Binding of Poly(ethylene glycol) to Samples of Forage Plants as an Assay of Tannins and Their Negative Effects on Ruminal Degradation†

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A procedure to measure poly(ethylene glycol) (PEG) binding to plant samples is described. The amount of PEG bound to a sample was found to be reproducible with a small coefficient of variation (0.5%). The PEG-binding capacity of plant samples was not affected by predrying of the samples at 90 °C, unlike the colorimetric methods for determination of tannins. Both the PEG-binding and the protein precipitation capacity of the plant samples were found to be useful in predicting the negative effects of tannins on ruminal degradation of the plant material. However, PEG binding to plant samples had an advantage over protein precipitation in samples in which formation of strong tannin−protein complexes results in low extractability of tannins. For this reason, the binding method was found to be preferable to the protein precipitation method in predicting the negative effect of tannins on ruminal degradation of forage plants.

Keywords: *Poly(ethylene glycol) (PEG); tannin; rumen; digestibility; forage plants; herbivore diet*

INTRODUCTION

Tannins are plant antinutritional factors which are widespread among dicotyledonous forbs, shrubs, and tree leaves and are thus ingested by many herbivorous mammals (Haslam, 1979). The quantification of tannins is important for predicting their negative effects on browsing animals (Hagerman and Butler, 1989). However, problems arise in the commonly used methods of colorimetric analysis, caused by the variable structures of tannin polymers and lack of satisfactory standards (Hagerman and Butler, 1989; Rickard, 1986). Consequently, variable specificity among the methods of analysis makes the nutritional and ecological interpretation of the tannin content difficult. The chemical interactions between tannins and proteins are generally similar for condensed and hydrolyzable tannins (Hagerman and Klucher, 1986). The physiological activities of tannins are attributed largely to their capacity to bind selectively to proteins, especially those that are large, conformationally open, and proline-rich (Hagerman *et al.*, 1992). Consequently, in some cases, protein precipitation assays have been found more useful for evaluating the antinutritional effects of tannins than their quantification by colorimetric methods (Martin and Martin, 1983; Rickard, 1986). Tannin extractability from plant tissues may be reduced by drying samples at temperatures above 50 °C and is dependent on many other factors, such as content and characteristic of plant proteins. Therefore, it is not feasible to recommend a single, optimal protocol for all plant samples (Hagerman, 1988). Tannin extractability may change dramatically within a single plant species according to seasonal changes in leaf morphology, moisture content, and chemical composition (Hagerman, 1988). Inextractable tannins may increase the amount of indigestible material in tannin-rich fodder (Rickard, 1986; Makkar *et al.*, 1995). Protein precipitation assays are, therefore, subject to limitations caused by the variable extractability of tannins from plant material.

Poly(ethylene glycol) (PEG), with a molecular weight of 4000, is a nonionic detergent which forms complexes with hydrolyzable and condensed tannins over a wide pH range (2−8.5) (Jones, 1965). Furthermore, protein may be released from the protein−tannin complex by exchange reaction with PEG (Jones and Mangan, 1977). The complex comprised of PEG and tannins is insoluble in boiling water, most organic solvents, and neutral and acidic detergent solutions. Such complexes do not respond to most colorimetric methods for determination of tannins (Jones, 1965; Jones and Mangan, 1977; Makkar *et al.*, 1995).

Hydrogen bonding between oxygen (ether) of the PEG chain and the phenolic hydroxyl group on the tannin moiety might explain the precipitation phenomena. It suggests that there is a considerable analogy between PEG−tannin and protein−tannin complexation (Jones, 1965). Thus, the information obtained from the amount of PEG binding (PEG-b) to a plant sample might be analogous to that obtained from protein precipitation capacity. Furthermore, because of the solubility of PEG in water and most organic solutions, PEG-b can be measured *in situ* without a need to pre-extract the tannins from the sample. PEG may react *in situ* with tannins that can not be extracted with conventional organic solvents because these tannins are bound to proteins and cell-wall components. Determination of tannins which are bound to cell components is possible in some cases (Terril *et al.*, 1992), but this method is too complicated for routine use or for screening large numbers of samples.

The purposes of the present experiment were (i) to determine whether the binding capacity of PEG to plant samples is related to the amount of tannins in the samples in a reproducible manner, (ii) to find the optimal conditions for measuring PEG-b to plant samples, (iii) to find how drying conditions, pH, and temperature during the reaction affected PEG-b, and (iv) to test the

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hypothesis that PEG-b provides useful information about the effects of tannins on the degradability of plants in the rumen environment. *In vitro* and *in situ* degradation of the plant samples provided reference values.

MATERIALS AND METHODS

Plants. The eight East Mediterranean browse plants used were as follows: *Calicotome vilosa* (pods), *Ceratonia siliqua* (leaves and pods), *Cistus* spp. (leaves), *Phillyrea media* (leaves), *Pistacia lentiscus* (leaves), *Pistacia palestina* (leaves), *Sarcopoterium spinosa* (regrowth), *Quercus calliprinos* (leaves), and *Rhmnus palestina* (leaves and fruits). Wheat straw was also used as a reference for tannin-free forage. A few hundred grams (fresh weight basis) of each of the above browse plants was gathered on the same day and from the same area (the western slopes of the Judeau Hills), dried at 40 °C (to minimize changes in tannin content and activity; Hagerman, 1988; Makkar and Singh, 1991) to constant weight, and then ground to pass through a 1-mm screen.

General Analytical Methods. Dry matter (DM), organic matter (OM) and nitrogen (Kjeldahl), and cell-wall fractions [NDF, ADF, and lignin (ADL)] were determined as described by Silanikove *et al.* (1994). Samples were extracted with 1% HCl in methanol (Hagerman, 1988). Total phenolic compounds were measured colorimetrically according to Swain and Hillis (1959), and the values are expressed as catechin equivalent. Condensed tannins in the extracts were measured by the butanol/HCl method (Porter *et al.*, 1986) using *Quebracho* (Trask Chemical Corp., GA)-condensed tannins as standard, after purification on a Sephadex LH-20 (Pharmacia) according to Asquith and Butler (1985). Protein precipitation capacity of nonextracted plant samples was determined according to Rickard (1986), except that protein was measured as nitrogen content (Kjeldahl × 6.25). The results are expressed as mg of protein precipitated by 1 g of sample. The *in vitro* digestibility of OM in each of the plant samples was determined according to Tilley and Terry (1963) using rumen fluid of a fistulated cow as an inoculum. The cow was fed a typical Israeli total mixed ration with 17% protein which was composed of 65% concentrates and 35% high-quality roughage. The *in situ* degradability was determined by weighing 4 g of DM of each of the plant samples into a dacron bag (external size, 14 × 16 cm; internal area, 144 cm^2; pore size, 40 μm), with or without 4 g of PEG. The samples were suspended separately in water for 24 h and then suspended in the rumen of a fistulated cow for 48 h. After incubation, the dacron bags were washed in a washing machine for 45−60 min without squeezing and with constant replacement of water, until the water became colorless. The samples were then air-dried at 105 °C and analyzed for OM and protein. The *in situ* degradation is the weight loss of OM after incubation in the rumen. In samples that were incubated with PEG, the amount of PEG that was bound to the sample was subtracted (as described below), under the assumption that the complex between PEG and tannins was indigestible (Makkar *et al.*, 1995).

PEG-b to Plant Samples. A stock solution contained 100 g/L PEG with molecular weight of 4000 (Sigma, Analytical Grade) in 0.05 M buffer Tris-BASE (Sigma), pH 7.1, spiked with 50 μCi of [^{14}C]PEG 4000 (Amersham, U.K.). A working solution was prepared by mixing 1 part of stock solution and 2 parts of distilled water. The ratio between plant sample weight to working solution was 1:15. Under the standard conditions the assays were run with plant samples of 1 g. The reaction was carried out in 50-mL centrifuge tubes. After the samples had been mixed with the working solution, the tubes were placed horizontal for equilibration for 24 h, with occasional mixing. The tubes were then centrifuged for 30 min at 2500*g*, 40 μL of the supernatant was decanted and added to 4 mL of liquid scintillator (Insta-Gel II, Packard), and radioactivity was counted in a β-scintillation counter for 10 min, resulting in total counts from 10 000 and above (*i.e.*, the precision was 1% or better). The radioactivity of 40 μL of the working solution in 4 mL of liquid scintillator served as a standard and that of 40 μL of buffer solution in 4 mL of liquid scintillatior served as a blank. The determination of the amount of PEG-b was based on the assumption that the radioactivity of labeled and non-^{14}C-labeled PEG exhibited the same binding capacity to the samples, according to

$$\text{PEG-b} = (C_{st} - C_{bl}) - (C_{sm} - C_{bl}) \times A_{PEG}/(C_{st} - C_{bl}) \times \text{Sw}$$

where C_{st}, C_{bl}, and C_{sm} are the ^{14}C counts of the standard, blank, and sample, receptively, A_{PEG} is the amount of PEG in the test tube, and Sw is the dry weight of the plant tissue. PEG-b was expressed as g/100 g of Sw.

The leaves of *Q. calliprinos* (oak) and *P. lentiscus* (pistacia) were used to study the optimal weight of plant sample, the effects of drying temperature of the samples, and the effects of pH and temperature during the binding reaction on the amount of PEG-b. The specific reaction conditions are indicated in the appropriate figure legends. PEG-b was also measured (in three replicates) as the difference between activity in the ^{14}C-labeled PEG introduced into the reaction

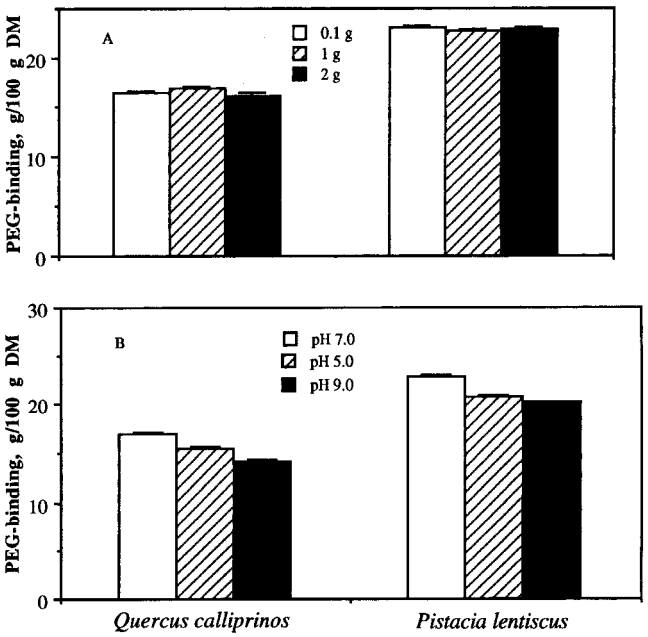

Figure 1. Effect of sample weight and pH on the binding of PEG (MW 4000) to *Q. caliprinos* and *P. lentiscus*. The samples were incubated for 24 h; the ratio of sample weight to solution was 1:15; the effect of pH was determined with samples weighing 1 g.

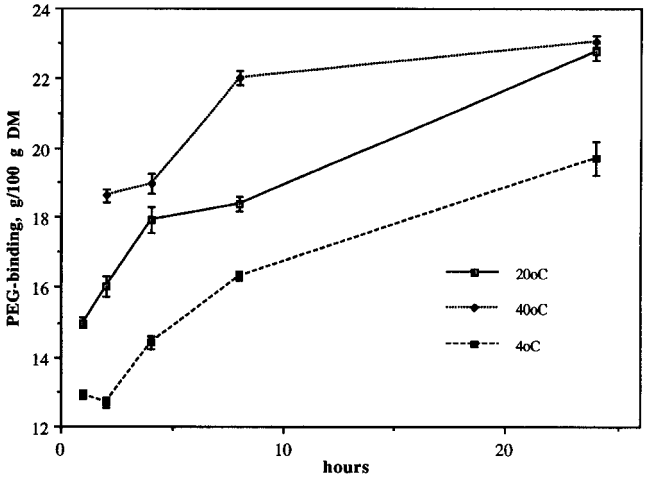

Figure 2. Effect of the temperature of incubation on the rate of binding of PEG (MW 4000) to *P. lentiscus*. Sample weight was 1 g.

Table 1. Chemical Composition of 11 Mediterranean Browse Plants and Wheat Straw (% of DM)[a]

species	type	OM	CP	NDF	ADF	ADL	TF	CT	PPC	PEG-b	D1	D2	D3	T-effect
wheat straw		90.1	4.8	79.9	56.9	21.1	0.3	0.1	0.0	0.0	39.9	40.7	40.9	0.2
Ph. media	pods	97.5	12.4	55.5	40.0	19.2	0.9	0.1	3.0	0.8	42.6	52.6	55.4	2.8
S. spinosum	regrowth	95.6	6.3	48.8	43.2	20.2	7.2	3.5	8.1	13.2	40.0	52.2	62.3	10.1
Ph. media	leaves	97.3	7.4	52.5	44.4	16.0	4.0	2.5	5.8	2.0	49.7	52.5	58.4	5.9
Q. caliprinos	leaves	95.5	7.3	50.9	48.7	21.4	4.1	9.9	25.2	8.5	31.9	35.0	54.2	19.2
Cistus spp.	leaves	95.1	7.5	44.7	29.9	22.9	10.2	13.6	51.5	17.9	59.7	71.5	91.0	19.5
	fruits	97.0	9.5	54.4	39.6	20.0	5.0	4.4	21.5	7.3	34.8	44.5	51.5	7.0
C. siliqua	leaves	97.1	7.9	57.5	54.8	25.1	1.8	0.6	3.0	11.1	46.3	50.2	64.5	14.3
	pods	97.2	9.5	48.7	44.1	20.3	1.5	0.9	3.1	8.7	59.4	64.2	78.3	14.1
R. palestina	leaves	95.5	9.1	43.9	34.8	16.3	5.0	25.4	22.1	7.5	51.7	64.6	72.4	7.8
P. lentiscus	leaves	96.9	5.2	63.4	52.5	33.4	8.5	23.5	65.1	22.1	25.2	42.0	70.2	28.2
	leaves	96.2	11.7	52.2	41.6	26.6	11.3	30.8	64.7	21.1	30.1	47.2	76.8	29.6

[a] OM, organic matter; CP, crude protein; NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin; TF, total phenolics; CT, condensed tannins; PPC, protein precipitation capacity; D1, *in vitro* degradability; D2, *in situ* organic matter degradation after 48 h of incubation in the rumen without PEG; D3, *in situ* organic matter degradation after 48 h of incubation in the rumen with PEG; T-effect, D3 minus D2.

Table 2. Linear Correlations (*r*) among Some of the Variables Listed in Table 1

	ADL	TF	CT	PPC	PEG-b	D1	D2	D3	T-effect
ADL			0.71***	0.49*	0.52*	−0.49*	−0.51*	0.61**	
TF			0.76***	0.78***	0.85***			0.54*	0.78***
CD				0.61**	0.69***			0.53*	0.72***
PPC								0.54*	0.79*** [b]
PEG-b						−0.50*	−0.52*	0.61**	0.94**** [c]
D1							0.96****		

[a] *$0.1 > P > 0.05$; **$0.05 > P > 0.01$; ***$P < 0.01$; ****$P < 0.001$. [b] T-effect = 5.7 + 0.33PPC. [c] T-effect = 1.6 + 1.16PEG-b. ADL, acid detergent lignin; TF, total phenolics; CT, condensed tannins; PPC, protein precipitation capacity; D1, *in vitro* degradability; D2, *in situ* organic matter degradation after 48 h of incubation in the rumen without PEG; D3, *in situ* organic matter degradation after 48 h of incubation in the rumen with PEG; T-effect, D3 minus D2.

tubes and that in the supernatant (nonbound PEG) following sequential washings. The washings were done as follows: The samples (1 g of DM) were mixed with 15 mL of water for 30 min; they were then centrifuged for 30 min at 2500*g*, and the activity of ^{14}C in the supernatant was determined as described above. This procedure was repeated five times, resulting in reduction of the ^{14}C activity in the supernatant to close to the blank value after the fourth washing. After the fifth washing, the samples were left horizontally for 24 h prior to measurement of ^{14}C activity in the supernatant from the final washing.

RESULTS AND DISCUSSION

Methodology. The PEG-b that was determined as the difference between the activity of the ^{14}C-labeled PEG introduced into the test tubes and that accumulated into supernatant by repeated washing was similar to that estimated from the equilibration procedure. This indicated that the complex formed between tannins and PEG was stable under assay conditions, which is consistent with the results of Jones (1965), Jones and Mangan (1977), and Makkar *et al.* (1995). Makkar *et al.* (1995) found that the binding between PEG of various molecular weight and tannins was more efficient than that between poly(vinylpyrrolidone)s and tannins and that the binding of tannins to PEG did not vary greatly with PEG of molecular weight range from 2000 to 35 000.

The repeatability of PEG-b on a particular plant sample was found to be very high (CV was 0.5% or lower). PEG-b was similar whether 0.1-, 1-, or 2-g leaf samples were used, and this was true of oak and pistacia (Figure 1, panel A). These results suggest methodologies that would allow flexibility in choice of sample size.

PEG-b was similar whether pistacia leaves were air-dried at 50 or 90 °C. With oak leaves, PEG-b following air-drying at 90 °C was slightly (1%) lower than at 50 °C ($P < 0.05$). However, the determined condensed tannin (CT) content was 50% lower for oak and 62% lower for pistacia when measured after drying at 90 °C than after drying at 50 °C (data not shown). The reduction in tannin extractability following air-drying at temperatures above 40−50 °C is a well-documented phenomenon (Hagerman, 1988; Makkar and Singh, 1991), although explanation is tenuous. Thus, the PEG-b technique has an advantage over methods that are based on pre-extraction because the results are less dependent on the way the samples are prepared, particularly with regard to the temperature during drying.

The rate of PEG-b was temperature-dependent: Maximal binding of tannins from pistacia and oak was obtained after 8 h of incubation at 40 °C, whereas approximately 24 h was needed when these samples were incubated at 20 °C. When the samples were incubated at 4 °C, PEG-b after 24 h of incubation was lower ($P < 0.01$) than with samples incubated at 20 °C (Figure 2). Because incubation at 40 °C slightly decreased ($P < 0.05$) maximal binding when oak samples were used, as compared with incubation at 20 °C, we chose incubation at 20 °C for 24 h as the preferred procedure.

PEG-b to tannins was maximal at neutral pH (Figure 1, panel B). With oak, PEG-b was 13.5% lower at pH 5 and 21.7% lower at pH 9 ($P < 0.01$). With pistacia, PEG-b was 8.9% lower at pH 5 and 11.5% lower at pH 9 ($P < 0.05$). Alkaline conditions inactivate tannins (Makkar and Singh, 1992); therefore, we chose to carry out the standard procedure at pH 7.1.

Usefulness. The chemical composition and the *in vitro* and *in situ* degradabilities of the browse sources that were investigated in the present study are summarized in Table 1. Total phenolics (TF), condensed tannins (CT), protein precipitation capacity (PPC), and PEG-b were determined for all the samples, except wheat straw. Similar variations in chemical composition and *in vitro* and *in situ* digestibilities have been found in studies of browse sources from Greece and the

Mediterranean part of France (Khazzal and Orskov, 1994; Khazzal *et al*., 1993).

There was a high correlation ($r = 0.96$; $P < 0.001$) between *in vitro* and *in situ* degradabilities (Table 2). The levels of lignin (ADL) and tannins (measured as PEG-b) negatively affected *in vitro* and *in situ* OM degradabilities (Table 2). However, the positive relation between the levels of ADL with the levels of CT, PPC, and PEG-b and the negative effect of tannin on rumen degradation suggest that a considerable part of the effect of lignin could be attributed to tannins and tannin−protein complexes that were determined as lignin (Silanikove *et al*., 1994; Makkar *et al*., 1995). The *in situ* degradability of NDF was negatively related to the NDF and ADF levels and positively related to protein levels. The *in situ* protein degradability was negatively related to the levels of PEG-b, NDF, and ADF and positively related to protein content (data not shown). Generally, the above relationships are consistent with previously reported biological effects of tannins, cell-wall components, and proteins (Van Soest, 1982) and confirm similar findings in previous studies (Reed, 1986; Rittner and Reed, 1992; Nsahlai *et al*., 1994).

One advantage of the PEG-b measurement over PPC in predicting the adverse effect of tannins can be related to the low levels of PPC obtained when tannins were strongly bound to proteins. In the present study, low levels of PPC were found for *C. siliqua* leaves and pods, despite the relatively large negative effect of tannin (Table 1). Prediction of tannin effects for *C. siliqua* leaves and pods based on eq 1 for PPC and eq 2 for PEG-b (Table 2) shows that PPC results underestimate the tannin effect, whereas PEG-b accurately predicts it. The low PPC of carob leaves and pods can be related to their resistance to extraction with conventional organic solutions, especially acidic methanol (Bravo *et al*., 1993; Silanikove *et al*., 1994). In agreement with the *in situ* data, the tannins in carob leaves induced significant negative effects on OM, protein, and NDF digestibilities *in vivo* (Silanikove *et al*., 1994, 1996). The high correlation between PEG-b and PPC (Table 2) sustains our suggestion that these two methods may provide equivalent results regarding the negative effects of tannins.

The increase in the *in situ* degradability of OM following incubation of the samples with PEG (T-effect) was significantly related, in order of increasing correlation coefficient to TF, CT, PPC, and PEG-b (Table 2). Similarly, an increase in gas evolution *in vitro*, or upon addition of PEG or poly(vinylpyrrolidone)s to the incubation medium was highly related with the tannin content and PPC (Makkar *et al*., 1995). Addition of PEG to tannin-free plants did not increase the *in vitro* gas production (Makkar *et al*., 1995), *in situ* degradability (present study), and *in vivo* digestibility (Silanikove *et al*., 1996). Thus, it may be concluded that the positive effect of PEG on organic matter degradation was related to neutralization of the adverse effects of tannins on ruminal degradation.

Conclusions. The effect of tannins on rumen degradation of plant tissue organic matter was found to be appreciable even in plants with relatively low tannin contents. PEG-b provides a tool to predict the adverse effects of tannins on rumen degradation. The information about plants obtained from PEG-b seems to be equivalent to that obtained from PPC. However, PEG-b offers advantages over PPC in cases where there is no alternative to air-drying for preservation of the samples and in cases where the tannin extractability is low.

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