Onderstepoort Journal of Veterinary Research, 61:215–222 (1994)

Photosensitivity in South Africa. VII. Chemical composition of biliary crystals from a sheep with experimentally induced geeldikkop

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ABSTRACT

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

Geeldikkop was induced in a sheep by oral administration of crude saponins from *Tribulus terrestris*. Centrifugation of the bile from this sheep gave a pale green sediment of crystalloid material which was insoluble in common organic solvents, but soluble in acetic acid. Analysis of the crystalloid material by ^{1}H and ^{13}C NMR, EDXA, TLC, LSIMS, and by acidic hydrolysis followed by TLC and GC-MS, revealed it to be composed principally of a 6:1 mixture of the calcium salts of the β-D-glucuronides of the steroidal sapogenins epismilagenin and episarsasapogenin. The administered saponin was found to contain glycosides of the steroidal sapogenins diosgenin, yamogenin, epismilagenin, tigogenin, neotigogenin, gitogenin and neogitogenin in the ratio 10:7:1:11:7:35:25. A metabolic pathway for the conversion of diosgenin and yamogenin saponins to the biliary glucuronides is proposed.

INTRODUCTION

Geeldikkop is one of the plant-induced hepatogenous photosensitization diseases of ruminants, characterized by the deposition of birefringent crystalloid material in the biliary system. The disease has long been associated with the consumption by sheep of *Tribulus terrestris*—especially of young wilted plants (Theiler 1918). In the 1950s, the possible involvement of the steroidal saponins of *T. terrestris* in the aetiology of geeldikkop was investigated (Henrici 1952). Since the end of that decade, however, interest in the role of saponins in geeldikkop has diminished because the disease could not be induced by dosing sheep with these compounds (Brown & De Kock 1959). Lately there has been a resurgence of interest in saponins, following reports of the experimental reproduction of two similar ovine hepatogenous photosensitizations by dosing with crude saponins from the causative plants (Patamalai 1988; Abdelkadar, Ceh, Dishington & Hauge 1984). Although coadministration of the mycotoxin sporidesmin

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Received 7 July 1994—Editor

and *T. terrestris* is known to induce geeldikkop in sheep (Kellerman, Van der Westhuizen, Coetzer, Roux, Marasas, Minné, Bath & Basson 1980), reproduction of the disease solely by administration of crude *T. terrestris* saponins has only recently been reported (Kellerman, Erasmus, Coetzer, Brown & Maartens 1991). The nature of the characteristic crystalloid material suspected of occluding the bile ducts during geeldikkop (Coetzer, Kellerman, Sadler & Bath 1983) has, nevertheless, remained unknown. Recently, the crystalloid material from the bile of sheep that became 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), *P. schinzii* (Lancaster, Vit & Lyford 1991; Miles, Munday, Holland, Lancaster & Wilkins 1992a; Miles *et al.* 1992b), and *Narthecium ossifragum* (Miles, Wilkins, Munday, Flåøyen, Holland & Smith 1993), was identified as being composed of insoluble (mainly calcium) salts of 5β-spirostan-3α-ol β-D-glucuronides derived from the saponins in the ingested plants. The aim of the present study was to determine the chemical nature of the biliary crystalloid material induced by the administration of crude *T. terrestris* saponins to sheep, in the expectation that this might throw light on the aetiology and pathogenesis of geeldikkop.

MATERIALS AND METHODS

General

Liquid surface-assisted ionization mass spectrometry (LSIMS) was performed by AECI Ltd on a Finnigan MAT TSQ700 instrument using an 8-keV Ar atom beam. Energy-dispersive X-ray analysis (EDXA) was performed as described previously (Miles *et al.* 1992b). ^{1}H and ^{13}C NMR spectra were obtained at 300,13 and 75,47 MHz, respectively, using a Bruker AC-300 spectrometer. NMR spectra of biliary crystals were recorded at 27 °C, or at 87 °C, from CD_3COOD solutions. NMR spectra of other compounds were obtained from $CDCl_3$ solutions at 27 °C. Thin-layer chromatography (TLC) was performed on silica gel plates (0,2 mm) (Machery-Nagel) using the following mobile phases: eluent A, chloroform-methanol-water 10:5:1; eluent B, chloroform-methanol-water 10:5:1 plus 1 % v/v acetic acid; eluent C, heptane-ethyl acetate 7:3; eluent D, chloroform-ethyl acetate 17:3. Sapogenin derivatives were visualized by spraying with anisaldehyde reagent (Holland *et al.* 1991). Standards of tigogenin, sarsasapogenin, and smilagenin were obtained from Upjohn Laboratories, diosgenin was obtained from Sigma Chemical Co., and epismilagenin was obtained from Steraloids Inc. Episarsasapogenin was prepared from sarsasapogenin (Miles *et al.* 1993), and epitigogenin was prepared from tigogenin by the method of Blunden, Jaffer, Jewers & Griffen (1979). The identity of each standard was confirmed by comparison of its ^{13}C NMR spectrum with that reported in the literature (Agrawal, Jain, Gupta & Thakur 1985). The calcium salt of epismilagenin β-D-glucuronide and specimens of neotigogenin and yamogenin were available from previous work in our laboratories (Miles *et al.* 1992b; 1993; Wilkins, Miles, Smith, Meagher & Ede 1994).

Gas chromatography-mass spectrometry (GC-MS) of acetylated spirostanols

A solution of pyridine-acetic anhydride (1:1, 200 μℓ) was added to a solution of the spirostanol(s) in chloroform (400 μℓ). The mixture was shaken, then allowed to stand for at least 3 h prior to GC-MS analysis using a 20 m x 0,25 mm i.d. HP-1 (Hewlett-Packard) methyl silicone capillary column installed on an HP5980 GC instrument interfaced to an HP5970B mass-selective detector. The GC column was temperature programmed from 200 °C (0,5 min hold) to 250 °C at 35 °C/min, and then to 285 °C at 2 °C/min (10 min hold). Total ion (mass range *m/z* 40–520) and a series of selected ion [*m/z* 139 (ring F fragment); *m/z* 396 (M^+-HOAc) and 282 for spirost-5-en-3-ol acetates); and *m/z* 458 (M^+), 344, 329, 315, 284, 269 and 255 for spirostan-3-ol acetates] mode aquisitions were undertaken (Wilkins *et al.* 1994).

Isolation and analysis of biliary sediment

A sheep was dosed daily with crude *T. terrestris* saponins, as described previously (Kellerman *et al.* 1991), at Onderstepoort. Approximately 6 h after its fourth dose the sheep was euthanazed, and the bile was recovered from the gall bladder for analysis. The bile, which was cloudy, was centrifuged, and the sediment was collected (the supernatant was retained for subsequent analysis). The sediment was washed (twice) by suspension in water followed by centrifugation, and then air-dried to give a pale green powder. A portion of this powder was washed successively with hot (80 °C) methanol-water (1:1, 1 mℓ), methanol (1 mℓ), and chloroform (1 mℓ), in a sealed vial. After each wash, the solution was cooled and the sediment was recovered by centrifugation, except that in the case of the chloroform wash it was necessary to add acetone (1 mℓ) to reduce the solvent density sufficiently for sedimentation to occur. The sediment was air-dried to give the biliary sediment as a colourless powder, a sample of which was examined by EDXA. The powder was taken up in acetic acid and filtered, and the filtrate

was evaporated *in vacuo* with the aid of the toluene azeotrope to leave a colourless solid. This solid was dissolved in acetic acid for LSIMS and TLC (eluents A and B), or was dissolved in CD_3COOD for NMR analysis. After NMR, a portion of the solution was added to ten volumes of 1 M hydrochloric acid, and heated to 100 °C for 4 h during which time a colourless precipitate formed. The hydrolysate was extracted with dichloromethane, dried ($MgSO_4$), and the solvent removed *in vacuo*. The residue was then examined by TLC (eluents C and D) and by GC-MS.

GC-MS analysis of the *T. terrestris* extract

A sample of the *T. terrestris* saponin extract (Kellerman *et al.* 1991) was hydrolyzed with 1 M hydrochloric acid at 100 °C for 1 h, and the hydrolysate was extracted with diethyl ether. The ethereal extract was washed with 0,1 M NaOH, then with water, and dried ($MgSO_4$). Solvent was removed *in vacuo* and the residue was acetylated. After analysis by GC-MS, the remainder of this material was used in the identification of the dihydroxyspirostanes (see below).

Identification of dihydroxyspirostanes

A portion of the acetylated *T. terrestris* sapogenin mixture (above) was separated on an alumina column (Brockman type II, BDH) using hexane-diethyl ether mixtures (40 mℓ each of 1:0, 9:1, 4:1, 2:1 and 1:1) to give a monoacetate (minor) and a diacetate (major) fraction. Separation of the diacetate fraction on a 5% $AgNO_3$-silica gel column using hexane-diethyl ether mixtures (20 mℓ each of 9:1, 4:1, 2:1 and 1:1) afforded two fractions, designated fractions A and B.

RESULTS

Geeldikkop was induced in a sheep by dosing it with crude saponins that had been extracted from *T. terrestris*. Biliary sediment was obtained from the sheep using methods developed for the isolation of crystalloid material from the bile of sheep with similar photosensitization diseases (Miles *et al.* 1992a; 1992b; 1993). Because both the biliary crystalloid material and the saponins in *T. terrestris* have been implicated in the aetiology of geeldikkop, the results presented here provide valuable information about the possible causes of this disease.

Analysis of biliary crystals

EDXA indicated that the sediment contained calcium, carbon and oxygen, and no other elements were detected. TLC analysis of the biliary crystals showed R_f values of 0,24 (major) and 0,17 (very minor), and 0,48 (major) and 0,35 (very minor) using eluents A and B, respectively. The very minor component was not identified. Using eluents A and B, a specimen of epismilagenin β-D-glucuronide had R_f values of 0,25 and 0,48, respectively. Positive ion LSIMS of the biliary crystals displayed a prominent quasimolecular ion at *m/z* 593,4, and a major fragment at *m/z* 417,3. The positive and negative ion LSIMS of the biliary crystals were indistinguishable from those obtained under identical conditions from a specimen of epismilagenin β-D-glucuronide. The NMR spectra of the biliary crystals showed the presence of two components, in a ratio of approximately 6:1. Epismilagenin β-D-glucuronide (major component) had: δ ^{1}H NMR (87 °C) 0,76 (3H, d, *J* 6,2 Hz, $CHCH_3$), 0,79 (3H, s, CH_3), 0,96 (3H, s, CH_3), 0,97 (3H, d, *J* 7,0 Hz, $CHCH_3$), 3,36 (1H, t, *J* 10,6 Hz, H-26α), 3,43-3,53 (2H, m, H-2' and H-26β), 3,60-3,80 (3H, br m, H-3', H-4' and H-3β), 3,99 (1H, m, H-5'), 4,45 (1H, br q, *J* 6,6 Hz, H-16), 4,58 (1H, br d, *J* 7,8 Hz, H-1'); d ^{13}C NMR (87 °C) 14,6 (C-21), 16,8 (C-27), 17,2 (C-18), 21,7 (C-11), 23,8 (C-19), 27,6 (C-2), 27,7 (C-7), 28,1 (C-6), 29,5 (C-24), 31,1 (C-25), 32,0 (C-23), 32,5 (C-15), 35,1 (C-4), 35,8 (C-10), 36,3 (C-1), 36,8 (C-8), 41,2 (C-12), 41,7 (C-13), 41,9 (C-9), 42,9 (C-20), 43,4 (C-5), 57,4 (C-14), 63,6 (C-17), 67,7 (C-26), 72,7 (C-4'), 74,5 (C-2'), 75,6 (C-5'), 77,1 (C-3'), 80,5 (C-3), 82,3 (C-16), 102,0 (C-1'), 110,8 (C-22), C-6' not observed. Some of the signals attributable to episarsasapogenin β-D-glucuronide (minor component) were adequately differentiated from those of the major component, including: δ ^{1}H NMR (87 °C) 0,99 (3H, d, *J* 6,7 Hz, $CHCH_3$), 1,05 (3H, d, *J* 6,9 Hz, $CHCH_3$); δ ^{13}C NMR (87 °C) 14,5 (C-21), 16,5 (C-27), 26,4 (C-24), 28,0 (C-25), 62,9 (C-17), 65,8 (C-26), 111,3 (C-22).

Analysis of biliary crystal hydrolysate

Hydrolysis of the biliary crystals using hydrochloric acid following the procedure of Miles *et al.* (1992b) yielded a lipophilic fraction that was extracted into dichloromethane. TLC analysis of the hydrolysate using eluent C showed one spot only, with R_f 0,28, while standards of smilagenin, epismilagenin, sarsasapogenin, episarsasapogenin, and tigogenin had R_f values of 0,34, 0,28, 0,34, 0,28, and 0,27, respectively. Using eluent D, the hydrolysate showed two spots at R_f values of 0,40 and 0,35, while standards of smilagenin, epismilagenin, sarsasapogenin, episarsasapogenin, and tigogenin had R_f values of 0,43, 0,38, 0,42, 0,34, and 0,38 respectively. GC-MS analysis of the acetylated hydrolysate showed a major and a minor (6:1 ratio) component to be present, with retention times of 18,23 and 18,58 min, respectively. The retention times of acetylated specimens of smilagenin, epismilagenin,

sarsasapogenin, episarsasapogenin, tigogenin, epitigogenin, neotigogenin, diosgenin and yamogenin were 17,88, 18,22, 18,24, 18,58, 19,42, 18,28, 19,81, 19,07, and 19,48 min, respectively, under identical conditions. The relative intensities of the ions of *m/z* 255, 269, 284, 315, 329, 344 and 458 in the mass spectra of the biliary crystal hydrolysate compounds were identical to those of epismilagenin acetate and episarsasapogenin acetate, and different from those of the acetates of smilagenin, sarsasapogenin, tigogenin, neotigogenin, epitigogenin and epineotigogenin (Wilkins *et al.* 1994).

Analysis of *T. terrestris* saponins

GC-MS analysis of the acetylated sapogenins from the hydrolyzed *T. terrestris* saponin mixture revealed the presence of diosgenin, yamogenin, epismilagenin, tigogenin, neotigogenin, gitogenin, and neogitogenin, in a ratio of 10:7:1:11:7:35:25.

Identification of dihydroxyspirostanes

The diacetate fractions were shown by ^{1}H and ^{13}C NMR analyses to be mixtures of gitogenin diacetate and neogitogenin diacetate, in a ratio of 4:1 (fraction A), and 2:3 (fraction B). Gitogenin diacetate had δ ^{13}C ($CDCl_3$, 27 °C) 13,0 (C-19), 14,5 (C-21), 16,5 (C-18), 17,2 (C-27), 21,2 (2 × $OCOCH_3$), 21,2 (C-11), 27,6 (C-6), 28,8 (C-24), 30,3 (C-25), 31,4 (C-23), 31,8 (C-15), 31,9 (C-7), 32,8 (C-4), 34,4 (C-8), 37,2 (C-10), 39,9 (C-12), 40,6 (C-13), 41,7 (C-20), 42,4 (C-1), 44,2 (C-5), 54,1 (C-9), 56,1 (C-14), 62,2 (C-17), 66,9 (C-26), 72,0 (C-2), 74,7 (C-3), 80,8 (C-16), 109,3 (C-21), 170,6, 170,7 (2 × $OCOCH_3$). Neogitogenin diacetate had δ ^{13}C ($CDCl_3$, 27 °C) 13,0 (C-19), 14,4 (C-21), 16,1 (C-27), 16,5 (C-18), 21,2 (2 × $OCOCH_3$), 21,2 (C-11), 25,8 (C-24), 26,0 (C-23), 27,2 (C-25), 27,6 (C-6), 31,8 (C-15), 31,9 (C-7), 32,8 (C-4), 34,4 (C-8), 37,2 (C-10), 39,9 (C-12), 40,6 (C-13), 42,2 (C-20), 42,4 (C-1), 44,2 (C-5), 54,1 (C-9), 56,1 (C-14), 62,0 (C-17), 65,2 (C-26), 72,0 (C-2), 74,7 (C-3), 80,9 (C-16), 109,8 (C-21), 170,6, 170,7 (2 × $OCOCH_3$). Alkaline hydrolysis of fraction B with refluxing ethanolic KOH for 30 min afforded a mixture of gitogenin and neogitogenin, the ^{1}H and ^{13}C NMR spectral features of which corresponded to those reported elsewhere for these compounds (Agrawal *et al.* 1985; Filho, Medeiros, Agra & Bhattacharyya 1989).

DISCUSSION

The crystalloid material in the livers of sheep with geeldikkop is of interest from the pathogenic and the aetiologic aspects of the disease. The biliary sediment from the sheep affected by geeldikkop had the same solubility characteristics as that isolated from sheep with *P. dichotomiflorum* or *P. schinzii* photosensitizations (identified as epismilagenin β-D-glucuronide calcium salt), being insoluble in common organic solvents, but soluble in acetic acid (Miles *et al.* 1992b). EDXA indicated the presence of calcium, carbon and oxygen in the sediment. Both the solubility characteristics and the EDXA are consistent with a calcium salt of an organic acid. The sediment was identical to epismilagenin β-D-glucuronide calcium salt when examined by TLC (eluents A and B) and LSIMS. However, hydrolysis with acid gave a sapogenin fraction which displayed two spots upon TLC (eluents C and D). One spot coeluted with epismilagenin, and the other spot with episarsasapogenin and tigogenin. GC-MS with selected-ion monitoring (Wilkins *et al.* 1994) conclusively demonstrated the presence of epismilagenin and episarsasapogenin, and the absence of tigogenin.

Examination of the biliary crystals by NMR spectroscopy confirmed the presence of two compounds, in a ratio of approximately 6:1. The ^{1}H and ^{13}C NMR resonances of the major compound corresponded to those reported for epismilagenin β-D-glucuronide (Miles *et al.* 1992b), while the resonances of the minor component corresponded to those previously observed for episarsasapogenin β-D-glucuronide (Miles *et al.* 1993). In the conventional ^{1}H NMR spectrum, secondary-methyl groups attributable to the major and minor compounds, were partially obscured by the tertiary methyl group signals. The secondary methyl group signals were, however, readily detected in a double quantum filtered ^{1}H NMR spectrum optimized for *J* 6,6 Hz. At 27 °C, the spirostanol ^{1}H and ^{13}C resonances were satisfactorily resolved, while the glucuronide resonances generally appeared as unresolved broad lines. Heating the sample to 87 °C improved the resolution sufficiently to permit the observation of all of the ^{1}H and ^{13}C resonances, with the sole exception of C-6' (the carboxyl carbon atom). We have previously observed that the line-widths of the resonances of epismilagenin β-D-glucuronide calcium salt vary, depending upon concentration and temperature, with the resonances of the atoms closest to the site of acid-salt exchange (i.e. C-6') being most affected. At 87 °C, ^{13}C NMR chemical shifts were typically 0,2–0,3 ppm higher than was the case at 27 °C (Miles *et al.* 1992b).

The biliary crystals of the sheep with experimentally induced geeldikkop are therefore shown to be composed of a 6:1 mixture of the calcium salts of the β-D-glucuronides of epismilagenin and episarsasapogenin (Fig. 1).

Spirostanol saponins and sapogenins are products of some plants, so it seems likely that the biliary

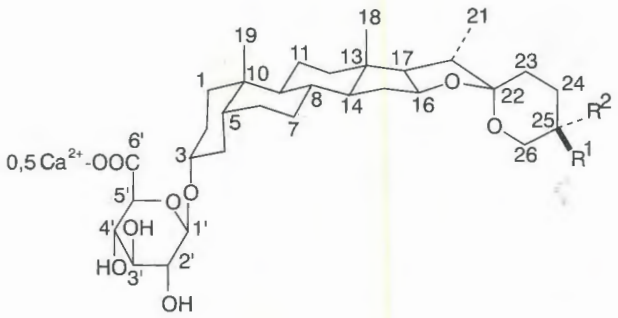

FIG. 1 Chemical structures of the components of the biliary sediment from a sheep with geeldikkop

crystals originate from the ingested plant saponins. GC-MS analysis of the hydrolyzed *T. terrestris* saponin mixture that was dosed to the animal revealed the presence of diosgenin, yamogenin, epismilagenin, tigogenin, neotigogenin, gitogenin, and neogitogenin in the ratio 10:7:1:11:7:35:25. The biliary crystals were derived from epismilagenin and episarsasapogenin, but the administered saponin mixture contained epismilagenin as a minor constituent only, and episarsasapogenin was not present at detectable levels in the extract. This suggests that the epismilagenin and episarsasapogenin present in the biliary crystals resulted primarily from ovine metabolism of the ingested diosgenin- and yamogenin-derived saponins. We have previously shown that ovine metabolism of diosgenin produces epismilagenin (Wilkins *et al.* 1994). The steps that are required during this conversion are: hydrolysis of the sugars from the plant saponin; reduction of the double bond at C-5; epimerization of the hydroxyl group at C-3 (i.e. 3β-OH to 3α-OH); and conjugation with glucuronic acid (Miles *et al.* 1992b; Wilkins *et al.* 1994). The same process would convert yamogenin-derived saponins into episarsasapogenin β-D-glucuronide. In the presence of calcium these glucuronides may then precipitate as their calcium salts, forming the characteristic biliary crystals. This sequence of reactions is depicted in Fig. 2, for a diosgenin-derived saponin.

It is remarkable that the biliary crystals are composed only of metabolites of spirost-5-en-3-ols and 5β-spirostan-3-ols; no metabolites of 5α-spirostan-3-ols (e.g. tigogenin and neotigogenin) were detected in the biliary crystals, even though tigogenin- and neotigogenin-containing saponins were abundant in the *T. terrestris* extract. Furthermore, no dihydroxyspirostanes were present in the biliary crystals, even though the plant extract contained higher levels of gitogenin and neogitogenin saponins than of diosgenin, yamogenin, tigogenin and neotigogenin saponins.

It has now been demonstrated that the characteristic crystalloid material deposited in the bile during four different plant-associated (*T. terrestris, P. dichotomiflorum, P. schinzii,* and *N. ossifragum*) photosensitization diseases is composed principally of insoluble salts of 5β-spirostan-3α-ol β-D-glucuronides, and that these glucuronides are derived by metabolism of the ingested plant saponins (Miles *et al.* 1992b; 1993). Furthermore, several other plants, including *Agave lecheguilla* (Camp, Bridges, Hill, Patamalai & Wilson 1988), *Nolina texana* (Mathews 1940), *Brachiaria decumbens* (Graydon, Hamid, Zahari & Gardiner 1991), *P. miliaceum* (Clare 1955), and *P. coloratum* (Bridges, Camp, Livingston & Bailey 1987), are associated with photosensitization diseases that are characterized by the deposition of birefringent crystalloid material in the biliary system. *A. lecheguilla* contains saponins derived from smilagenin (Camp *et al.* 1988), whereas the biliary crystals fom a sheep that consumed this plant contained a sapogenin that was tentatively identified as smilagenin (Camp *et al.* 1988), although a recent analysis of the published mass spectral data indicates that the sapogenin was epismilagenin (Holland *et al.* 1991). *P. coloratum* (Patamalai, Hejtmancik, Bridges, Hill & Camp 1990), *P. miliaceum* (Miles *et al.* 1993), *P. coloratum* var. bambatze, and *B. decumbens* (Wilkins *et al.* 1994) have also been shown to contain steroidal saponins. Thus, with the exception of *N. texana*, the presence of steroidal saponins has now been demonstrated in all the plants associated with photosensitization accompanied by biliary crystals. Furthermore, the rumen of a sheep photosensitized on *B. decumbens* has been shown to contain episarsasapogenin and epismilagenin (Abdullah, Lajis, Bremner, Davies, Mustapha & Rajion 1992; Lajis, Abdullah, Khan, Jalaludin, Salim & Bremner 1993), whereas this plant is reported to contain saponins derived from yamogenin and diosgenin (Smith & Miles 1993; Wilkins *et al.* 1994). Thus, ruminal metabolism of plant saponins appears to occur during *B. decumbens* photosensitization, and may be a general phenomenon that is important in the aetiology of not only geeldikkop, but also the other related photosensitization diseases.

In geeldikkop, phylloerythrin is believed to be retained as a result of the occlusion of bile ducts by crystalloid material. This hypothesis is supported by histopathological evidence that bile ducts are often occluded by the crystalloid material, and that the lesions in the portal tracts are consistent with those of biliary occlusive disease. Lamellar periductal fibrosis was found both in sheep with geeldikkop, and in sheep in which the bile ducts had been ligated

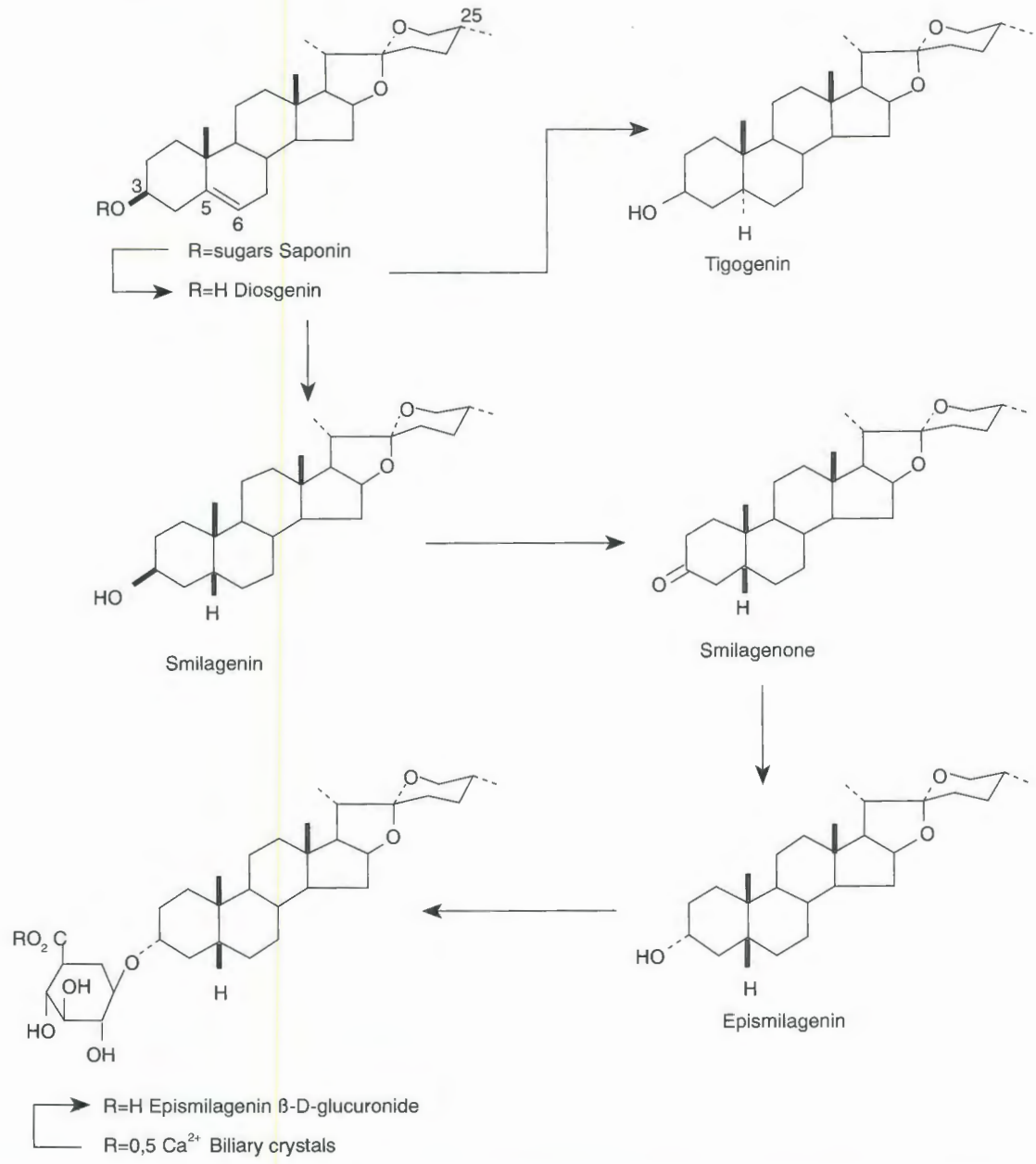

FIG. 2 Proposed pathway for metabolic conversion of diosgenin-containing saponins from *T. terrestris* into the major component of the geeldikkop biliary crystals. An analogous series of transformations would convert the yamogenin-containing saponins of *T. terrestris* into the minor component of the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

(Coetzer *et al*. 1983). Some doubts have, nevertheless, been expressed about the role of the crystalloid material in the pathogenesis of the so-called biliary occlusive photosensitizations (Kellerman, Miles, Erasmus, Wilkins & Coetzer 1994). The suggestion of Flåøyen, Borrebaek & Nordstoga (1991) that parenchymal damage might play an important role in phylloerythrin retention in alveld may be true also for geeldikkop, as Kellerman *et al*. (1991) failed to see biliary occlusive microliths in a sheep photosensitized with crude saponins from *T. terrestris*. It has been suggested that the toxins responsible for crystal-associated hepatogenous photosensitizations (such as that induced by *Panicum* spp.) act primarily on the membranes of the bile canaliculi, in a manner similar to that of pentacyclic triterpenes in *Lantana* poisoning (Seawright 1989). It should be noted, however, that lesions typical of pentacyclic triterpene poisoning have not yet been reported in geeldikkop.

If geeldikkop and allied diseases are indeed due to occlusion of the biliary system by the crystalloid material, then the finding that the crystalloid material is a metabolite of the ingested plant saponins, constitutes proof that the plant saponins are the toxic agents responsible for the photosensitization. Steroidal saponins have been further implicated as causes of geeldikkop, alveld, and *Agave lecheguilla* poisoning by the experimental reproduction of these diseases, by administration to sheep of crude saponins from the plants in which they occur (Kellerman *et al.* 1991; Abdelkader *et al.* 1984; Patamalai 1988). The induction of photosensitization (Patamalai 1988) and of intrabiliary crystals (Patamalai 1988; Flåøyen, Smith & Miles 1993) in sheep dosed with the sapogenin, diosgenin, provides further proof that saponins play a role in hepatogenous photosensitization. It has, nevertheless, yet to be demonstrated that plant saponins are solely responsible for crystalloid-associated photosensitization diseases, and it is possible that the photosensitization is caused by some component, other than the saponins, that is present in the crude plant-saponin extracts.

A mycotoxin contribution to the cause of geeldikkop and alveld has been postulated (Kellerman *et al.* 1980; Aas & Ulvund 1989), in an attempt to account both for the sporadic nature of outbreaks of these diseases, and for the difficulties often experienced in reproducing these diseases solely by the administration of crude plant saponins to sheep. Indeed, Kellerman *et al.* (1980) have presented evidence that the mycotoxin sporidesmin (the causative agent of facial eczema) can precipitate geeldikkop in sheep grazing *T. terrestris* in the karoid areas of South Africa. However, sporidesmin is unlikely to have contributed in the present experiment because, owing to its instability (White, Mortimer & Di Menna 1977), it is unlikely to have survived the procedure used by Kellerman *et al.* (1991) for saponin extraction. Further evidence that sporidesmin (apart, possibly, from acting as a triggering agent) is not directly involved in geeldikkop, comes from the observation of pathologic differences between the hepatic lesions of crystalloid-associated photosensitizations, and those of facial eczema (Coetzer *et al.* 1983). Additional support for this contention comes from the observation that photosensitization induced by *P. coloratum* (Bridges *et al.* 1987), *P. schinzii* (Button, Paynter, Shiel, Colson, Paterson & Lyford 1987), *N. ossifragum* (Di Menna, Flåøyen & Ulvund 1992), *B. decumbens* (Graydon *et al.* 1991; Baber 1989; Smith & Miles 1993) and *T. terrestris* (Jacob & Peet 1987) can apparently occur in the absence of sporidesmin. Furthermore, Muchiri (1977, cited by Button *et al.* 1987) found that the application of two antifungal agents to *P. coloratum* pastures did not prevent the development of photosensitization in lambs. Although not the primary cause of geeldikkop and related diseases, sporidesmin has been shown to exacerbate both *P. dichotomiflorum* (P.H. Mortimer, Parker Road, RD 1, Huntly, N.Z., unpublished observations 1987) and *T. terrestris* (Kellerman *et al.* 1980) photosensitizations.

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.

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