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Formation of complexes between polyvinyl pyrrolidones or polyethylene glycols and tannins, and their implication in gas production and true digestibility in *in vitro* techniques

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Various tannin-complexing agents have been used to study the potential adverse effects of tannins on rumen metabolism. Using a method based on turbidity formation, the binding of various tannin-complexing agents (polyvinyl polypyrrolidone (PVPP), polyethylene glycol (PEG) of molecular weights 2000 to 35000, and polyvinyl pyrrolidone (PVP) of molecular weight 10000, 40000 and 360000) to tannins (tannic acid, purified tannins from quebracho (*Aspidosperma quebracho*) and leaves of trees and shrubs (*Acioa barteri*, *Dichostachys cinerea*, *Guiera senegalensis*, *Piliostigma reticulatum*)) was investigated at different pH values. The binding of all the tannins with PVPP was highest at pH 3–4 and lowest at pH 7. For all the pH range (3–7) studied, the binding of PEG was higher than that of PVP. For all the tannins except tannic acid, the binding to PVP was the same from pH 4·7 to 7. Similar results were observed for the PEG of molecular weight 6000 or higher for all the tannins except quebracho tannins for which the binding increased as the pH increased from 3 to 7. The binding with PEG 2000 decreased to a greater extent as the pH reached near neutral and for PEG 4000 this decrease was slightly lower. Addition of these tannin-complexing agents to the *in vitro* gas system resulted in higher gas production from tannin-rich feeds (increase varied from 0 to 135%). The PEG were the most effective followed by PVP and PVPP. The PEG 35000 was least effective. The efficiency of other PEG was similar. The PEG 6000 was preferred to PEG 2000 or 4000 as its binding to tannins was higher at near neutral pH values. The gas production increased with an increase in the amount of PEG 6000 up to 0·6 g/40 ml rumen-fluid-containing medium containing 0·5 g tannin-rich feed, beyond which no increase was observed. The percentage increase in gas value at 24 h fermentation correlated significantly with tannin values, the highest correlation (*r* 0·95) being with protein precipitation capacity of tannins. The increase in gas production was associated with higher production of short-chain fatty acids with little change in their molar proportions, suggesting an increase in organic matter digestibility by inclusion of the PEG in tannin-rich feeds. However, apparent and true digestibilities were lower on addition of the PEG, due to the presence of PEG–tannin complexes in the residues. The use of this bioassay (percentage increase in gas production in the presence of PEG 6000) along with other tannin assays would provide a better insight into the nutritional significance of tannins.

Polyethylene glycol: Polyvinyl pyrrolidone: Tannins: Gas production: Digestibility

Tannins are widely distributed in higher plants and occur at high levels in various feeds and foods. The presence of tannins has been associated with lower biological availability of various nutrients. At high levels of intake these cause toxicity. On the other hand, tannins control bloat, and improve protein utilization in ruminants (Makkar *et al.* 1987).

Tannins bind to polyvinyl polypyrrolidone (insoluble polyvinyl pyrrolidone, PVPP) and polyethylene glycol (PEG), and this property has been exploited for various purposes such as quantification of tannins (Barry & Manley, 1986; Makkar *et al.* 1993), extraction of

enzymes (Badran & Jones, 1965), use as an HPLC stationary phase (Glenn *et al.* 1972; Percival, 1986) and in TLC plates for separation of a variety of compounds (Wrolstad, 1968), and in alleviating the adverse effects of foods and feeds rich in tannins (Barry & Duncan, 1984; Horigome *et al.* 1984; Laurena *et al.* 1984; Barroga *et al.* 1985; Foley & Hume, 1987; Garrido *et al.* 1991; Pritchard *et al.* 1992). The use of PVPP in studying the plant–microbe association has also been suggested (Doner *et al.* 1993). Recently, Khazaal & Ørskov (1993) investigated the use of the *in vitro* gas technique using PVPP for assessing antinutritional factors in some browse. The present study describes (1) formation of complexes of polyvinyl pyrrolidone (PVP) and PEG of different molecular weights and PVPP with purified tannins under different conditions, (2) effects of this complexation on gas production using the *in vitro* gas method (Menke *et al.* 1979), (3) solubility of the complexes in detergent solutions used for fibre analysis, and (4) their significance in the determination of true digestibility as determined by two-stage *in vitro* rumen fermentation in which the second stage is the use of neutral-detergent solution (not acid–pepsin) (Marten & Barnes, 1980).

MATERIALS AND METHODS

Materials

The leaves used were air dried and ground to pass a 1 mm sieve. The PVP, PVPP and polyethylene-glycol compound (PEG-compound; consists of 2 mol PEG of molecular weight 6000–7000 joined by an epoxide; cat no. P2263) were from Sigma Chemical Co. (Deisenhofen, Germany), and the other PEG used were from Merck Chemical Co. (Darmstadt, Germany). Tannic acid was obtained from Merck and spray-dried quebracho (*Aspidosperma quebracho*) extract was a kind gift from Trask Chem Corporation, 3200 West Somerset Court, Marietta, Georgia 30067, USA.

Binding of tannins and tannin-complexing agents at different pH values

Citrate–phosphate buffers (0·05 M) of pH values 3, 4, 4·7, 5·5, 6, 6·6 and 7 were used in the present study. The tannins from quebracho and leaves of *Acioa barteri*, *Dichostachys cinerea*, *Piliostigma reticulatum* and *Guiera senegalensis* were purified as described by Makkar & Becker (1994). The tannins were dissolved in distilled water. The tannins of *D. cinerea*, *P. reticulatum* and *G. senegalensis* did not dissolve easily in water. A suspension of these tannins was prepared using a sonicator.

Binding with PVPP

The binding of tannins with PVPP was studied essentially as described by Doner *et al.* (1993). In brief, a portion (0·24 ml) of tannic acid or the purified tannins (1 mg/ml in distilled water) was added to 12 ml buffer. This tannic acid solution was pipetted (3 ml) in triplicate and 0·1 ml of the PVPP suspension (25 mg/ml in distilled water) was added to each. The PVPP suspension was withdrawn while the solution was being stirred. The contents were stirred for 30 min at 4° and centrifuged to collect the supernatant fraction. The absorbance of the supernatant fraction was recorded at 280 nm and compared with that of the untreated tannin solution.

Binding with PEG and PVP

A method based on formation of turbidity was used for studying the binding of these tannin-complexing agents. This method was essentially that of Asquith *et al.* (1987) who studied interaction of tannic acid and proteins by monitoring turbidity at 500 nm. The

assay was performed in disposable cuvettes. First, 0·1 ml tannin solution in distilled water (1 mg/ml for the tannic acid and 2 mg/ml for other tannins) was pipetted followed by 1·5 ml buffer and then a portion (0·05 ml) of the tannin-complexing agent (0·5 mg/ml in distilled water for the PEG and 2·5 mg/ml for the PVP). Absorbance was recorded at different time intervals at 500 nm. The absorbances presented here were recorded when the absorbance was at plateau. Under the conditions used here, the absorbance was at plateau at 30 min for tannic acid and quebracho tannins when complexed with PEG or PVP and at 5 min for the tannins of *A. barteri* and *D. cinerea*. Using PEG, the absorbance was at maximum at 5 and 10 min respectively for tannins of *P. reticulatum* and *G. senegalensis*, and on using PVP, the maximum absorbance was recorded at 10 min for both these tannins. An increase in the amount of tannin-complexing agent in the assay increased the turbidity suggesting that the tannins were not limiting in the assay.

In vitro *gas production*

The feed samples (500 mg) were incubated in 100 ml calibrated glass syringes essentially by the procedure of Menke *et al.* (1979). The tannin-complexing agents were weighed and transferred to syringes similar to those used for the feed samples. The feedstuffs with and without tannin-complexing agents were incubated in triplicate. The rumen fluid and particulate matter were collected before the morning feed from two cattle fed on a roughage diet, homogenized, strained and filtered through glass wool. The glassware used was kept at approximately 39° and flushed with CO_2 before use. As the amount of feed taken was 500 mg, composition of the medium was according to Tilley & Terry (1963). Menke *et al.* (1979) reduced the rumen buffer volume per syringe by half as they used 200 mg of the substrate because of the limited volume of the syringes and the inconvenience of emptying the syringes. In the present study, besides recording the gas volume, we were interested to use the fermented material for various analyses; therefore the amount of substrate was kept at 500 mg, as described by Tilley & Terry (1963). There is an inherent error associated with gravimetric determination which is large if 200 mg feed is taken in place of 500 mg. We generally prepared medium for sixty syringes (40 ml/syringe) plus 10% extra. The rumen fluid (660 ml) was added to warm (about 39°) and reduced medium consisting of 1095 ml distilled water, 730 ml rumen buffer solution (35·0 g $NaHCO_3$ and 4 g NH_4HCO_3 made up to 1 litre with distilled water), 365 ml macromineral solution (6·2 g KH_2PO_4, 5·7 g Na_2HPO_4, 2·22 g NaCl and 0·6 g $MgSO_4.7H_2O$ made up to 1 litre with distilled water), 0·23 ml micromineral solution (10·0 g $MnCl_2.4H_2O$, 13·2 g $CaCl_2.2H_2O$, 1 g $CoCl_2.6H_2O$, 8·0 g $FeCl_3.6H_2O$ and made up to 100 ml with distilled water), 1 ml resazurine (0·1 g made up to 100 ml with distilled water) and 60 ml freshly prepared reduction solution containing 580 mg $Na_2S.9H_2O$ and 3·7 ml 1 M-NaOH. The mixture was kept stirred under CO_2 at 39° using a magnetic stirrer fitted with a hot plate. A portion (40 ml) of the rumen-fluid medium was transferred into each syringe and incubated in a water bath at 39° as described by Blümmel & Ørskov (1993). Addition of tannin-complexing agents to blanks did not affect the gas production from blanks, suggesting that these agents are inert.

Bound proanthocyanidin analysis

These were determined in residues of neutral-detergent fibre (NDF) and acid-detergent fibre (ADF) as described in Makkar & Singh (1991) but with the addition of Fe as suggested by Porter *et al.* (1986). In brief, 10 mg NDF or ADF was weighed into a test tube, 3 ml butanol–HCl reagent (95:5, v/v) and 100 μl of the Fe reagent (20 g/l ammonium iron sulphate in 2 M-HCl) were added. The tubes were capped with marbles and heated at 97° for 1 h in a heating block. The tubes were cooled and the contents centrifuged to collect the supernatant fraction. The absorbance of the supernatant fraction was recorded at 550 nm.

The same procedure was followed for determination of proanthocyanidins bound to PVPP (see below).

Other analyses

The fibre fractions (NDF and ADF) were determined according to Goering & van Soest (1970), omitting decahydronaphthalene and sodium sulphite.

The PEG from the PEG–tannin complexes present in the NDF fractions was extracted by stirring 50–100 mg NDF in 4 ml 1 M-NaOH for 30 min. The contents were centrifuged to collect the supernatant fraction. TCA–$BaCl_2$ reagent (2 ml) (Hydén, 1955) was added to 2 ml of the supernatant fraction. The freshly prepared TCA–$BaCl_2$ reagent had turbidity which was removed by standing overnight followed by centrifugation. This clear reagent was used. High levels of NaOH interfered with the method (produced precipitate in the blank); however, 2 ml 1 M-NaOH in the assay did not interfere. Efforts were not made to recover the PEG quantitatively.

Total phenols (TP), tannins, soluble proanthocyanidins (PA) and protein precipitation capacity (PPC) were determined as described by Makkar & Becker (1993) and Makkar *et al.* (1993).

The short-chain fatty acids were determined in the supernatant fractions obtained on centrifuging (20000 ***g***) the syringe contents, using a Hewlett Packard gas–liquid chromatograph (Cafantaris, 1981).

The PVPP–tannin complexes were prepared by suspending the PVPP in aqueous solutions of purified tannins or the tannic acid and stirring the contents for 20 min in the cold. The PVPP–tannin complexes were recovered in a crucible and washed thoroughly with distilled water until absorbance of the filtrate at 280 nm reached zero.

Apparent digestibility was determined by centrifuging (20000 ***g***) the syringe contents at 24 h incubation. The pellet obtained was dried and weighed. The weight of apparently unfermented residue was the difference in pellet weight for the syringe contents where the feed was incubated, and the blank. True digestibility was determined at 24 h by treating the syringe contents with neutral-detergent solution to obtain NDF. Apparently-digested substrate was the weight of the substrate taken for incubation minus the weight of the apparently unfermented residue, and the truly digested substrate was the difference in weight between substrate and NDF following 24 h fermentation.

The model used for calculation of digestion variables using the gas values followed Ørskov & McDonald (1979).

The significance of differences between means was compared using Duncan's multiple range test after ANOVA for one-way classified data with the aid of the SAS/STAT program (SAS, 1988). For comparison of means of two groups, Student's t test was applied.

In order to have clarity in the plots, the error bars have not been presented. The individual values did not deviate from the mean by more than 5%.

RESULTS

Binding of tannin-complexing agents with tannins at different pH values

Understanding of the factors affecting binding of PVPP or PEG to tannins would help in exploiting the full potential of these tannin-binding agents in various biological applications.

Binding of PVPP. For all the tannins studied the binding was maximum at pH 3 and minimum at pH 7. For tannins of *D. cinerea* and *P. reticulatum* the binding was approximately similar from pH 4 to 6·6, for quebracho tannins the binding was similar

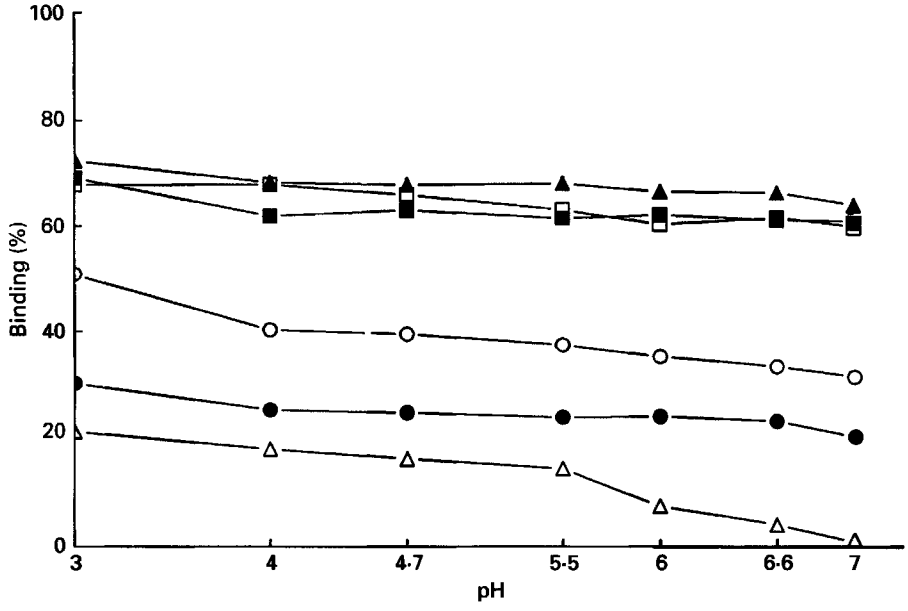

Fig. 1. Binding of polyvinyl polypyrrolidone to tannic acid and purified tannins from quebracho (*Aspidosperma quebracho*) and various leaves, at different pH values. (□), Tannic acid; (■), quebracho tannins; (△), *Acioa barteri*; (▲), *Dichostachys cinerea*; (○), *Guiera senegalensis*; (●), *Piliostigma reticulatum*.

from pH 4 to 7 and for other tannins it decreased gradually from pH 3 to 7. The binding affinity was of similar order for tannic acid, quebracho tannins and *D. cinerea* tannins. The binding affinities for these tannins were much higher than for other tannins. The binding was lowest for *A. barteri* tannins (Fig. 1).

Binding of PEG. The binding, as measured by turbidity development, was minimum for PEG 2000 compared with other PEG. For all the tannins except quebracho tannins the curves (Fig. 2(a), (b), (c)) became broader with increase in the molecular weight of PEG. With PEG 6000 the binding affinities were approximately similar from pH 4·7 to 7 for all the tannins except quebracho tannins, whereas with PEG 4000 the binding affinity was lower at pH 7 compared with pH 6 or 6·6 for tannins of *A. barteri* and *G. senegalensis*. For quebracho tannins the affinity increased from pH 3 to 7. This tannin also differed from others in another respect, in that its affinity was lowest at pH 3 whereas for others the affinity was maximum at this pH for all the PEG studied (Fig. 2(a), (b), (c)). For PEG 10000, PEG 20000, PEG 35000 and PEG-compound, the affinities were similar to those of PEG 6000 at all the pH values (the curves virtually overlap and the results are therefore not shown).

Binding of PVP. The turbidity observed for various tannins using PVP 10000 (PVP-10), PVP 40000 (PVP-40) and PVP-360000 (PVP-360) is presented in Fig. 3. Using the PVP-40 and PVP-360 the turbidity was approximately similar from pH 4·7 to 7 for all the tannins except tannic acid. For tannic acid the turbidity was lowest at pH 7 with all three PVP, but became higher and higher as the molecular weight of PVP increased. The turbidity values were much lower using PVP compared with PEG even though the concentration of the former was five-times higher in the assay (see pp. 898–899).

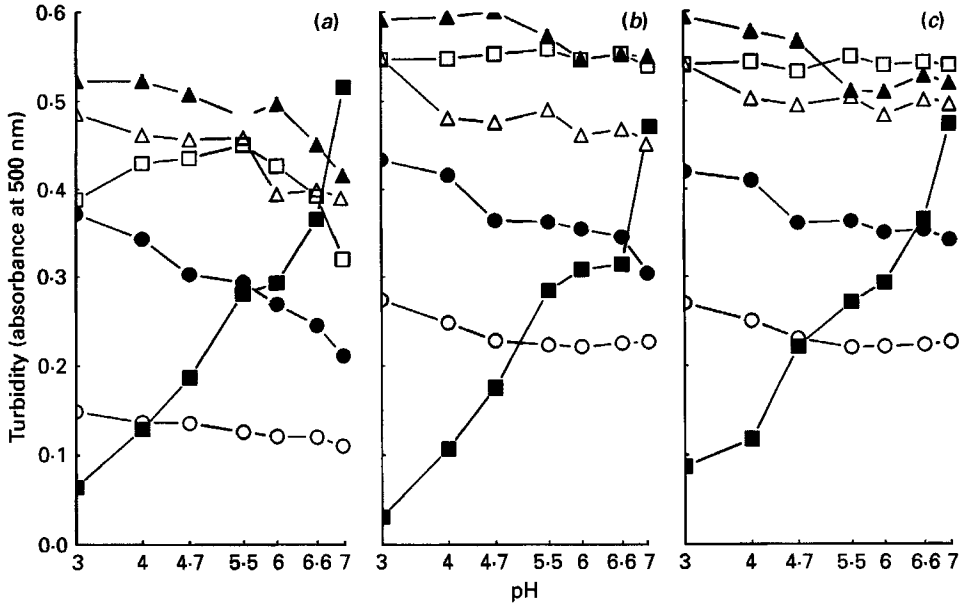

Fig. 2. Binding of polyethylene glycol (PEG) of different molecular weights to tannic acid and purified tannins from quebracho (*Aspidosperma quebracho*) and various leaves at different pH values. (a), PEG 2000; (b), PEG 4000; (c), PEG 6000; (□), tannic acid; (■), quebracho tannins; (△), *Acioa barteri*; (▲), *Dichostachys cinerea*; (○), *Piliostigma reticulatum*; (●), *Guiera senegalensis*.

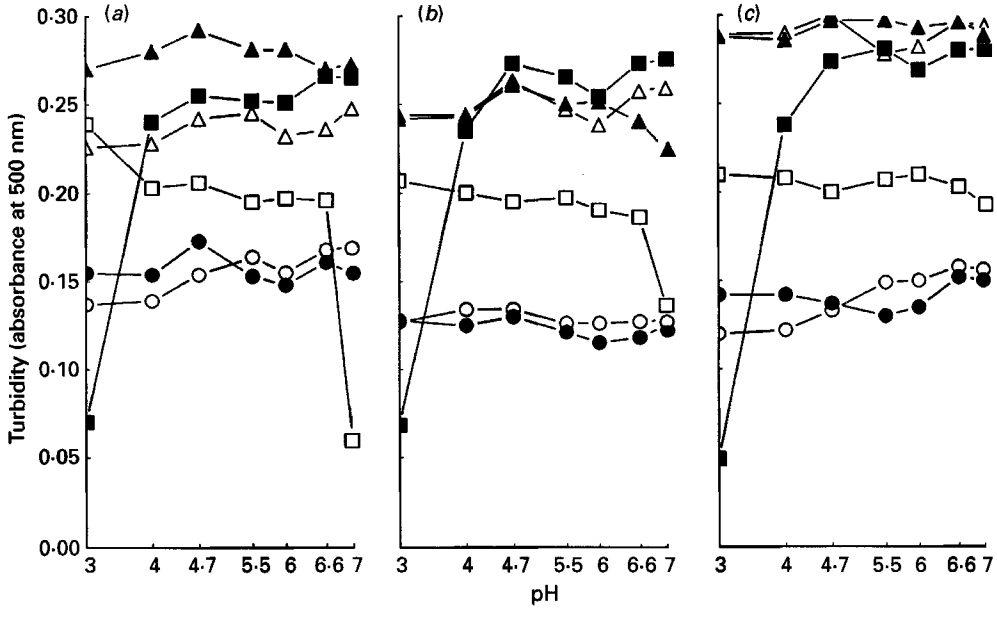

Fig. 3. Binding of polyvinyl pyrrolidone (PVP) of different molecular weights to tannic acid and purified tannins from quebracho (*Aspidosperma quebracho*) and various leaves, at different pH values. (a), PVP 10000; (b), PVP 40000; (c), PVP 360000; (□), tannic acid; (■), quebracho tannins; (△), *Acioa barteri*; (▲), *Dichostachys cinerea*; (○), *Piliostigma reticulatum*; (●), *Guiera senegalensis*.

Table 1. *Binding (measured as turbidity at 500 nm) of polyethylene glycols (PEG) or polyvinyl pyrrolidones (PVP) of different molecular weights with tannins at pH 6·6**

(Mean values with their standard errors for three observations)

Tannin...	TA		QT		Ab		Dc		Pr		Gs	
	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
PEG 2000	0·520	0·006	0·293	0·003	0·399	0·006	0·451	0·006	0·120	0·001	0·267	0·002
PEG 4000	0·610	0·005	0·314	0·005	0·496	0·004	0·552	0·005	0·225	0·005	0·345	0·001
PEG 6000	0·605	0·004	0·293	0·009	0·501	0·010	0·528	0·006	0·234	0·007	0·340	0·004
PEG 10000	0·592	0·002	0·290	0·004	0·525	0·005	0·560	0·005	0·240	0·005	0·328	0·008
PEG 20000	0·613	0·005	0·303	0·002	0·558	0·004	0·532	0·006	0·225	0·004	0·358	0·005
PEG 35000	0·600	0·005	0·287	0·010	0·542	0·010	0·501	0·005	0·242	0·002	0·339	0·010
PEG compound	0·632	0·004	0·310	0·005	0·513	0·005	0·578	0·006	0·258	0·002	0·352	0·006
PVP 10000	0·226	0·010	0·266	0·090	0·236	0·005	0·270	0·006	0·168	0·001	0·161	0·002
PVP 40000	0·202	0·002	0·280	0·010	0·257	0·006	0·240	0·010	0·127	0·008	0·118	0·006
PVP 360000	0·214	0·005	0·273	0·004	0·296	0·006	0·296	0·004	0·158	0·006	0·152	0·003

TA, tannic acid; QT, quebracho (*Aspidosperma quebracho*) tannins; Ab, *Acioa barteri*; Dc, *Dichostachys cinerea*; Pr, *Piliostigma reticulatum*; Gs, *Guiera senegalensis*.

* The assay used 100 μl tannin solution (2 mg/ml) and 50 μl PEG (0·5 mg/ml) or 50 μl PVP (2·5 mg/ml). For further details, see pp. 898–899.

Affinities of PEG and PVP with tannins at pH 6·6

Turbidity was compared at pH 6·6 (Table 1) as pH of the medium used in the gas method was from 6·8 to 7·0 at the start and 6·7–6·85 at 24 h fermentation. Among the PEG the affinity was minimum for PEG 2000 for all the tannins. For the rest of the PEG the affinity was approximately similar. For PVP the affinities were more or less similar but lower compared with those for PEG.

Effect of PEG and PVP on gas production

PEG and PVP bind tannins. An increase of gas production on inclusion of these tannin-binding agents into the gas system, represents the potential adverse effect of tannins present in the feed.

Table 2 shows the effect of incubation of 0·5 g PVP or PEG or the PVPP with 0·5 g feed sample in a syringe containing 40 ml medium. All the PEG studied significantly increased the gas produced in 24 h. Amongst these tannin-complexing agents the PEG 35000 and PEG-compound were least effective whereas the others were equally effective. The PVP were not as effective as the PEG. The effectiveness of these PVP decreased with increase in their molecular weight. The PVPP was also not very effective. At 24 h the percentage increase in the gas volume for *D. cinerea* was from 34 to 42 using the PEG and from 3 to 12 using the soluble PVP. These values for *A. barteri* and *Cassia sieberiana* were from 19 to 24% and 2 to 7%, and from 4 to 11% and 1 to 6% respectively. Using PVPP the increases in gas production were 1, 2 and 5% for *D. cinerea*, *A. barteri* and *C. sieberiana* respectively. We selected PEG 6000 for further studies. Some additional studies were also conducted using PVPP, which has already been used (Khazal & Ørskov, 1993), in order to have a comparison between these two tannin-complexing agents.

The addition of PEG 6000 (1 g) and PVPP (0·5 g) did not affect the gas production from wheat straw (0·5 g) and grass hay (0·5 g) samples which were free of tannins (results not shown).

Incubation of 0·5 g of the PVPP–tannin complexes (see p. 899) with wheat straw and

Table 2. *Effect of addition of polyethylene glycol (PEG) and polyvinyl pyrrolidone (PVP) of different molecular weights and polyvinyl polypyrrolidone (PVPP) on gas production at 24 h from some tannin-rich feeds**

(Mean values from three observations)

	Dichostachys cinerea		*Acioa barteri*		*Cassia sieberiana*	
Agents	Gas (ml)	Increase (%)	Gas (ml)	Increase (%)	Gas (ml)	Increase (%)
Control	25·33^{d}		20·50^{d}		43·33^{a}	
PEG 2000	35·33^{a}	39·5	25·00^{a,b}	22·0	48·17^{b}	11·2
PEG 4000	35·33^{a}	39·5	25·10^{a,b}	22·4	48·17^{b}	11·2
PEG 6000	36·00^{a}	42·1	25·43^{a}	24·1	48·00^{b}	10·8
PEG 10000	35·83^{a}	41·4	25·33^{a}	23·6	48·33^{b}	11·5
PEG 20000	35·23^{a}	39·1	24·63^{a,b}	20·2	46·93^{b,d}	8·3
PEG 35000	33·83^{b}	33·6	24·37^{b}	18·9	44·83^{a,e}	3·5
PEG compound	33·92^{b}	33·9	24·60^{a,b}	20·0	45·17^{c,e,f}	4·3
PVP 10000	28·42^{e}	12·2	21·83^{e}	6·5	45·83^{c,d,e,g}	5·8
PVP 40000	26·17^{d}	3·32	21·20^{e,d}	3·4	45·00^{a,f,g}	3·9
PVP 360000	26·00^{d}	2·7	20·83^{d}	1·6	43·67^{a,f}	0·8
PVPP	25·56^{d}	0·9	21·00^{e,d}	2·4	45·30^{a,f,g}	4·5
SEM	0·401		0·311		0·523	

^{a,b,c,d,e,f,g} Mean values within a column not sharing a common superscript letter were significantly different, $P < 0·05$.

* For details of procedures, see pp. 898–899.

grass hay samples did not have any influence on gas production, suggesting that tannins bound to PVPP are innocuous (results not shown).

Effect of different concentrations of PEG 6000 on gas production

Different quantities (0·2 g to 1 g) of PEG 6000 were used per syringe containing 0·5 g feed and 40 ml fluid at pH 6·85. Fig. 4 shows the effect on gas production up to 24 h. For *D. cinerea* and *A. barteri* the gas production increased up to 0·6 g and for *C. sieberiana* the increase was up to 0·4 g, whereafter no increase in gas production was observed. The curves for 0·8 or 1 g PEG 6000 virtually overlap those for 0·6 g PEG, and for this reason these curves are not shown in Fig. 4. For the digestion-variable studies, 1 g PEG was used in each syringe.

Effect of incubation of PEG 6000 with various leaves on digestion variables

Addition of the PEG to tannin-rich leaves increased gas production. Table 3 presents the increase in gas values at 24 h and the area under the curve up to 24 h fermentation together with contents of TP, tannins, PA and PPC. Percentage increase in gas values and area were quite similar, varying from 0 to 135% for different leaves (Table 3). For all the samples except the leaves of *A. barteri*, percentage increase in gas was almost constant from 12 to 60 h, a slight decrease being observed at 72 and 96 h fermentation. For *A. barteri* leaves the percentage increase in gas was almost the same (135%) at 12 and 24 h, thereafter a drastic decrease was observed (75, 55, 49, 47, 44 and 42% at 36, 48, 54, 60, 72 and 96 h respectively). The percentage increase in area for all the samples except *A. barteri* leaves was similar up to 24 and 36 h fermentation, with a slight decrease when area up to 96 h was taken (results not shown). The correlations between increase in these variables and TP, tannins, soluble PA and PPC are shown in Table 4. A significant positive correlation was

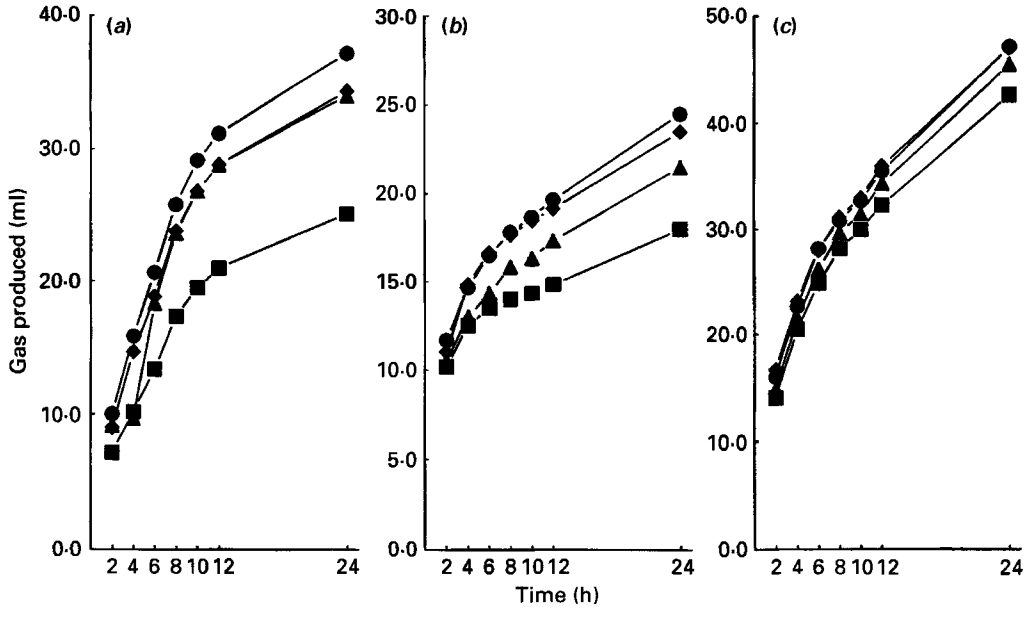

Fig. 4. Effect of different concentrations of polyethylene glycol 6000 on the production of gas from leaves of (a) *Dichostachys cinerea*, (b) *Acioa barteri* and (c) *Cassia sieberiana*, during fermentation with rumen fluid. (■), Control; (▲), 0·2 g; (◆), 0·4 g; (●), 0·6 g.

observed between percentage increase in gas at 24 h and the PPC, TP, tannins and soluble PA. Similar results were obtained for increase in rate and extent of digestion and area under the curves (Table 4).

Effect of PEG 6000 and PVPP on apparent and true digestibilities

The apparent digestibilities were lower using the PEG (Table 5) for tannin-rich feedstuffs. The true digestibilities were also lower using PVPP or the PEG. Addition of PVPP or the PEG (Table 5) had no effect on the true digestibilities of wheat straw and hay samples (Table 5). The decrease in the digestibilities observed could be due to the presence of PVPP–tannin or PEG–tannin complexes in the residues. The presence of these complexes was investigated (see below). Addition of the PEG increased the short-chain fatty acid (SCFA) production from the tannin-rich leaves by 44·6, 17·1, 28·2 and 10·4% respectively for *D. cinerea*, *C. sieberiana*, *A. barteri* and *Robinia pseudoacacia*. The PEG did not affect the molar proportions of SCFA production (Table 6).

Presence of PEG–tannin complexes in NDF and ADF

Fermented samples. The NDF obtained following 24 h fermentation in the gas method was studied for the presence of tannins and PEG. The PA present in the NDF obtained from the syringes using PEG were higher than those when the PEG was not used. Similarly, PEG was present in the NDF obtained after incubation of tannin-rich feeds with the PEG (Table 7), suggesting the presence of PEG–tannin complexes in the NDF. On subjecting the NDF to acid-detergent solution (ADS), the ADF for the PEG–tannin complex containing NDF was also higher (Table 7). Similar results were obtained when ADF was directly determined following fermentation; the values obtained were higher compared with the ADF values obtained sequentially. As for NDF, the PA content of ADF was higher for the PEG containing samples (Table 7).

Table 3. *Increase in gas production at 24 h and area under the curve up to 24 h fermentation on incubation with polyethylene glycol 6000, total phenols (TP), tannins, soluble proanthocyanidins (PA) and protein precipitation capacity (PPC) of some leaves**

(Mean values for three observations)

	% Increase					PPC (mg BSA precipitated/mg leaf)	
	Gas (ml)	Area	TP†	Tannins†	PA‡	Dye method§	BSA method‖
Piliostigma reticulatum	12·99	11·44	5·02	4·05	3·02	0·185	0·086
Guiera senegalensis	18·87	12·30	6·01	4·05	0·53	0·161	0·078
Panicum maximum	1·28	0·90	1·11	0·22	0·05	ND	ND
Dialium guineense	23·26	22·11	5·34	3·91	5·23	0·161	0·106
Quercus incana	39·23	28·73	10·82	9·94	2·65	0·507	0·279
Sesbania sesban (accession 10865)	1·35	1·60	2·56	1·38	0·28	0·0339	ND
Sesbania sesban (accession 15019)	0	0·60	4·01	2·28	2·07	0·0193	ND
Sesbania sesban (accession 15036)	4·22	5·00	5·11	2·86	4·44	0·0484	0·0357
Sesbania sesban (accession 15007)	2·14	2·24	6·51	3·30	8·39	0·0734	0·0520
Milletia thonningii	0	0·66	3·65	1·10	0·18	ND	ND
Acioa barteri (leaves)	135·40	105·30	10·67	6·80	14·64	0·604	0·634
Acioa barteri (leaves, twigs and stems)	20	11·31	3·55	2·54	3·54	0·258	0·154
Robinia pseudoacacia	9·58	11·79	3·48	2·05	6·01	0·0549	0·0264
Dichostachys cinerea	41·51	38·30	9·15	7·05	3·63	0·507	0·287
Cassia sieberiana	11·32	8·66	4·05	2·65	0·55	0·067	0·043

BSA, bovine serum albumin; ND, not determined.
* For details of procedures, see pp. 898–900.
† As tannic acid equivalent.
‡ As leucocyanidin equivalent.
§ Asquith & Butler (1985).
‖ Makkar *et al.* (1988).

Table 4. *Correlations between tannin levels and increase in the gas produced at 24 h, rate and potential extent of digestion and area under the gas production curve up to 24, 36 and 96 h for different feed plants*

(Correlation coefficients for fifteen observations)

	TP	Tannin	PA	PPC dye method†	PPC BSA method‡
% Increase in:					
Gas at 24 h	0·74**	0·64*	0·75**	0·84***	0·98***
Rate	0·64*	0·49	0·78**	0·69**	0·90***
Extent	0·67**	0·75**	0·32	0·85***	0·71**
Area up to 24 h	0·74**	0·64*	0·77**	0·84***	0·97***
Area up to 36 h	0·77***	0·69**	0·74**	0·87***	0·98***
Area up to 96 h	0·81***	0·78**	0·64*	0·94***	0·96***

TP, total phenols; PA, proanthocyanidins; PPC, protein precipitation capacity; BSA, bovine serum albumin.
* $P < 0·05$, ** $P < 0·01$, *** $P < 0·001$.
† Asquith & Butler (1985).
‡ Makkar *et al.* (1988).

Table 5. *Effect of polyethylene glycol 6000 (PEG) on apparent and true digestibilities and of polyvinyl polypyrrolidone (PVPP) on true digestibility† of various feedstuffs‡*

(Mean values with their standard errors for three observations)

	% Digestibility			
	Apparent		True	
	Mean	SE	Mean	SE
Dichostachys cinerea				
Control	8·9	0·58	43·8	1·51
With PEG	0·1***	0·03	35·5*	1·09
With PVPP	—	—	41·2	2·11
Cassia sieberiana				
Control	22·0	0·88	44·9	1·50
With PEG	17·6**	1·15	44·5	1·73
With PVPP	—	—	44·7	1·99
Acioa barteri				
Control	11·5	0·52	31·7	1·21
With PEG	2·7***	0·07	32·1	1·44
PVPP	—	—	31·9	0·99
Robinia pseudoacacia				
Control	29·1	1·0	67·3	1·67
With PEG	28·5	1·74	61·9**	2·31
Wheat straw				
Control	—	—	42·8	2·10
With PEG	—	—	42·2	1·46
Grass hay				
Control	—	—	78·3	2·89
With PEG	—	—	80·1	3·50

Mean values were significantly different from those for control: * $P < 0·05$; ** $P < 0·01$; *** $P < 0·001$.

† After taking into account the neutral-detergent fibre of PVPP, which was 990 g/kg dry matter.

‡ For details of procedures, see pp. 898–900.

Unfermented samples. The tannin-rich feed and the PEG were incubated overnight in 40 ml distilled water and the NDF was determined. In the second set these were not allowed to react (the feed and the PEG were weighed in a beaker and neutral-detergent solution (NDS) added) and NDF was determined. In the third set NDF was determined for the feed samples (Table 8). The NDF contents were higher when the PEG was present, and the NDF contents for *D. cinerea* and *C. sieberiana* did not differ significantly between the first and the second set. For *A. barteri* the NDF was highest when the tannins were allowed to react with the PEG. These observations suggested formation of PEG–tannin complexes even in the presence of NDS. This was substantiated by addition of lyophilized samples of purified tannins to tannin-free feed samples (wheat straw and grass hay) and the PEG. Higher NDF contents (Table 9) on addition of tannins further suggested the formation of PEG–tannin complexes in the presence of NDS. The highest increase in NDF was obtained when the PEG and purified tannins of *A. barteri* were present, followed by the PEG and tannic acid. No difference in NDF was observed for the PEG and the purified quebracho tannins which could be due to the compact and less accessible structures of quebracho tannins (Hagerman & Robbins, 1993). The PA were also present in the NDF obtained when the PEG and purified tannins of *A. barteri* were present.

Table 6. *Short-chain fatty acid (SCFA) production in 24 h, on incubation of various leaves with rumen fluid with and without polyethylene glycol 6000 (PEG)*†

(Mean values with their standard errors for three observations, with molar proportions given in parentheses)

	SCFA production (μmol/syringe)						
	Acetate		Propionate		Butyrate		
	Mean	SE	Mean	SE	Mean	SE	Total
Dichostachys cinerea							
Without PEG	375·8 (79·1)	10·97	99·1 (20·9)	2·60	0		474·9
With PEG	526·3*** (76·6)	4·91	135·8*** (19·8)	1·10	24·6 (3·6)	0·37	686·7
Cassia sieberiana							
Without PEG	595·9 (80·3)	12·99	135·9 (18·3)	3·92	10·5 (1·4)	2·08	742·3
With PEG	697·3** (80·2)	12·12	157·7* (18·1)	2·08	14·1 (1·6)	0·92	869·1
Acioa barteri							
Without PEG	240·3 (78·1)	4·85	67·5 (21·9)	3·0	0		307·8
With PEG	302·6* (78·0)	23·90	85·3* (22·0)	3·46	0		387·9
Robinia pseudoacacia							
Without PEG	1029·6 (77·9)	16·45	255·0 (19·3)	4·62	36·3 (2·7)	1·33	1320·9
With PEG	1114·2 (76·4)	48·09	277·3 (19·0)	9·99	66·1** (4·5)	2·14	1457·6

Mean values were significantly different from those for PEG-free sets: * $P < 0·05$; ** $P < 0·01$; *** $P < 0·001$.

† For details of procedures, see pp. 898–900.

Demonstration of PVPP–tannin complexes in NDF and ADF was handled in a different way. The PVPP–purified tannin complexes formed (see p. 900) were subjected to NDS and ADS. The residue obtained, and the PVPP–tannin complexes before subjecting to the NDS or ADS treatment, were analysed for PA. About 45–70% of the PA were present in the 'NDF' and 32–38% in the 'ADF', suggesting that tannins complexed with PVPP appear in the NDF or ADF creating difficulty in the determination of true digestibilities.

DISCUSSION

Tannin-complexing agents find applications in various biological processes. However, little is known about their interactions with tannins as a function of, for example, pH, temperature and ionic strength. The binding of PVPP, an insoluble polymer, to phenols (Andersen & Sowers, 1968) and flavonoids (Doner *et al.* 1995) at different pH values has been studied. This is the first report which deals with the binding of this insoluble matrix to polyphenols/tannins. Furthermore, lack of an assay system prevented workers from studying the interactions of soluble tannin-complexing agents with phenols or tannins. A procedure based on the formation of turbidity resulting from these agents and tannins was developed which enables studies to be made on their interactions as a function of various reaction conditions. We investigated the effect of pH using this procedure.

Table 7. *Contents of neutral-detergent fibre (NDF) and acid-detergent fibre (ADF) and their proanthocyanidins (PA) following fermentation of tannin-rich leaves for 24 h*†

(Mean values with their standard errors for three observations)

	NDF‡ (mg)		PA (A550 nm/ 10 mg NDF)		ADF (mg)		PA (A550 nm/ 10 mg ADF)		ADF§ (mg)		PA (A550 nm/ 10 mg ADF§)	
Leaves	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE	Mean	SE
Dc	264·1	7·51	0·323	0·03	283·4	2·71	1·54	0·05	223·4	4·62	0·21	0·006
Dc+PEG	303·2*	11·54	1·09***	0·05	323·9**	5·66	2·04**	0·12	235·5	5·25	0·50***	0·02
Cs	259·2	3·52	1·30	0·03	256·9	0·53	2·59	0·08	220·5	5·89	0·67	0·02
Cs+PEG	261·4	11·14	1·32	0·03	263·4*	1·96	2·63	0·06	227·6	2·94	0·71	0·03
Ab	319·5	17·38	0·61	0·05	309·6	4·05	2·59	0·18	270·2	7·51	0·21	0·02
Ab+PEG	317·0	4·68	0·90*	0·05	349·4*	9·41	3·68**	0·13	271·3	4·62	0·50***	0·04
Rp	152·0	2·37	0·72	0·02	115·4	1·15	1·82	0·13	—		—	
Rp+PEG	177·1**	5·89	1·35***	0·08	118·1	1·79	3·18	0·14**	—		—	

Dc, *Dichostachys cinerea*; Cs, *Cassia sieberiana*; Ab, *Acioa barteri*; Rp, *Robinia pseudoacacia*; PEG; polyethylene glycol 6000; A550, absorbance at 550 nm.

Mean values were significantly different from those for PEG-free set: * $P < 0{\cdot}05$, ** $P < 0{\cdot}01$, *** $P < 0{\cdot}001$.

† For details of procedures, see pp. 898–900.

‡ NDF obtained for samples incubated with the PEG in the gas method was positive for PEG.

§ Determined sequentially (from NDF).

Table 8. *Contents of neutral-detergent fibre (NDF) and its proanthocyanidins (PA) from tannin-rich feeds after overnight incubation with polyethylene glycol 6000 (PEG) in water*†

(Mean values and the standard error of the mean for three observations)

	NDF (mg)	PA (A550 nm/10 mg NDF)
Dichostachys cinerea		
(1) leaves+PEG+water	355·0[a]	2·16[a]
(2) leaves+PEG	359·8[a]	2·11[a]
(3) leaves	289·4[b]	1·05[b]
SEM	3·66	0·06
Cassia sieberiana		
(1) leaves+PEG+water	291·2[a]	2·95[a]
(2) leaves+PEG	286·4[a]	2·91[a]
(3) leaves	272·7[b]	2·25[b]
SEM	2·07	0·08
Acioa barteri		
(1) leaves+PEG+water	363·7[a]	3·62[a]
(2) leaves+PEG	349·1[b]	3·14[b]
(3) leaves	323·5[c]	1·93[c]
SEM	2·09	0·06

[a,b,c] For each leaf sample, values in a column with different superscripts were significantly different, $P < 0{\cdot}05$.

† For details of procedures, see pp. 898–900.

Table 9. *Neutral-detergent fibre (NDF) and its proanthocyanidin (PA) content on incubation of purified tannins with tannin-free feeds and polyethylene glycol 6000 (PEG)**

(Mean values with their standard errors for three observations)

	NDF (mg)		PA (A550 nm/10 mg NDF)	
	Mean	SE	Mean	SE
Grass hay	206·1[a]	1·44	0	—
Grass hay + PEG	207·2[a]	1·22	—	—
Grass hay + TA	208·9[a]	2·23	—	—
Grass hay + PEG + TA	211·5[a]	2·77	—	—
Grass hay + QT	210·8[a]	2·19	0·023	0·001
Grass hay + PEG + QT	210·7[a]	2·24	0·033	0·002
Grass hay + Dc	208·4[a]	1·41	0·013	0·001
Grass hay + PEG + Dc	224·1[b]	0·69	0·314	0·006
Wheat straw	364·1[a]	0·12	0	—
Wheat straw + PEG	366·0[a]	1·92	—	—
Wheat straw + TA	368·5[a]	1·59	—	—
Wheat straw + PEG + TA	376·1[b]	3·44	—	—
Wheat straw + QT	365·6[a]	1·62	0·012	0·0006
Wheat straw + PEG + QT	368·2[a]	0·68	0·009	0·0005
Wheat straw + Dc	374·3[b]	1·02	0·016	0·002
Wheat straw + PEG + Dc	388·7[c]	1·61	0·192	0·006

QT, purified tannins of quebracho (*Aspidosperma quebracho*); Dc, purified tannins from leaves of *Dichostachys cinerea*; TA, tannic acid; A550, absorbance at 550 nm.

[a,b,c] For each set, values in a column with different superscripts were significantly different, $P < 0·05$.

* The weights of tannin-free feeds, PEG and purified tannins were 0·47 g, 0·5 g and 50 mg respectively.

The binding of PVPP and phenols takes place via H bonding which increases with the number of phenolic hydroxyl groups and is dependent on their position (Andersen & Sowers, 1968; Dower *et al.* 1993). Different tannins bind to PVPP to different extents (Fig. 1). This could be due to the different number of phenolic hydroxyl groups, to their different positions on the nucleus or to different conformation of these tannins. Analogous to the binding of tannins with proteins (Hagerman & Butler, 1981), binding of tannins to PVPP could be higher for open and random-coiled tannins. Similar to the binding of PVPP to phenols, its binding to polyphenols is also highest at pH values of 3 to 4 and lowest at pH 7 (Fig. 1). The binding of soluble PVP to tannins at different pH values differed in some respects; the binding of all the tannins except tannic acid and quebracho tannins was almost the same from pH 3 to 7. For tannic acid the binding was lowest at pH 7 and for quebracho tannins it was lowest at pH 3 (Fig. 3). The difference in binding of PVPP to quebracho tannins could not be attributed to the cross-linking of soluble PVP, which is performed commercially to produce insoluble PVPP, as a similar pattern (increase in binding as pH increased from 3 to 7) in binding of this tannin to the soluble PEG was observed (Fig. 2). This difference could be due to the branched chain nature of quebracho tannins (Hemingway, 1989). Comparison of the binding data revealed: (1) for most of the tannins, increase of the molecular weight of the PEG from 2000 to 4000 increased the binding, thereafter not much change was observed, (2) increase of molecular weight of PEG up to 6000 and of PVP made the binding curves broader with higher binding at the extreme pH values studied, and (3) the binding efficiency of PEG was higher than that of PVP. These results suggest that PEG should be preferred to PVP for complexing tannins.

The comparative effectiveness of the addition of PEG and PVP on gas production was essentially the same as their order of binding to tannins. The PEG were more effective than PVP at the same concentration in the medium while the PVPP was least effective (Table 2). The comparative binding of this insoluble matrix with the soluble tannin-complexing agents cannot be compared because of the different nature of the two assays. Amongst different PEG, PEG 35000 and PEG-compound were comparatively less effective in increasing gas production, although the binding studies showed these to be as effective as PEG of other molecular weights except PEG 2000. There was no difference in the effectiveness of PEG 2000 and the PEG of molecular weights 4000, 6000, 10000 and 20000 on gas production. These differences in effectiveness of PEG in binding studies and the gas production may be due to the different conditions of the binding assay and the gas method (possibly different ionic strength and the temperature). These studies showed that any of the PEG of molecular weight 2000–20000 can be used in the *in vitro* gas method. We selected PEG 6000 because the curves were much broader compared with PEG 4000 in the binding studies (Fig. 2(a), (b), (c)). Although 0·6 g of this PEG was sufficient to produce the maximum gas from the system, we suggest the use of 1 g PEG as the tannin content of the samples could be higher than those used in the present study.

The increase in digestion variables (calculated from gas values) obtained on incubation with the PEG was significantly correlated with the tannin values (Table 3), suggesting that the increase in gas was due to the binding of this tannin-complexing agent with tannins, thereby inactivating them. The *in vivo* beneficial effects of incorporating PEG 4000 in tannin-rich feeds (Horigome *et al.* 1984; Foley & Hume, 1987; Pritchard *et al.* 1992) are mediated by these mechanisms. In addition, the increase in gas production shows that tannins inhibit enzymes and micro-organisms in the rumen. It is concluded from the higher correlations observed between increase in digestion variables and the functional tannin assays (protein precipitation assays, PPA) compared with the chemical assays that: (1) the PPA predict the biological value of the tannin-rich feedstuffs better, and (2) the gas method is a useful tool for studying the potentially adverse effects of tannins. The optimal conditions for this bioassay of tannins are: incubation of 0·5 g feed sample without and with PEG 6000 (1 g) in 40 ml rumen-fluid-containing medium at 39°. The percentage increase in gas value at 24 h fermentation as the easiest to record amongst all the digestion variables investigated represents the adverse effects of tannins; the higher the percentage increase in the gas production, the higher the adverse effects of tannins. This bioassay along with other tannin assays would provide a better understanding of the nutritional and physiological significance of tannins. The relationship between PPC (mg bovine serum albumin precipitated/mg leaf) determined by Makkar *et al.* (1988) and percentage increase in gas volume (% V) was: % V = 196·5 PPC − 1·92 (r 0·95), and when the PPC was determined by Asquith & Butler (1985) the relationship was: % V = 142·9 PPC − 4·12 (r 0·84). Another factor which mimics the effect of the PEG is the protein of the feed. Increase in gas production on addition of PEG from feeds containing drastically different protein contents is not expected to give a good correlation with PPC. In the present study the protein content of the samples (12–15% on a dry-matter basis) did not differ to a large extent. More studies are required to understand the full potential of this bioassay.

The percentage increase in gas observed on addition of PVPP for some browses (Khazal & Ørskov, 1993) was much lower compared with values observed in the present study. The present study clearly demonstrated the superiority of using PEG 4000 or 6000 over PVPP in the *in vitro* gas method. PEG 4000 or 6000 should also be preferred over PVPP for inactivating tannins in various biological applications due to their higher binding affinity over PVPP.

The increase in gas production on incubation with PEG could be due either to an

increase in SCFA production or to changes in the molar proportions of these acids or to both (Blümmel & Ørskov, 1993). In the present study there was a substantial increase in SCFA production without much change in their molar proportions (Table 6), suggesting that the removal of adverse effects of tannins by PEG increased the digestibility of tannin-rich feeds. However, both the apparent and true digestibilities were found to be lower on addition of PEG (Table 5). Higher PA in the NDF obtained in the presence of tannin or tannin-rich feed and PEG, and the presence of PEG in NDF (Tables 7, 8 and 9) confirmed that the PEG–tannin complexes come together with the NDF as 'artifact NDF', which leads to lower true digestibilities. These complexes were also present in ADF (Table 7). The determination of NDF by presoaking a tannin-rich sample in PEG as suggested by McArthur (1988) should not be adopted as this would overestimate NDF content (Table 8). The increase in true digestibility observed using HCl–pepsin (Garrido *et al.* 1991) suggests that the solubility of tannin–PEG complexes is higher in HCl–pepsin than in NDS or ADS. However, the digestibility values using HCl–pepsin could be lower because of the presence of some tannin–PEG complexes in the residue obtained following HCl–pepsin treatment. The presence of these complexes in ADF points towards this. Strong binding of PEG with tannins could also decrease the recovery of the PEG in faeces when PEG is incorporated into the tannin-rich feed to alleviate the adverse effects of tannins. Research is needed to investigate these aspects. The results of the present study highlight problems associated with NDF and ADF determination for tannin-rich feedstuffs which should be kept in mind while interpreting data from *in vitro* or *in vivo* studies where tannin-complexing agents are used.

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