Onderstepoort Journal of Veterinary Research, 61:351–359 (1994)

Photosensitivity in South Africa. VIII Ovine metabolism of *Tribulus terrestris* saponins during experimentally induced geeldikkop

C.O. MILES[1], A.L. WILKINS[2], G.L. ERASMUS[3] and T.S. KELLERMAN[3, 4]

ABSTRACT

MILES, C.O., WILKINS, A.L., ERASMUS, G.L. & KELLERMAN, T.S. 1994. Photosensitivity in South Africa. VIII. Ovine metabolism of *Tribulus terrestris* saponins during experimentally induced geeldikkop. *Onderstepoort Journal of Veterinary Research*, 61:351–359

Geeldikkop was induced in a sheep by dosing it orally with a crude extract of the steroidal saponins from *Tribulus terrestris*. GC-MS analysis of the sheep's ruminal contents, bile, faeces and urine for free and conjugated sapogenins, revealed the general features of the metabolic pathway by which diosgenin and yamogenin glycosides were converted into the glucuronides of epismilagenin and episarsasapogenin, the major constituents of the biliary crystals that usually form during geeldikkop. Other steroidal saponins in the *T. terrestris* extract, including those derived from tigogenin, neotigogenin, gitogenin and neogitogenin, appear to be non-lithogenic. The implications of these findings are discussed.

INTRODUCTION

Geeldikkop is one of a number of hepatogenous photosensitization diseases associated with the consumption of certain plants by ruminants, and characterized by the deposition in the biliary system of optically active, birefringent, crystalloid material (Miles, Wilkins, Erasmus, Kellerman & Coetzer 1994). Recently we demonstrated that geeldikkop can be induced in sheep by oral administration of a crude extract of the saponins from *T. terrestris* (Kellerman, Erasmus, Coetzer, Brown & Maartens 1991), and that the resulting biliary crystals are the calcium salts of the β-D-glucuronides of epismilagenin [1] and episarsasapogenin [2] (Fig. 1) (Miles *et al*. 1994).

Two similar ovine hepatogenous photosensitization diseases (caused by *Agave lecheguilla* and *Narthecium ossifragum*) have also been induced by dosing sheep with crude extracts of the saponins from the causative plants (Abdelkadar, Ceh, Dishington & Hauge 1984; Patamalai 1988). The crystalloid material present in the bile of sheep photosensitized while grazing *Panicum dichotomiflorum* (Holland, Miles, Mortimer, Wilkins, Hawkes & Smith 1991; Miles, Munday, Holland, Smith, Embling & Wilkins 1991; Miles, Wilkins, Munday, Holland, Smith, Lancaster & Embling 1992b), *Panicum schinzii* (Lancaster, Vit & Lyford 1991; Miles, Munday, Holland, Lancaster & Wilkins 1992a; Miles *et al*. 1992b), and *Narthecium*

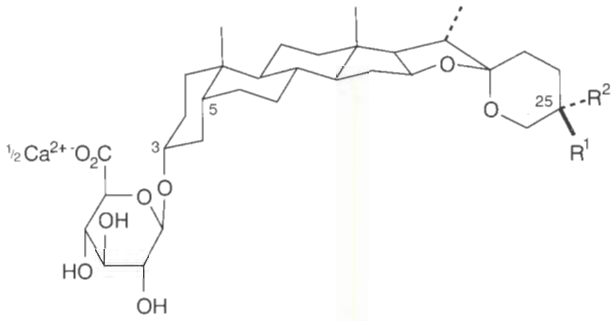
[1] Fungal and Plant Toxin Research Group, New Zealand Pastoral Agriculture Research Institute Ltd, Ruakura Agricultural Research Centre, Private Bag 3123, Hamilton, New Zealand

[2] Chemistry Department, The University of Waikato, Private Bag 3105, Hamilton, New Zealand

[3] Division of Toxicology, Onderstepoort Veterinary Institute, Private Bag X5, Onderstepoort, 0110 South Africa

[4] Honorary Professor, Department of Pharmacology and Toxicology, Faculty of Veterinary Science, University of Pretoria, Pretoria, 0001 South Africa

Accepted for publication 20 September 1994—Editor



1 R^1=CH_3, R^2=H Epismilagenin β-D-glucuronide calcium salt

2 R^1 = H, R^2 = CH_3 Episarsasapogenin β-D-glucuronide calcium salt

FIG. 1 Chemical structures of the components of the biliary sediment from a sheep with geeldikkop

ossifragum (Miles, Wilkins, Munday, Flåøyen, Holland & Smith 1993) was also found to be composed of insoluble salts of epismilagenin and episarsasapogenin β-D-glucuronides (Fig. 1). Epismilagenin [5] and episarsasapogenin [6] (Fig. 2) have also been identified in the ruminal contents of a sheep photosensitized while grazing *Brachiaria decumbens* (Abdullah, Lajis, Bremner, Davies, Mustapha & Rajion 1992; Lajis, Abdullah, Khan, Jalaludin, Salim & Brem ner 1993). However, for each of these diseases, including geeldikkop (Miles *et al.* 1994), saponins derived from epismilagenin and episarsasapogenin were not significant constituents of the causal plants.

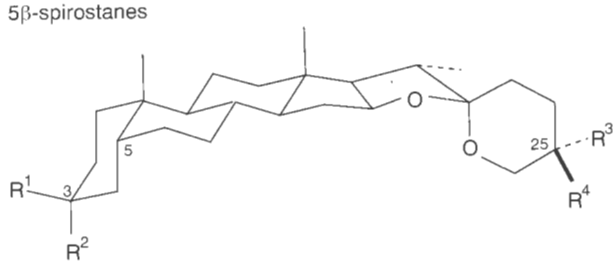
It has been proposed that the epismilagenin [5] and episarsasapogenin [6] in the biliary crystals that form during geeldikkop, result mainly from the metabolism of the diosgenin- [13] and yamogenin- [14] containing saponins present in *T. terrestris* (Miles *et al.* 1994). The aim of the present study was to elucidate the metabolism of *T. terrestris* saponins in sheep during geeldikkop, in order to provide information about the aetiology of the disease.

MATERIALS AND METHODS

General

Standards of smilagenin [3], sarsasapogenin [4] and tigogenin [7] were obtained from Upjohn Laboratories. Diosgenin [13] was obtained from Sigma Chemical Co., and epismilagenin [5] was obtained from Steraloids Inc. Episarsasapogenin [6] was prepared from sarsasapogenin (Miles *et al.* 1993), and epitigogenin [9] was prepared from tigogenin [7] by the method of Blunden, Jaffer, Jewers & Griffen (1979). Similarly, oxidation and then reduction of diosgenin, gave a mixture of diosgenin [13] and epidiosgenin [15]. The identity of each standard was confirmed by comparing its ^{13}C NMR spectrum with that reported in the literature (Agrawal, Jain, Gupta & Thakur 1985).

5β-spirostanes



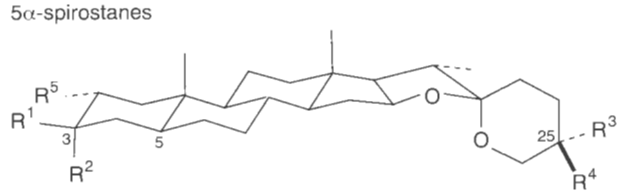
3 R^1 = OH, R^2 = R^3 = H, R^4 = CH_3 smilagenin

4 R^1 = OH, R^2 = R^4 = H, R^3 = CH_3 sarsasapogenin

5 R^1 = R^3 = H, R^2 = OH, R^4 = CH_3 epismilagenin

6 R^1 = R^4 = H, R^2 = OH, R^3 = CH_3 episarsasapogenin

5α-spirostanes



7 R^1 = OH, R^2 = R^3 = R^5 = H, R^4 = CH_3 tigogenin

8 R^1 = OH, R^2 = R^4 = R^5 = H, R^3 = CH_3 neotigogenin

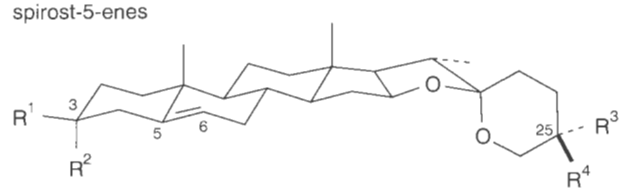
9 R^1 = R^3 = R^5 = H, R^2 = OH, R^4 = CH_3 epitigogenin

10 R^1 = R^4 = R^5 = H, R^2 = OH, R^3 = CH_3 epineotigogenin

11 R^1 = R^5 = OH, R^2 = R^3 = H, R^4 = CH_3 gitogenin

12 R^1 = R^5 = OH, R^2 = R^4 = H, R^3 = CH_3 neogitogenin

spirost-5-enes



13 R^1 = OH, R^2 = R^3 = H, R^4 = CH_3 diosgenin

14 R^1 = OH, R^2 = R^4 = H, R^3 = CH_3 yamogenin

15 R^1 = R^3 = H, R^2 = OH, R^4 = CH_3 epidiosgenin

16 R^1 = R^4 = H, R^2 = OH, R^3 = CH_3 epiyamogenin

FIG. 2 Chemical structures of steroidal sapogenins mentioned in the text

TABLE 1 Relative content[a] of free and conjugated sapogenins[b] in samples from a sheep with geeldikkop

Sapogenin	Conc[d]	25*R*-sapogenins						25*S*-sapogenins[c]					OH (%)
		Dio	Smil	Epi-smil	Tigo	Gito	25*R* (%)	Yam	Epi-sars	Neo-tigo	Neo-gito	25*S* (%)	
T. terrestris extract (conjugates)[e]	+++	10,1	ND	0,8	11,5	36,6	59,0	7,6	ND	7,0	26,4	41,0	37,0
Ruminal sapogenins	++	0,2	0,1	2,5	2,3	53,8	58,9	0,1	4,2	1,7	35,0	41,1	11,1
Ruminal conjugates	+++	2,4	0,1	0,2	18,3	34,3	55,3	1,8	ND	15,6	27,3	44,7	38,4
Biliary crystal conjugates[ef]	++	ND	ND	86,5	ND	ND	86,5	ND	13,5	ND	ND	13,5	100,0
Bile supernatant sapogenins	+	ND	6,0	12,9	26,3	21,7	66,9	ND	4,1	9,1	19,9	33,1	58,4
Bile supernatant conjugates	+++	0,7	0,1	26,8	1,6	32,7	61,9	0,2	13,3	0,6	24,0	38,1	43,3
Faecal sapogenins	+++	1,6	1,3	24,2	8,6	24,0	59,7	0,9	11,2	6,2	21,9	40,3	54,0
Faecal conjugates	+	2,2	0,4	13,6	17,3	25,5	59,0	1,2	4,6	11,3	23,8	41,0	50,6
Urinary conjugates[f]	+	ND	ND	25,5	ND	59,8	85,3	ND	5,9	ND	8,8	14,7	31,4

[a] Expressed as % of total mono- and dihydroxy-sapogenins identified in each extract

[b] Abbreviations
Dio = diosgenin [13]
Smil = smilagenin [3]
Epismil = epismilagenin [5]
Tigo = tigogenin [7]
Gito = gitogenin [11]
Yam = yamogenin [14]
Episars = episarsasapogenin [6]
Neotigo = neotigogenin [8]
Neogito = neogitogenin [12]
OH (%) = % monohydroxy sapogenin isomers
25*R* (%) = % 25*R*-sapogenins
25*S* (%) = % 25*S*-sapogenins
ND = not detected

[c] Sarsasapogenin [4] was not detectable in any samples (see Results)

[d] Relative concentration of sapogenins + = trace; ++ = moderate; +++ = high

[e] From Miles *et al.* (1994)

[f] No free sapogenins detected in the biliary crystals or urine

The calcium salt of epismilagenin β-D-glucuronide [1] and specimens of neotigogenin [8], yamogenin [14], gitogenin [11], and neogitogenin [12] were available from previous work in our laboratories (Miles *et al.* 1992b; 1993; 1994; Wilkins, Miles, Smith, Meagher & Ede 1994). Spirostanols were acetylated and analysed by GC-MS by use of standard methods (Miles *et al.* 1994; Wilkins *et al.* 1994).

Extraction of free and conjugated sapogenins from ovine samples

The lyophilized bile supernatant was that obtained from a sheep photosensitized with crude saponins from *T. terrestris* during the experiment described by Miles *et al.* (1994). Samples of ruminal contents, faeces, and urine were also obtained during the same experiment, and were immediately frozen and lyophilized. Subsamples of the lyophilized material were extracted with hexane over a period of 6 h by means of a Soxhlet apparatus. The resulting extract, which contained free (unconjugated) sapogenins, was worked up and acetylated as described previously for hydrolysed *T. terrestris* saponin extracts and biliary crystal hydrolysates (Miles *et al.* 1994). The extracts were then analysed by selected ion GC-MS. The residue which remained after the hexane extraction was air-dried, refluxed for 3 h with ethanol-water (85:15) containing 1 % acetic acid, and then filtered. The filtrate contained conjugated sapogenins, which were hydrolysed, extracted, washed, acetylated and then analysed by GC-MS, in the manner described by Miles *et al.* (1994) for plant saponins.

RESULTS

The identities of the acetylated sapogenins in the extracts were determined by comparing their GC-MS retention times and fragmentation patterns with those of authentic acetylated specimens of smilagenin, epismilagenin, sarsasapogenin, episarsasapogenin, tigogenin, epitigogenin, neotigogenin, diosgenin, epidiosgenin, yamogenin, gitogenin, and neogitogenin. The results of the GC-MS analysis for free and conjugated sapogenins in the ruminal content, bile supernatant, faeces and urine, are reported in Table 1, along with those previously determined by Miles *et al.* (1994) for the biliary crystalloid material and the administered *T. terrestris* extract. Results are given as a percentage of the total spirostanol content of each sample.

Upon GC-MS analysis, the acetates of sarsasapogenin [4] and epismilagenin [5] coeluted (Miles *et al.* 1994; Wilkins *et al.* 1994), so that the peak observed was due to the sum of the contributions of these two components. Analysis of the ratio of the intensities of the ions at *m/z* 284 and *m/z* 344 for this peak, permits quantitation of [4] in the presence of [5] (Wilkins *et al.* 1994), but only when [4] is present in a concentration greater than *c.* 10 % of that of [5]. The concentration of sarsasapogenin [4] did not exceed this threshold in any of the samples examined, though it may have been present at low levels.

DISCUSSION

The saponins present in *T. terrestris* have been implicated in the aetiology of geeldikkop (Kellerman *et*

al. 1991; Kellerman, Miles, Erasmus, Wilkins & Coetzer 1994; Miles *et al.* 1994). Geeldikkop is thought to be caused by occlusion of the bile ducts by biliary crystalloid deposits (Coetzer, Kellerman, Sadler & Bath 1983), so it is particularly notable that epismilagenin [5] and episarsarsapogenin [6]—the only genins present in the biliary crystalloid material deposited during geeldikkop—were present only at very low levels in the administered saponin extract (Table 1). This indicates that metabolism plays a crucial role in the conversion of the ingested *T. terrestris* saponins into the biliary crystals (Miles *et al.* 1994). Detailed examination of the data in Table 1 reveals much about ovine metabolism of steroidal saponins, and how it leads to the formation of the biliary crystals.

Ruminal metabolism

The concentration of free sapogenins in the rumen was approximately 20% that of conjugated saponins, indicating rapid hydrolysis of the ingested saponins. The GC-MS profile of the conjugated sapogenins (i.e. saponins) recovered from the rumen was generally similar to that of the sapogenins identified in the hydrolysed *T. terrestris* extract [the dominant saponins were identified as conjugates of tigogenin [7] (18,3%), neotigogenin [8] (15,6%), gitogenin [11] (34,3%) and neogitogenin [12] (27,3%)], suggesting that metabolic transformation of the steroidal nucleus occurs primarily after removal of the sugar moiety.

Epismilagenin [5] (2,5%) and episarsasapogenin [6] (4,2%) were found to be significant components of the free (non-conjugated) ruminal sapogenins, while less epismilagenin (0,2%) and no episarsasapogenins were detected in the conjugated ruminal material. Also of note is that only low levels of free diosgenin [13] (0,2%) and yamogenin [14] (0,1%) were present in the rumen, even though most of the diosgenin- and yamogenin-containing saponins originally present in the administered extract had been deconjugated. This suggests that the ruminal microflora rapidly converts most of the diosgenin and yamogenin liberated by hydrolysis to epismilagenin and episarsasapogenin.

We have already shown (Wilkins *et al.* 1994) that metabolism of orally administered diosgenin [13] in the sheep, generates epismilagenin [5], tigogenin [7], and smilagenin [3], along with traces of smilagenone. The present experiment indicates that these metabolites are generated by the action of the ruminal flora on diosgenin, and that diosgenin itself is rapidly liberated from diosgenin-containing saponins in the rumen. The conversion of diosgenin into epismilagenin requires reduction of the 5(6)-double bond, and epimerization of the hydroxyl at C-3 (conversion of 3β-OH to 3α-OH) (see Fig. 3). The same sequence of reactions would convert yamogenin [14] (the 25*S*-isomer of diosgenin) into episarsasapogenin [6] (the 25*S*-isomer of epismilagenin).

Ruminal reduction of the 5(6)-double bond of diosgenin [13], to give smilagenin [3] and tigogenin [7] derivatives, presumably occurs mainly after hydrolysis, because the diosgenin:smilagenin ratio is very much higher in the conjugated sapogenin fraction (23:1) than in the free sapogenin (2:1) extract from the rumen. It is possible that some reduction of the 5(6)-double bond of conjugated diosgenin also occurs, because smilagenin conjugates were present in the rumen, but were not detected in the administered saponin extract. The absence of detectable levels of epidiosgenin [15] from the rumen suggests that epimerization of the hydroxyl group (3β-OH to 3α-OH) takes place after the 5(6)-double bond has been reduced.

Interestingly, tigogenin [7] and neotigogenin [8] do not undergo epimerization in the rumen, as shown by the absence of detectable levels of epitigogenin [9] and epineotigogenin [10]. These observations are consistent with the view that ruminal metabolism of diosgenin yields smilagenin [3] [*via* β-face (upper face) hydrogenation of the 5(6)-double bond] and/or tigogenin [7] [*via* α-face (lower face) hydrogenation of the 5(6)-double bond] (Fig. 3). The C-3 hydroxyl group of smilagenin [3] is axially inclined with respect to ring A, whereas that of tigogenin [7] is equatorially inclined. Oxidation of the foregoing pair of spirostanols by ruminal oxido-reductases would afford the corresponding spirostanones (smilagenone and tigogenone, respectively). It is well known that axial hydroxyl groups are more sterically congested than equatorial hydroxyl groups, and that reduction of ketones generally proceeds preferentially to give the less sterically congested alcohols. Thus, ruminal reduction of the spirostanones would be expected to yield spirostanols in which the C-3 hydroxyl group is equatorially inclined with respect to ring A. It might therefore be anticipated that reduction of tigogenone would afford tigogenin [7], whereas reduction of smilagenone would afford epismilagenin [5] (rather than smilagenin). Similarly, ruminal metabolism of yamogenin [14] would afford episarsasapogenin [6] and neotigogenin [8].

Alternatively, the apparent non-epimerization of tigogenin [7] could be due to 5α-spirostan-3-ols being poor substrates for the oxido-reductases present in the rumen of the experimental animal. This view is consistent with the absence of detectable levels of epitigogenin [9] in the rumen; if non-epimerization of tigogenin were due solely to thermodynamic considerations, then small but significant amounts of epitigogenin should have been present.

Ruminal hydrolysis of tigogenin [7] and neotigogenin [8] saponins appears to be slower than hydrolysis of saponins derived from diosgenin [13] and yamogenin [14]. For example, the administered plant extract contained 10,0% diosgenin saponins, but of the saponins remaining in the rumen, only 2,3% were derived

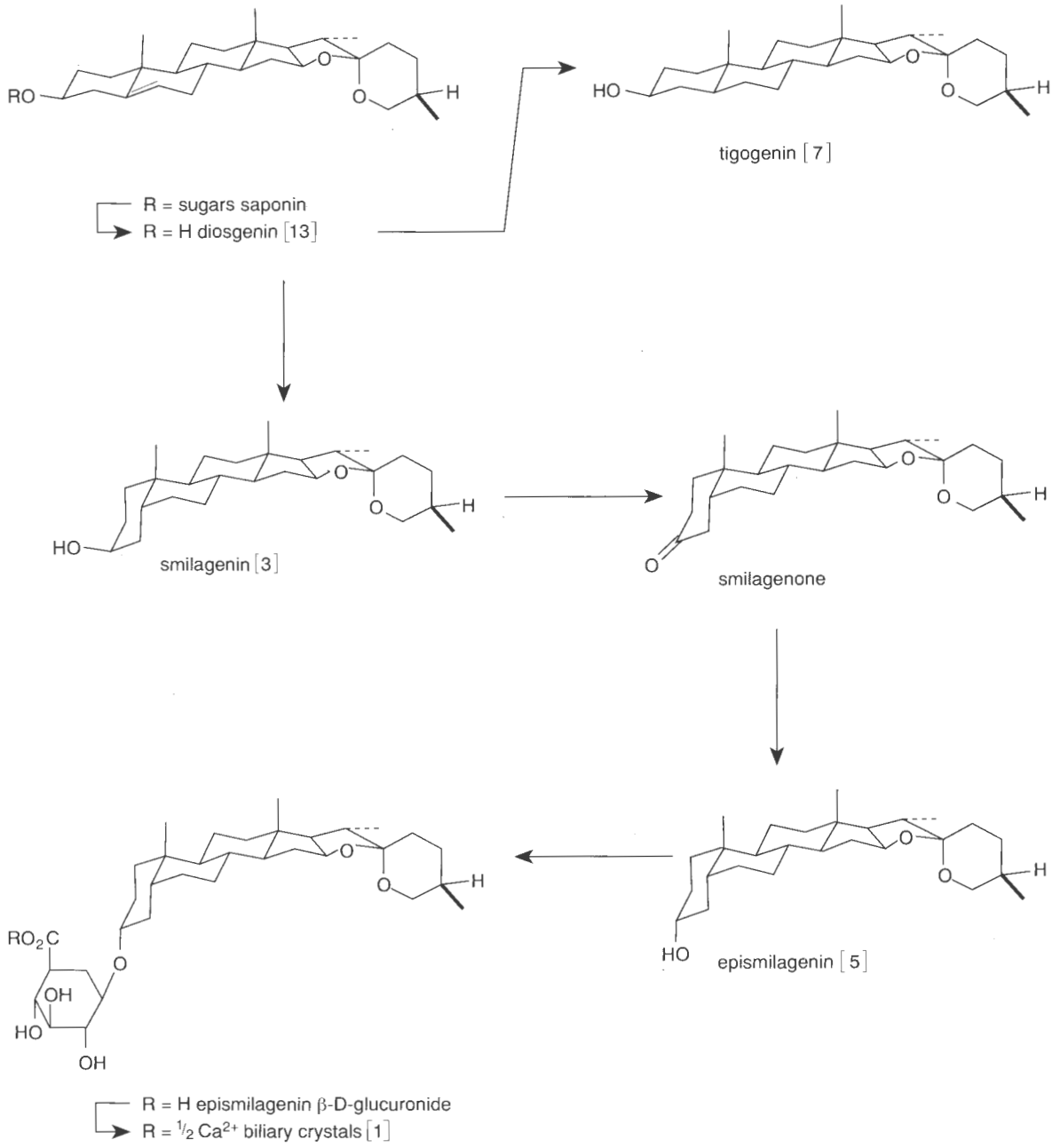


FIG. 3 Proposed pathway for metabolic conversion of diosgenin-containing saponins from *T. terrestris* into the major component [1] of the geeldikkop biliary crystals. An analogous series of transformations would convert the yamogenin-containing saponins of *T. terrestris* into the minor component [2] of the biliary crystals

* This figure is a corrected version of Fig. 2 in the previous article in this series (*Onderstepoort Journal of Veterinary Research*, 1994, 61:22) in which the arrow was inadvertently reversed

from diosgenin. In contrast, the plant extract contained 11,5% of tigogenin-derived saponins, and tigogenin saponins still constituted 18,0% of the saponins remaining in the ruminal extract approximately 6 h after dosing. Another intriguing aspect of ruminal metabolism is that monohydroxy spirostanes constitute about a third of the sapogenin conjugates (saponins) present in the crude *T. terrestris* extract (37,0%) and rumen (38,4%), whereas monohydroxy spirostanes constituted only 11,1% of the free sapogenins in the rumen. This suggests that free monohydroxy sapogenins are more rapidly removed from (or degraded in) the rumen than are dihydroxy sapogenins.

Amongst the monohydroxy sapogenins, epismilagenin [5] appears to be more readily removed from the

rumen than does episarsasapogenin [6], since it is only in the free ruminal sapogenin fraction that the level of episarsasapogenin (4,2 %) exceeds that of epismilagenin (2,5 %). In each of the other samples (bile, faeces, urine, etc.), the level of epismilagenin is typically two to six times greater than that of episarsasapogenin (Table 1).

Biliary metabolism

High concentrations of conjugated sapogenins were present in the bile of the sheep, but low levels of free sapogenins were also present. The major unconjugated sapogenins were epismilagenin [5] (12,9 %), tigogenin [7] (26,3 %), gitogenin [11] (21,7 %), neotigogenin [8] (9,1 %) and neogitogenin [12] (19,9 %). The proportions of tigogenin and neotigogenin in the free sapogenin fraction were much larger than in the conjugated fraction, where tigogenin (1,6 %) and neotigogenin (0,6 %) were only minor constituents. The reverse of this situation occured for epismilagenin [5] and episarsasapogenin [6], which constituted 26,8 and 13,3 %, respectively, of the conjugated biliary sapogenins, but only 12,9 and 4,1 %, respectively, of the free biliary sapogenins. Possibly this is because conjugation of 5α-spirostan-3β-ols (tigogenin and neotigogenin) is less efficient than that of 5β-spirostan-3α-ols (epismilagenin and episarsasapogenin), or because tigogenin and neotigogenin conjugates are more rapidly removed from the bile than are epismilagenin and episarsasapogenin conjugates.

Another notable feature of the bile is the relatively high level of free smilagenin [3] (6 %). This could possibly reflect comparatively inefficient conjugation of smilagenin, or formation of smilagenin by hepatic metabolism of epismilagenin [5]. The latter proposition is supported by our observation (data not presented) of low, but significant, levels of spirostan-3-ones (e.g. smilagenone, tigogenone) in the unconjugated sapogenin fraction from the bile supernatant.

In contrast to the bile supernatant, the biliary crystals contained only epismilagenin [5] (86,5 %) and episarsasapogenin [6] (13,5 %) glucuronides (i.e. [1] and [2], respectively); no derivatives of diosgenin [13], smilagenin [3], tigogenin [7], gitogenin [11], yamogenin [14], neotigogenin [8], or neogitogenin [12] could be detected (Miles *et al.* 1994), even though conjugates of these sapogenins were present in the bile from which the crystals had precipitated (Table 1). Another feature of the biliary crystals is that their 25*R*:25*S* ratio is almost 6:1, whereas the ratio for the administered plant extract was 6:4. Because the ratio of diosgenin:yamogenin (the precursors of epismilagenin and episarsasapogenin) in the plant extract was also almost 6:4, it appears that (25*R*)-spirost-5-en-3-ols (and hence also (25*R*)-5β-spirostan-3-ols) may be more lithogenic than their 25*S*-isomers. This is supported by the results of Miles *et al.* (1993), who found that lambs fed *N. ossifragum* containing smilagenin [3] and sarsasapogenin [4] (1:9), produced biliary crystals derived from epismilagenin [5] and episarsasapogenin [6] in a ratio of 1:4.

Excretion and metabolism of sapogenins in the faeces

Free sapogenins predominated in faeces recovered from the colon, although appreciable quantities of conjugated sapogenins were also present. As might be expected, the colonic microflora appear to be capable of deconjugating the conjugated sapogenins. Again, there was a 5β-spirostan-3α-ol/5α-spirostan-3β-ol dichotomy, with epismilagenin [5] (24,2 %) predominating over tigogenin [7] (8,6 %) in the free sapogenin fraction, while tigogenin (17,3 %) was more abundant than epismilagenin (13,6 %) in the conjugated fraction. The same dichotomy also applies to their respective 25*S*-isomers, episarsasapogenin [6] and neotigogenin [8].

Urinary excretion of sapogenin metabolites

In accord with the earlier work of Flåøyen, Smith & Miles (1993b), only very low levels of conjugated sapogenin derivatives, and no free sapogenins, were detected in the urine.

The urine contained conjugates of epismilagenin [5] (25,5 %), episarsasapogenin [6] (5,9 %), gitogenin [11] (59,8 %) and neogitogenin [12] (8,8 %). No conjugates of tigogenin [7], neotigogenin [8], diosgenin [13], or yamogenin [14] were detected. In this respect, the composition of the urine parallels that of the biliary crystals, except that the urine also contained conjugates of gitogenin and neogitogenin. This is not unexpected, because conjugates of dihydroxyspirostanes are likely to be more hydrophilic than their monohydroxy analogues (and hence more water-soluble) and so would be more easily excreted in the urine. The 25*R*/25*S* discrimination observed for the biliary crystals (86,5 % 25*R*) is also present in the urinary sapogenin conjugates (85,4 % 25*R*).

A remarkable feature of the urine extract is the absence of tigogenin [7] and neotigogenin [8] conjugates. Given their presence in both free and conjugated forms in both the bile and faeces, it is difficult to account for their absence from the urine.

Implications of the results

The present study shows that rapid hydrolysis of ingested saponins to form free sapogenins, occurs in the rumen. Subsequent reduction of the 5(6)-double bonds of diosgenin [13] and yamogenin [14] also takes place in the rumen. This reduction affords a mixture of both the 5α- and 5β-spirostanols; reduction of diosgenin, for example, gives both smilagenin

[13] and tigogenin [7]. Although the conversion of diosgenin to tigogenin cannot be detected in the present dosing experiment due to the presence of tigogenin in the *T. terrestris* extract, Wilkins *et al.* (1994) identified conjugates of both smilagenin and tigogenin in the bile of a lamb dosed with diosgenin (Flåøyen *et al.* 1993b). Analogous reactions occur for the 25*S*-isomer of diosgenin, yamogenin [14] glycosides being converted to sarsasapogenin [4] and neotigogenin [8].

Smilagenin [3] and sarsasapogenin [4] are then epimerized to form their thermodynamically more stable isomers, epismilagenin [5] and episarsasapogenin [6], respectively. Again, this process takes place in the rumen, and Wilkins *et al.* (1994) presented evidence that smilagenone is an intermediate in the epimerization of smilagenin during ovine metabolism of diosgenin.

The present study shows that all of the metabolic steps shown in Fig. 3, with the exception of glucuronide conjugation and calcium salt formation, occur in the rumen. The metabolic processes identified are consistent with the observations of Wilkins *et al.* (1994), in their study of the ovine metabolism of diosgenin (Flåøyen *et al.* 1993b). The ovine ruminal metabolism of saponins (see Fig. 3) in some respects parallels the metabolism of the steroidal glycoalkaloids α-solanine and α-chaconine by bovine ruminal fluid *in vitro* (King & McQueen 1981). These alkaloids were rapidly converted to their aglycone (solanidine), followed by reduction of the double bond at C-5 of the steroidal nucleus, to give 5β-solanidan-3β-ol. However, neither reduction to 5α-solanidan-3β-ol, nor epimerization at C-3 (to give solanidan-3α-ols)—which by anology with our results might have been expected—were observed by King & McQueen (1981).

Recently, other authors (Abdullah *et al.* 1992; Lajis *et al.* 1993) reported that epismilagenin [5] and episarsasapogenin [6] were present in the rumen of a sheep photosensitized while grazing *B. decumbens*, whereas this plant contained saponins derived from yamogenin [14] and diosgenin [13] (Smith & Miles 1993; Wilkins *et al.* 1994). As with geeldikkop, ruminal metabolism of diosgenin and yamogenin appears to play a crucial role in *B. decumbens* photosensitization. The ruminal metabolism of plant steroidal saponins therefore appears to be a general phenomenon that is important in the aetiology of crystal-associated photosensitization diseases.

Conjugation of the spirostanols presumably occurs in the liver, and the resulting 5β-spirostan-3α-ol glucuronides can then precipitate throughout the biliary system in the form of their insoluble calcium salts. Remarkably, the biliary crystals are composed only of the glucuronides of epismilagenin [5] and episarsasapogenin [6], even though conjugates of other sapogenins (e.g. tigogenin [7], neotigogenin [8], gitogenin [11], neogitogenin [12]) were the dominant saponin constituents of the dosed *T. terrestris* extract, and of the bile from which the crystals had precipitated. It appears, therefore, that 5α-spirostan-3-ols such as tigogenin and neotigogenin, and 5α-dihydroxyspirostanes such as gitogenin and neogitogenin, are not lithogenic. This is an important observation which may eventually provide some control options for alleviating the animal welfare and economic problems which crystal-associated hepatogenous photosensitizations such as geeldikkop and alveld pose to farmers throughout the world.

If biliary crystals are ultimately responsible for the retention of phylloerythrin, as has been suggested (Coetzer *et al.* 1983; Kellerman *et al.* 1994; Miles *et al.* 1994), then saponins derived from these non-lithogenic 5α-spirostanols and dihydroxyspirostanes would not cause photosensitization. Equally, it follows that plants containing saponins derived from diosgenin [13], epidiosgenin [15], yamogenin [14], epiyamogenin [16], smilagenin [3], epismilagenin [5], sarsasapogenin [4], or episarsasapogenin [6]—all of which can be metabolized by sheep to give to epismilagenin or episarsasapogenin β-D-glucuronides (i.e. [1] or [2])—would be capable of causing biliary crystal-associated hepatogenous photosensitization. In addition, plants containing 25*R*-spirostanols (e.g. smilagenin [3]) would be more toxic than those containing equivalent amounts of their 25*S*-isomers (e.g. sarsasapogenin [4]), because the former appear to be more lithogenic. Furthermore, 5β-spirostan-3-ol (e.g. smilagenin [3]) glycosides would be expected to be more toxic than their spirost-5-en-3-ol analogues (e.g. diosgenin [13]), because a proportion of the latter are converted into non-lithogenic 5α-spirostan-3-ols (e.g. tigogenin [7]) during passage through the rumen. It is therefore noteworthy that two populations of *T. terrestris* are believed to occur in Australia (Morrison & Scott 1993). That which occurs in southern Australia is believed to have originated from South Africa (Morrison & Scott 1993), and is considered to be responsible for periodic outbreaks of photosensitivity (Jacob & Peet 1987), whereas no photosensitivity problems appear to have been reported from the *T. terrestris* population in Queensland. Wilkins *et al.* (1994) reported that a specimen of *T. terrestris* from Queensland contained saponins derived from tigogenin [7] and neotigogenin [8], both of which are non-lithogenic. In contrast, saponins derived from diosgenin [13], yamogenin [14] (both of which are lithogenic), tigogenin [7] and neotigogenin [8] (both non-lithogenic) were present in a *T. terrestris* specimen from a site in southern Australia.

Although our experiments have established that steroidal saponins are involved in the aetiologies of geeldikkop and other crystal-associated photosensitizations, it has not yet been demonstrated that these

compounds are solely responsible for these diseases. The possible involvement of mycotoxins in the aetiologies of geeldikkop (Kellerman, Van der Westhuizen, Coetzer, Roux, Marasas, Minné, Bath & Basson 1980) and alveld (Aas & Ulvund 1989; Flåøyen, Di Menna, Collins & Smith 1993a) was proposed in order to account for the sporadic nature of these photosensitizations, and for the difficulties sometimes encountered (e.g. Flåøyen, Tønnesen, Grønstøl & Karlsen 1991) in reproducing these diseases by administration of saponins extracted from the causative plants. However, it is equally possible that variations in the quantitative and qualitative saponin content of the plants, possibly in combination with variations in the activity of the ruminal flora, may be responsible for these features of this group of diseases.

There is anecdotal evidence that young foliage from *T. terrestris* (Theiler 1918; Kellerman *et al.* 1980) and *B. decumbens* (Low, Bryden, Jephcott & Grant 1993) is more toxic than mature foliage. It is interesting, therefore, to note that the concentration of lithogenic saponins in young foliage from an Australian specimen of *B. decumbens* was almost six times that present in mature foliage from the same plant (Wilkins *et al.* 1994). Similarly, Patamalai (1988) reported that immature *P. coloratum* contained higher levels of sapogenins than did the mature plant; and Ender (1955, cited by Stabursvik 1959) found higher levels of saponins in the leaf tips and newer foliage of *N. ossifragum* than in the older leaves. If this difference in saponin content with age proves to be a general phenomenon, it would help not only to explain the apparently sporadic nature of these photosensitization diseases, but also to raise the possibility of using grazing management to assist in disease control.

The observation that many saponins are non-lithogenic, coupled with the large intraspecies variation in plant-saponin content that has been observed (Wilkins *et al.* 1994), suggests that it may be possible to breed plants that contain no, or only non-lithogenic, saponins. Such an approach would not be feasible for adventitious species such as *T. terrestris*, *N. ossifragum* or *P. dichotomiflorum,* but for species such as *B. decumbens*, *P. maximum*, *P. schinzii* or *P. coloratum*, which are cultivated for fodder, it might prove practical.

In summary, the general features of the metabolic processes by which ingested *T. terrestris* saponins are converted to biliary crystals in sheep with geeldikkop have been revealed. Many of the saponins in *T. terrestris* appear to be non-lithogenic, and a number of strategies have been proposed for reducing the intake of lithogenic saponins by stock grazing this and other plants that cause crystal-associated cholangiohepatopathy. However, it has not yet unequivocally been proved that the biliary crystals—and hence the lithogenic plant saponins—are solely responsible for causing geeldikkop.

ACKNOWLEDGEMENTS

We thank the Agricultural Research Council for Research Fellowships enabling C.O.M. and A.L.W. to visit Onderstepoort Veterinary Institute, the Foundation for Research and Development for assistance with travel expenses for A.L.W., and the University of Waikato for study leave for A.L.W.

REFERENCES

AAS, O. & ULVUND, M.J. 1989. Do microfungi help to induce the phototoxic disease alveld in Norway? *Veterinary Record*, 124: 563.

ABDELKADAR, S.V., CEH, L., DISHINGTON, I.W. & HAUGE, J.G. 1984. Alveld-producing saponins. II. Toxicological studies. *Acta Veterinaria Scandinavica*, 25:76–85.

ABDULLAH, A.S., LAJIS, N.H., BREMNER, J.B., DAVIES, N.W., MUSTAPHA, W. & RAJION, M.A. 1992. Hepatotoxic constituents in the rumen of *Brachiaria decumbens* intoxicated sheep. *Veterinary and Human Toxicology*, 34:154–155.

AGRAWAL, P.K., JAIN, D.C., GUPTA, R.K. & THAKUR, R.S. 1985. Carbon 13 NMR spectroscopy of steroidal sapogenins and steroidal saponins. *Phytochemistry*, 24:2479–2496.

BLUNDEN, G., JAFFER, J.A., JEWERS, K. & GRIFFIN, W.J. 1979. Epi-neotigogenin and epi-tigogenin, two new steroidal sapogenins from *Cordyline cannifolia* leaves. *Journal of Natural Products*, 42:478–482.

COETZER, J.A.W., KELLERMAN, T.S., SADLER, W. & BATH, G.F. 1983. Photosensitivity in South Africa. V. A comparative study of the pathology of the ovine hepatogenous photosensitivity diseases, facial eczema and geeldikkop (*Tribulosis ovis*), with special reference to their pathogenesis. *Onderstepoort Journal of Veterinary Research*, 50:59–71.

FLÅØYEN, A., TØNNESEN, H.H., GRØNSTØL, H. & KARLSEN, J. 1991. Failure to induce toxicity in lambs by administering saponins from *Narthecium ossifragum*. *Veterinary Research Communications*, 15:483–487.

FLÅØYEN, A., DI MENNA, M.E., COLLINS, R.G., & SMITH, B.L. 1993a. *Cladosporium magnusianum* (Jaap) M. B. Ellis is probably not involved in alveld. *Veterinary Research Communications*, 17:241–245.

FLÅØYEN, A., SMITH, B.L. & MILES, C.O. 1993b. An attempt to reproduce crystal-associated cholangitis in lambs by the experimental dosing of sarsasapogenin or diosgenin alone and in combination with sporidesmin. *New Zealand Veterinary Journal*, 41:171–174.

HOLLAND, P.T., MILES, C.O., MORTIMER, P.H., WILKINS, A.L., HAWKES, A.D. & SMITH, B.L. 1991. Isolation of the steroidal sapogenin epismilagenin from the bile of sheep affected by *Panicum dichotomiflorum* toxicosis. *Journal of Agricultural and Food Chemistry*, 39:1963–1965.

JACOB, R.H. & PEET, R.L. 1987. Poisoning of sheep and goats by *Tribulis terrestris* (caltrop). *Australian Veterinary Journal*, 64:288–289.

KELLERMAN, T.S., VAN DER WESTHUIZEN, G.C.A., COETZER, J.A.W., ROUX, C., MARASAS, W.F.O., MINNÉ, J.A., BATH, G.F. & BASSON, P.A. 1980. Photosensitivity in South Africa. II. The experimental production of the ovine hepatogenous photosensitivity disease geeldikkop (*Tribulosis ovis*) by the simultaneous ingestion of *Tribulus terrestris* plants and cultures of *Pithomyces chartarum* containing the mycotoxin sporidesmin. *Onderstepoort Journal of Veterinary Research*, 47:231–261.

KELLERMAN, T.S., ERASMUS, G.L., COETZER, J.A.W., BROWN, J.M.M. & MAARTENS, B.P. 1991. Photosensitivity in South Africa. VI. The experimental induction of geeldikkop in sheep with crude steroidal saponins from *Tribulus terrestris*. *Onderstepoort Journal of Veterinary Research*, 58:47–53.

KELLERMAN, T.S., MILES, C.O., ERASMUS, G.L., WILKINS, A.L. & COETZER, J.A.W. 1994. The possible role of steroidal saponins in the pathogenesis of geeldikkop, a major hepatogenous photosensitization of small stock in South Africa, in *Poisonous plants of the world: agricultural, phytochemical and ecological aspects*, edited by S.M. Colegate, & P.R. Dorling. *Proceedings of the 4th International Symposium on Poisonous Plants, Fremantle, Australia, 27th September–1st October, 1993*. CAB International. In press.

KING, R.R. & MCQUEEN, R.E. 1981. Transformation of potato glycoalkaloids by rumen microorganisms. *Journal of Agricultural and Food Chemistry*, 29:1101–1103.

LAJIS, N.H., ABDULLAH, A.S.H., KHAN, M.N., JALALUDIN, S., SALIM, S. & BREMNER, J.B. 1993. *Epi*-sarsasapogenin and *epi*-smilagenin: Two steroidal sapogenins isolated from the rumen contents of sheep intoxicated by *Brachiaria decumbens*. *Steroids*, 58:387–389.

LANCASTER, M.J., VIT, L. & LYFORD, R.L. 1991. Analysis of bile crystals from sheep grazing *Panicum schinzii* (sweet grass). *Australian Veterinary Journal*, 68:281.

LOW, S.G., BRYDEN, W.L., JEPHCOTT, S.B. & GRANT, I. McL. 1993. Photosensitization of cattle grazing Signal grass (*Brachiaria decumbens*) in Papua New Guinea. *New Zealand Veterinary Journal*, 41:220–221.

MILES, C.O., MUNDAY, S.C., HOLLAND, P.T., SMITH, B.L., EMBLING, P.P. & WILKINS, A.L. 1991. Identification of a sapogenin glucuronide in the bile of sheep affected by *Panicum dichotomiflorum* toxicosis. *New Zealand Veterinary Journal*, 39: 150–152.

MILES, C.O., MUNDAY, S.C., HOLLAND, P.T., LANCASTER, M.J. & WILKINS, A.L. 1992a. Further analysis of bile crystals from sheep grazing *Panicum schinzii* (sweet grass). *Australian Veterinary Journal*, 69:34.

MILES, C.O., WILKINS, A.L., MUNDAY, S.C., HOLLAND, P.T., SMITH, B.L., LANCASTER, M.J. & EMBLING, P.P. 1992b. Identification of the calcium salt of epismilagenin β-D-glucuronide in the bile crystals of sheep affected by *Panicum dichotomiflorum* and *Panicum schinzii* toxicoses. *Journal of Agricultural and Food Chemistry*, 40:1606–1609.

MILES, C.O., WILKINS, A.L., MUNDAY, S.C., FLÅØYEN, A., HOLLAND, P.T. & SMITH, B.L. 1993. Identification of insoluble salts of the β-D-glucuronides of episarsasapogenin and epismilagenin in in the bile of lambs with alveld, and examination of *Narthecium ossifragum*, *Tribulus terrestris*, and *Panicum miliaceum* for sapogenins. *Journal of Agricultural and Food Chemistry*, 41:914–917.

MILES, C.O., WILKINS, A.L., ERASMUS, G.L., KELLERMAN, T.S. & COETZER, J.A.W. 1994. Photosensitivity in South Africa. VII. Chemical composition of biliary crystals from a sheep with experimentally induced geeldikkop. *Onderstepoort Journal of Veterinary Research*, 61:215–222.

MORRISON, S.M. & SCOTT, J.K. 1993. Assessment of the origins of *Tribulus terrestris* in Australia. *Proceedings of the 10th Australian Weeds Conference and 14th Asian Pacific Weed Science Society Conference 6th–10th September 1993*, Brisbane, Australia: 388–391.

PATAMALAI, B. 1988. Identification of hepatotoxins in kleingrass and a study of their toxicity. (Abstr.) *Dissertation Abstracts International*, B., 49:2080.

SMITH, B.L. & MILES, C.O. 1993. A role for *Brachiaria decumbens* in hepatogenous photosensitization of ruminants?: A letter to the editor. *Veterinary and Human Toxicology*, 35:256–257.

STABURSVIK, A. 1959. A phytochemical study of *Narthecium ossifragum* (L.) Huds. Oslo University Press. (*Norges Tekniske Vitenskapsakademi*, series 2, no. 6).

THEILER, A. 1918. Geeldikkop in sheep (*Tribulosis ovis*). *Report on Veterinary Research, Union of South Africa*, 7 and 8:1–56.

WILKINS, A.L., MILES, C.O., SMITH, B.L., MEAGHER, L.P. & EDE, R.M. 1994. GC-MS method for analysis of plant and animal samples associated with ovine photosensitization, in *Poisonous plants of the world: agricultural, phytochemical and ecological aspects*, edited by S.M. Colegate, & P.R. Dorling. *Proceedings of the 4th International Symposium on Poisonous Plants, Fremantle, Australia, 27th September–1st October, 1993*. CAB International. In press.