricidia sepium* $>$ *Panicum maximum* $>$ *Leucaena leucocephala* $>$ *L. trichandra* $>$ *L. pallida* $>$ *Calliandra calothyrsus* $>$ *Acacia boliviana*. PEG increased IVDMD for *A. boliviana*, decreased it for *L. pallida* and had no effect on the other species ($p>0.05$). The CIVDMD values for the legumes were all higher with + PEG by a mean of 8.6% units, though the species ranking remained the same. IVND varied from 82% for *G. sepium* to 49% for *C. calothyrsus* in the absence of PEG. The rankings were similar to those for IVDMD. PEG increased the IVND of all species except the grass and reduced the range from 86.6% with *G. sepium* to 75.5% with *P. maximum*. There are no condensed tannins in *P. maximum* and so PEG had no effect on IVDMD or IVND. PEG binding of the legume species in mg/g DM ($x$) was negatively related to IVND% ($y$) in the absence of PEG: $y=92.3-0.322x$; $r^2=0.941$, and to the IVND difference between the + and – PEG treatments ($y$): $y=-7.5+0.303x$; $r^2=0.923$.

The results show that the tannins in these shrub species can have a large effect on IVDMD and an even larger effect on IVND, consequently the potential value of *A. boliviana* and *C. calothyrsus* for...
Improving animal production should be questioned. The digestibility of the psyllid-tolerant *L. pallida* and *L. trichandra* was also shown to be lower than that of the proven high quality but psyllid susceptible *L. leucocephala*. © 2000 Published by Elsevier Science B.V.

**Keywords:** Tropical browse; In vitro digestibility; PEG binding; Condensed tannins; PEG 4000

1. **Introduction**

Polyethylene glycol (PEG) is known to preferentially bind with condensed tannins and thus prevent the formation of potentially indigestible tannin–protein complexes (Jones, 1965). When PEG was used in vitro, the expected improvement in in vitro nitrogen digestibility (IVND) of tanniniferous forages was obtained, however, there was little or no improvement in in vitro dry matter digestibility (IVDMD) (Jones et al., 2000b). This puzzling result could have been due to the presence of insoluble tannin–PEG complexes remaining in the residues even after digestion in acid pepsin. The availability of ¹⁴C-labelled PEG 4000 enables an assessment to be made of the magnitude of any PEG remaining in the residues following the Tilley and Terry (1963) in vitro digestion.

Previous in vitro studies (Tilley and Terry, 1963) with ¹⁴C-labelled PEG showed that differences in PEG binding of plant samples, and of the residue after Stage 1 digestion, varied with species and with the method of preparation of the samples (Palmer and Jones, 2000). In this study we measured the in vitro digestibility of a wider range of tanniniferous tropical browse legumes and the non-tanniniferous grass *Panicum maximum* in the presence and absence of polyethylene glycol (PEG) 4000 spiked with ¹⁴C-PEG 4000. The aim was to rank the species for digestibility; to measure differences in digestibility between the species in the presence and absence of PEG, and to relate these differences, by regression analysis, to measures of PEG binding of the plant samples and of the residues after Stage 1 digestion.

2. **Materials and methods**

The plant materials listed in Table 1 were obtained from actively growing, well established plants at the CSIRO Lansdown Pasture Research Station, 50 km south of Townsville in tropical north Queensland (19°40′S, 146°51′E). The shrub samples consisted of the first five fully expanded leaves on actively growing shoots (about 1 m long). The grass leaves were plucked from young, actively growing tillers. The samples chosen had a range of condensed tannin levels as measured previously by the butanol/HCl and vanillin/HCl assays (Jackson et al., 1996).

Harvested leaves were placed on dry ice, then freeze dried in the laboratory, ground to pass a 1 mm sieve and stored in sealed bottles.

The experiment compared the seven forages in the presence and absence of PEG with two replications in a randomised block design. Controls (no plant samples) with (+) or without (−) PEG were used in each replicate.
Samples of 0.4 g DW were placed in glass centrifuge tubes and 2 ml of PEG 4000 spiked with 14C-labelled PEG 4000 (Amersham, UK) were added to provide 64 mg PEG/tube (160 mg/g DM). Forty milliliters rumen fluid-buffer (1:4) was then added to duplicate samples of each species. Tubes were stoppered and incubated in an anaerobic chamber at 39°C for 72 h using a modified Tilley and Terry (1963) in vitro technique described in more detail by Jones et al. (2000a).

After incubation, tubes were centrifuged for 20 min at 2500 g, and two samples, each of 1 ml were withdrawn from the supernatant and added to 10 ml of scintillant (OptiPhase ‘HiSafe’ 3; Fisher Chemicals, England). Samples were subsequently counted in a β scintillation counter (Wallac 1410, Pharmacia, Finland). Residues were washed with water on a vortex mixer, then centrifuged for 20 min, the supernatant discarded and the process repeated before adding 40 ml of acid pepsin (2 g 1;10,000 pepsin in 1 l HCl (0.1N)). Each tube was then thoroughly mixed on the vortex mixer and incubated at 39°C for 24 h (Stage 2). Tubes were centrifuged, the supernatant discarded and the residues washed, dried at 80°C for 48 h and weighed.

Plant samples and residues were analysed for nitrogen to enable the calculation of in vitro dry matter digestibility (IVDMD), corrected IVDMD (CIVDMD) and in vitro nitrogen digestibility (IVND) of seven forage species.

<table>
<thead>
<tr>
<th>Species</th>
<th>Cultivar or CPI</th>
<th>IVDMD (%)</th>
<th>CIVDMD (%)</th>
<th>IVND (%)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>L. leucocephala</td>
<td>Cunningham</td>
<td>70.2</td>
<td>71.3</td>
<td>70.2</td>
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<tr>
<td>L. pallida</td>
<td>84581</td>
<td>61.4</td>
<td>59.4</td>
<td>61.4</td>
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<tr>
<td>L. trichandra</td>
<td>46568</td>
<td>64.3</td>
<td>64.7</td>
<td>64.3</td>
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<tr>
<td>C. calothyrsus</td>
<td>115690</td>
<td>56.1</td>
<td>57.0</td>
<td>56.1</td>
</tr>
<tr>
<td>G. sepium</td>
<td>60796</td>
<td>78.3</td>
<td>77.4</td>
<td>78.3</td>
</tr>
<tr>
<td>A. boliviana</td>
<td>40175</td>
<td>42.8</td>
<td>56.2</td>
<td>42.8</td>
</tr>
<tr>
<td>P. maximum</td>
<td>Local</td>
<td>75.8</td>
<td>75.9</td>
<td>75.8</td>
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<tr>
<td>Mean</td>
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<td>64.1</td>
<td>66.0</td>
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Table 1
Effect of Polyethylene Glycol (PEG) 4000 on in vitro dry matter digestibility (IVDMD), corrected IVDMD (CIVDMD) and in vitro nitrogen digestibility (IVND) of seven forage species.

<table>
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<tr>
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<td></td>
<td>64.1</td>
<td>66.0</td>
<td>64.1</td>
</tr>
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*LSD for the Species × PEG interactions: IVDMD 5% 1.8, 1% 2.5; CIVDMD 5% 2.0, 1% 2.8; IVND 5% 3.4, 1% 4.8 (Error DF=13).*

*Commonwealth Plant Introduction Number (Australia).*
The statistical significance of treatment differences was measured by ANOVA. IVND was regressed on PEG binding of the samples, and the improvement in IVND due to PEG regressed on the PEG binding of the residue measured after Stage 1, and the PEG binding of the original plant samples using standard linear regression models.

3. Results

3.1. In vitro dry matter digestibility (IVDMD)

The effects of PEG, species and the PEG × species interaction were all highly significant \((p<0.001)\). There was no effect of PEG on *L. leucocephala, L. trichandra, C. calothyrsus, G. sepium* or *P. maximum* \((p>0.05)\) (Table 1). PEG increased IVDMD with *A. boliviana* \((p<0.01)\) and marginally decreased IVDMD with *L. pallida* \((p<0.05)\) (Table 1).

3.2. Corrected IVDMD (CIVDMD)

Treatment effects and their interaction were all highly significant. The PEG × species interaction was due to the absence of a response to PEG by the grass *P. maximum*. All other species responded positively to PEG, though to varying degrees; *G. sepium* gave the smallest response and *A. boliviana* the greatest (Table 1).

3.3. In vitro N digestibility (IVND)

Differences in IVND were similar to, but greater, than those measured for CIVDMD. Again there was no response to PEG by the grass, and varying responses by the legumes. *A. boliviana* and *C. calothyrsus* were the most responsive (>30% units) and *G. sepium* the least responsive (4% units) to PEG (Table 1).

3.4. PEG binding

PEG binding for the 1 mm ground samples was strongly related to the PEG binding of the Tema samples \((r^2=0.995)\). Tema samples had values at 6% higher (not significant \(p>0.05\)). The mean PEG binding values for the two sample treatments were used in the regressions involving PEG binding of plant samples discussed below.

Plant samples differed widely in PEG binding \((p<0.001)\); the grass had low or zero values and *A. boliviana* values were greater than 100 mg/g DM. PEG binding measured on the plant samples was higher than for the measurements on the residue after Stage 1, though strongly correlated: \(y=5.62+1.090x\) \((r^2=0.965)\).

In the absence of PEG, IVND was negatively related to the capacity of the plant samples to bind with PEG \((r^2=0.941)\) (Fig. 1). There was no significant \((p>0.05)\) relation between IVND in the presence of PEG and PEG binding of the samples since there was then little difference between treatments (Fig. 1).

IVND improvement was positively related to PEG binding \((r^2=0.923)\) (Fig. 2).
Fig. 1. The relation between in vitro nitrogen digestibility (IVND) and PEG binding for six tanniniferous shrub legumes in the presence (open circles) or absence of PEG 4000 (solid circles). In the absence of PEG the regression equation is: $$y = 92.3 - 0.322x \quad (r^2=0.941, \ p<0.01).$$ The data for *P. maximum* was not used in the regression.

Fig. 2. The relation between the improvement in in vitro nitrogen digestibility (IVND) due to PEG 4000 and PEG binding of six tanniniferous shrub legumes. The relationship is expressed by the equation: $$y = -7.52 + 0.303x \quad (r^2=0.923, \ p<0.01).$$ The data for *P. maximum* was not used in the regression.
4. Discussion

4.1. Species comparisons

The ranking of the species in the absence of PEG is similar to that obtained in other in vitro studies with these plant materials (Jones et al., 2000a,b), and with nylon bag studies with dried leaf (Bamualim et al., 1980; Balogun et al., 1998). On the basis of these results, G. sepium, and L. leucocephala cv. Cunningham would be regarded as having potentially high nutritive value and C. calothyrsus and A. boliviana low nutritive value as the sole feed. The other Leucaena species were intermediate. It is of interest that the psyllid-resistant L. pallida and L. trichandra had lower digestibility of DM and N than the higher quality, though psyllid susceptible, L. leucocephala cv. Cunningham. This needs to be considered in any proposal to use them as replacement species. Although animal production comparisons have not been made with all of these shrub legumes, what evidence is available suggests that the rankings for digestibility are in good agreement with animal studies, the results of which are discussed in an earlier paper (Jones et al., 2000a).

4.2. PEG effects

The reasons for the lower PEG binding values with the residue compared with those for the plant samples are not known. It may have been anticipated that the reverse would have been the case since the samples being digested were in the presence of PEG for 72 h at 39°C compared with 24 h at ≈25°C for the plant samples. However, the correlation between the two estimates was very high.

The improvement in CIVDMD and IVND with PEG for all the legume species, but not the grass, emphasises the negative effect of tannins on digestibility. Without the use of labelled PEG, the adverse effect of tannins on DMD would have been masked because the tannin/PEG complexes in the residue resulted in underestimation of the IVDMD. These effects were greater for those species with high tannin levels (C. calothyrsus and A. boliviana). The effects of PEG on IVND were greater than those for CIVDMD and, as discussed in earlier papers (Palmer and Jones, 2000; Jones et al., 2000a), are not influenced by the tannin/PEG complexes in the residue after digestion. Thus, there is no need to use labelled PEG to make any correction for IVND estimates in routine screening of plant samples for condensed tannin activity.

The absence of a response to PEG with the tannin-free grass strongly indicates that the PEG is binding only to the tannins in the shrub species, and that there is no adverse effect of using PEG in these in vitro studies.

The good relations between PEG binding of the samples and both IVND and the improvement in IVND due to PEG, confirm the usefulness of the PEG binding technique as a tannin assay (Silanikove et al., 1996). In this respect it is far better than either the butanol/HCl or the vanillin/HCl methods when assessing a range of different genera and species. These two methods gave far poorer correlations with the IVND improvement due to PEG of $r^2=0.0007$ and 0.578, respectively, with the same plant samples (Jones et al., 2000a) compared with a value of 0.923 with PEG binding in this study. The very strong
relationship between PEG binding and the improvement in IVND due to PEG show that either method could be used as a screening procedure for tanniniferous forages. Where facilities are available for measuring radioactivity, the PEG binding method (Silanikove et al., 1996) is much faster. In the absence of such facilities, measuring IVND in the presence and absence of PEG to assess the improvement due to PEG may be more appropriate.

The low IVND, even after Stage 2 digestion in acid pepsin, shows that the protein in some of these shrub legumes is over-protected by the tannins and so may not be readily available in the small intestine. Treatment with PEG greatly improves the IVND, though the practical and economic use of such a strategy to improve nutritive value of these shrubs in the tropics is yet to be proven.

Acknowledgements

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References