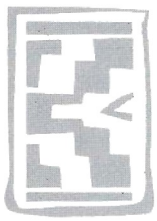

Morphological changes associated with the development of the rumino-reticulum in growing lambs fed different rations

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ABSTRACT

SWAN, G.E. & GROENEWALD, H.B. 2000. Morphological changes associated with the development of the rumino-reticulum in growing lambs fed different rations. *Onderstepoort Journal of Veterinary Research*, 67:105–114

Morphological changes associated with rumino-reticular development was compared in two groups of SA Mutton Merino lambs ($n = 12$) fed different diets at 3–5 weeks, 17–19 weeks and 31–33 weeks of age. Diet groups were identified as MMH or MHH according to the sequence at which the full-milk replacement (M) or hay (H) diet were fed to the lambs over the three study phases (phases I, II and III) preceding each age period. Prominent differences in the morphology (size and volume) and function (pH, proteolytic activity and microbial population) of the rumino-reticulum were observed in indicator lambs ($n = 2$) killed at every age period and also between milk-fed and hay-fed lambs. The size of the rumino-reticulum was rudimentary at 3–5 weeks of age and remained underdeveloped in lambs at 17–19 weeks of age which had received a full-milk replacement diet during phase II. One lamb, slaughtered at 3 weeks of age, showed a large distended rumen with severe sloughing of the surface cells of the stratum corneum. The size of the rumino-reticulum increased in size (2 x) in lambs which were fed hay relative to the milk-fed lambs during phase II and reached adult proportions in all lambs at 31–33 weeks of age. Ultrastructural examination showed that rumen papillae were more developed in lambs fed hay during phase II when compared to those of milk-fed lambs. Rumen papillae were best developed in phase III lambs.

Keywords: Growing lambs, hay-fed, milk-fed, morphological changes, rumen development

INTRODUCTION

The forestomach, consisting of the rumen, reticulum and omasum of ruminants is rudimentary and non-functional at birth (Church 1988). By contrast, the abomasum is well developed and highly functional. The rumino-reticulum has a considerably larger percentage growth relative to the total stomach tissue from birth to adulthood.

Development of the forestomach in size and function can be divided into three distinct phases: a non-ruminant phase from birth to 3 weeks of age; a transitional phase from 3–8 weeks of age; and an adult stage from 8 weeks onward (Church 1988). During the non-ruminant phase, the rumen is small and flaccid, with rudimentary papillae in calves and slightly larger papillae in lambs. The reticulum of lambs is a small elastic sac, about one third the size of the rumen, and has a differentiated polygonal surface structure similar to that of the adult, but with rudimentary papillae on the floor and walls of the surface structure. During the transitional phase, the rumen grows to 4–8 times its birth mass, but without attaining the rumen wall thickness characteristic of the adult phase. Rumino-reticular papillae and the spaces between the laminae of the omasum become larger and more distinct with advancing age. At 8 weeks of age in lambs and 12 weeks in the calf, the proportions of

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Accepted for publication 19 January 2000—Editor

the forestomachs are similar to those of the adult animal. The body mass of calves doubles and that of lambs quadruples by 8 weeks of age. There is an increase in rumen capacity and a proliferation of the smooth musculature of the rumen in the adult stage, giving it a heavy muscular appearance. Rumen papillae, particularly in the atrium and recessus ruminis, become large and prominent (Amasaki & Diago 1988; Arias, Cabrera & Valencia 1978; Church 1988; Hendrikson 1970). The reticulum remains a pliable sac without the heavy musculature of the rumen and accounts for only 4–7 % of the total stomach capacity and 6 % of its volume. In contrast, the omasum continues to increase in size and mass up to 36–38 weeks of age. Salivary secretion, rumino-reticulum motility, internal rumino-reticulum conditions, such as pH and microbial population, and blood supply change concurrently with the transition of the forestomach development from the rudimentary to adult stages (Church 1979).

The surface of the rumino-reticulum is covered by numerous papillae, which vary in shape and size within the various ruminal sacs and between the dorsal and ventral compartments (Scott & Gardner 1973). Papillae in the rumen cover the entire surface, including the ruminal pillars. At the cranial entrance to the reticulum, there is a distinct change in the papillae from a rudimentary honey-comb appearance to a hexagonal arrangement of thick-walled crests. The papillary forms range from heavily-furrowed or grooved tongue-shaped forms in the ventral, atruim ruminis and recesses ruminis sacs, to less grooved, ridged, flattened, flap-like forms over the dorsal ruminal surfaces, and finally to smoother conical, heavily keratinised forms in the reticulum and omasum. At the reticulo-omasal opening, there are peculiar cornified, unguliform papillae. Mechanical forces appear initially to shape the form of the papillae, but subsequently it depends on the nature of the diet fed to ruminants. Papillae increase the surface area for absorption and thus play an important role in absorptive capacity. Larger papillae also provide friction for movement of liquid ingesta. Papillae consist of a core of connective tissue covered by stratified squamous, non-glandular epithelium typically organized into the *stratum basale, spinosum, granulosum and corneum* (Hyden & Sperber 1965; Lavker, Chalupa & Dickey 1969). Demarcation of the various layers is not distinct in the intermediate epithelium layers of the rumino-reticulum. The ultrastructure of the epithelium is similar in the various compartments of the forestomach.

A continuous, extremely convoluted basal cell membrane (approximately 0,025–0,04 µm thick), adjacent to the basal lamina, separates the basal cells and the connective tissue. The basal lamina does not always follow the contours of the basal cell membrane (Lavker *et al.* 1969). Rare cytoplasmic infoldings of the basal cells, observed as finger-like processes, project into the connective tissue. Where the borders of adjacent basal cells are in contact with the basement membrane, the infoldings occur more frequently. According to Hyden & Sperber (1965), processes that frequently interdigitate with other similar processes may be a characteristic of cells located near capillaries. Smooth lateral and apical cell membranes of the basal cells are often closely apposed to those of adjacent cells, but they may also form wide intercellular spaces between small contact areas where the membrane has a more wavy appearance. Intercellular bridges or desmosomes occur infrequently in the basal strata. Blood capillaries are located below the basal cell membrane and in most instances are less than 0,5 µ from the basal epithelial cells. The thickness of the capillary endothelium, which is often very tenuous, varies widely. An abundant number of microcytic, pinocytotic-like vesicles occur in the endothelial cytoplasm.

The cell borders of the intermediate spinosum and granulosum strata are generally very similar to those of the basal layer, except that the plasma membranes form an abundance of intercellular bridges or desmosomes. Intercellular spaces are usually wide. In the cells near the cornified layer, plasma membranes show smooth interdigitating processes and have narrow intercellular spaces. Desmosomes are also abundant. Numerous granular to fold-like protrusions, similar to the finger-like structures of the basal layer, are present on the cornified layer (Tamate, Kikuchi, Onodera & Nagatani 1971). The protrusions appear to be formed during the typical keratinization process of the rumen epithelium under the influence of the rumen environment. They serve to enhance the absorption of certain substances, such as sodium and volatile fatty acids. They also represent attachment sites of desmosomes (Scott & Gardner 1973).

The absorptive function of the rumino-reticulum epithelium is primarily restricted to the basal cells (Hyden & Sperber 1965; Lavker *et al.* 1969). Intercellular spaces appear to allow fairly unrestricted diffusion of water and solutes. Free diffusion between the cells of the basal layers occur. The finger-like processes protruding into the intercellular space facilitate absorption from these and may also increase the selective passage of solutes through the basal layer. Specific and non-specific absorption of nutrients at the basal lamina are evident from the presence of both smooth and "fussy-coated" micropinocytotic vesicles (Lavker *et al.* 1969).

The normal growth and rate of development of the ruminant forestomachs are dependent on the type of feed ingested, its physical form and the nutritional status of the animal (Church 1979; McGavin & Morrill 1976). Animals fed solely on milk diets show a lack

of development in contrast to those fed on concentrates or hay. Calves fed exclusively milk remain in a non-ruminant phase for prolonged periods of time. Little papillary growth occurred in calves fed only milk for 16 weeks, while extensive papillary growth was present when solid foods were included in the diet (Church 1988). Even under natural grazing conditions, development will depend on the amount of milk consumed by the neonate for normal growth as determined by the availability and consumption of readily digestible feedstuffs. A pronounced increase in the growth of the rumen and reticulum occurs when there is access to solid foods in grazing lambs and become the most dominant of the stomachs by 12–16 weeks of age (Church 1979). Although the presence of dietary fibre in a relatively coarse form may be required for normal rumen development, the increased forestomach capacity of hay-fed diets may be due to stretching rather than an increase in tissue growth.

Groenewald & Booth (1992) and Groenewald (1992; 1993) described large, milk-filled rumens with severe sloughing of the surface epithelium cells in Karakul lambs carrying a lethal genetical factor and hypothesized that a decrease in innervation to the parts concerned might be the cause.

The current study examines the morphological changes associated with the development of the rumino-reticulum in growing lambs fed exclusively on milk in contrast to those fed on hay and to those changed from a milk-fed to hay-fed diet. It formed part of a larger study designed to determine the effect of the rumino-reticulum development in growing lambs on the absorption and disposition of halogenated salicylanilides (Swan 1997).

MATERIALS AND METHODS

Animals

Twelve healthy South African Mutton Merino lambs were selected at random from a group of twenty lambs. These lambs served as indicators to monitor the morphological changes associated with rumino-reticulum growth in a comparative pharmacokinetic study with rafoxanide, a halogenated salicylanilide, in growing lambs at three ages and fed diets to either stimulate or inhibit rumino-reticulum development (Swan 1997). All experimental animals were kept under experimental housing conditions from 1–3 weeks of age throughout the study period.

Study design

A randomized, parallel two-group, three-phase study design was utilized to compare the physical and ultrastructural changes associated with rumino-reticular growth at three ages (3–5 weeks, 17–19 weeks and 31–33 weeks) and in two groups of lambs fed different diets. The intervals preceded each age, representing the phases of the study. At the time of selection, the lambs (1–3 weeks of age) were randomly allocated, by means of a table of random numbers to two diet groups of ten animals each. Within each diet group, the lambs were fed a diet intended to either inhibit or stimulate the development of the rumino-reticulum during the different phases. During Phase I, following the selection of the lambs and for a period of 2 weeks, all lambs received a full milk-replacement with the purpose of inhibiting rumino-reticulum development. One diet group in Phase II was kept on the full milk-replacement, whereas a second group was provided with a standard diet of hay 14 days after the commencement of Phase II. This diet was intended to stimulate rumino-reticulum development. Both diet groups were provided access to hay during Phase III. The diet groups were named according to the sequence of milk or hay supplied over the three study phases, *viz.* a milk(M)-milk(M)-hay(H)-fed group and a milk(M)-hay(H)-hay(H)-fed group.

Housing and feeding

The animals were housed in a conventional, temperature-controlled, small stock housing facility, in the Onderstepoort Veterinary Academic Research Unit, Faculty of Veterinary Science, University of Pretoria. They were housed individually in pens, each of 2 m^2 in size. The different dietary groups of lambs were separated from each other in pens by brick walls. Water was supplied *ad libitum.* The lambs were docked and vaccinated for bluetong and pulpy kidney disease prior to selection and introduction to the experimental facilities.

Milk replacement consisted of a mixture of whole cow's milk (1 ℓ), cream (10 mℓ) and egg powder (1 desert spoon, i.e. *c.* 10 g), fed at a rate of *c.* 1 ℓ/kg in 3–5 portions during the day (a maximum of 600 mℓ was fed at each feeding). Chopped lucerne hay was offered at approximately 30 g/kg. The amounts of milk and hay were adapted according to live weight determined at weekly intervals.

Morphological development of the rumino-reticulum

Two indicator lambs from each diet group were euthanased by the intravenous administration of pentobarbitone (Euthapent, Kyron) at *c.* 60 mg/kg following each dietary phase and treatment. The indicator lambs were allocated randomly at the start of the trial. Immediately after death the rumino-reticulum was tied off at the cardia and at the reticulo-omasal orifice to prevent any loss of contents and then removed from the carcass. The volume of rumino-reticular content was measured by emptying the content into a measuring cylinder and an aliquot of 50 mℓ taken

for evaluation of rumen microbial activity. In addition, the capacity of the rumino-reticulum was estimated by determining the volume of phosphate-buffered saline (PBS) at a pH of 7,4 required to fill it to atmospheric pressure measured with a water manometer. The dry-mass (dried in an oven at 100 °C overnight) of the total rumino-reticulum wall was determined for each animal.

Evaluation of ruminal microbial activity was performed immediately after collection of the aliquot of rumino-reticulum content. Evaluation included measurement of pH using a pH meter (PHB 82 Standard, Radiometer, Copenhagen), determination of proteolytic activity using the methylene blue reduction test (Dirksen 1979), and light microscopic examination in order to determine the concentration (low, moderate and high), movement (sluggish, fairly good and rapid) and type (large, medium and small) of rumen protozoa.

Tissue samples of the ruminal wall were collected for transmission electron microscopy (TEM) from corresponding areas of the dorsal and ventral ruminal sacs of the indicator lambs within 30 min of euthanasia. One square centimetre (cm^2) samples were taken from the same areas for scanning electron microscopy (SEM). The tissue samples for TEM were rinsed in phosphate- buffered saline (PBS) at pH 7,4. Small blocks of tissue were immersion-fixed in 4 % glutaraldehyde in sodium cacodylate buffer for at least 24 h at 4 °C. Thereafter, the blocks were rinsed in sodium cacodylate buffer, post-fixed for 1 h at room temperature in similarly buffered 1 % osmium tetroxide and given two final buffer washes. The samples were dehydrated through a graded ethanol series (25 %, 50 %, 75 %, 96 % and 2 x 100 %—10 min per step), cleared in propylene oxide and embedded in epoxy resin (Embed 812). Semi-thin sections (0,5 µm) were cut from each sample and stained with toluidine blue to determine suitable areas for ultrathin sectioning. Thin sections (0,1 µm) were cut with a diamond knife on an ultramicrotome (Reichert OmU4), stained with uranyl acetate for 30 min (Watson 1958a; b) and lead citrate for 4 min (Reynolds 1963). The sections were examined in a transmission electron microscope (Philips 301 or Philips CM10)

TABLE 1 Rumino-reticulum function and organ size measurements

Diet group	Age (w)	Mean rumino-reticulum measurements (range)						
		pH	Reduction (s)	Volume of contents (ℓ)	Size of organ		Micro-organism	
					Capacity (ℓ)	Mass (g)	Type[3]	Motility[4]
MMH[1]-fed	3–5	6,8 (6,7–6,9)	195 (135–255)	0,09 (0,07–0,1)	0,37 (0,31–0,43)	5,9 (5,4–6,3)	1	0,5 (0–1)
	17–19	6,85 (6,85–6,85)	165 (150–180)	1,45 (1,4–1,5)	4,4 (3–5,8)	96	1	1 (1–1)
	31–33	6,93 (6,85–7,0)	150 (150–150)	6,4 (5,6–7,1)	13,8 (12,5–15,1)	173,5 (168–179)	3	4 (4–4)
MHH[2]-fed	3–5	5,5 (4,3–6,7)	0–120	0,6 (0,21–1)	0,96 (0,72–1,2)	13,4 (8,1–18,7)	1	0–1
	17–19	6,68 (6,6–6,75)	52,5 (45–60)	3,1 (2,5–3,7)	8,8 (8–9,6)	148,6 (136,9–160,3)	2	2,5 (2–3)
	31-33	7,08 (7,05–7,1)	105 (90–120)	5,6 (4,9–6,3)	11,3 (11–11,5)	240,5 (227–254)	3	3,5 (3–4)

[1] Milk-Milk-Hay diet
[2] Milk-Hay-Hay diet
[3] 1 = small
2 = small and medium
3 = small, medium and large micro-organisms
[4] 0 = no organisms
1 = low numbers of organisms with no movement
2 = low numbers of organisms with sluggish movement
3 = moderate numbers of organisms with fairly good movement
4 = high numbers of organisms with fairly good movement
5 = high numbers of organisms with very rapid movement

operated at 80 kV. Samples for SEM were pinned with insect pins to wax squares to prevent them from curling and fixed in 4% glutaraldehyde in sodium cacodylate buffer. After fixation they were dehydrated through a graded ethanol series (as for the TEM samples), and dried in a critical-point drier (Polaron) with liquid carbon dioxide. The dried samples were mounted on stubs and gold coated in a coating unit (Polaron E5100) and studied with a scanning electron microscope (Phillips XL20) operated at 10 kV.

Statistical analysis

The mean and range (n = 2) of all morphological measurements and results of functional tests of the rumino-reticulum of lambs within each group and at each age interval were determined and graphically illustrated. Linear regression analysis was performed of the volume and size of rumino-reticulum in relation to the age of the lambs within each group.

RESULTS

Size and function of the rumino-reticulum

Rumino-reticulum function and organ size measurements for both diet groups at the end of each study phase are summarized in Table 1. Changes in the mean volume of contents and size of the rumino-reticulum (capacity and mass) versus age of the lambs within each diet group are graphically illustrated in Fig. 1.

The volume of contents and size of the rumino-reticulum (capacity and mass) increased linearly with age in lambs of both diet groups (r^2 = 0,96 ± 0,04 for all parameters and both diet groups). Rumino-reticulum development was delayed in milk-fed (MMH-fed) lambs relative to the hay-fed (MHH-fed) lambs up to 17–19 (18) weeks of age (Fig. 1). The volume of the contents, capacity and wall mass of the rumino-reticulum of the milk-fed lambs was *c.* half that of the hay-fed lambs at 17–19 (18) weeks of age. From 17–19 (18) weeks to 31–33 (32) weeks of age, when all lambs were fed hay, a larger increase in the volume of contents and capacity of the rumino-reticulum occurred in the MMH-fed lambs relative to the MHH-fed lambs. The mass of the rumino-reticulum wall was greater in MHH-fed lambs at 17–19 weeks and 31–33 weeks of age compared to the MMH-fed lambs.

Very few ruminal micro-organisms were present in the lambs at 3–5 weeks of age, and in the MMH-fed lambs at 17–19 weeks of age. Ruminal micro-organisms were present in MHH-fed lambs at 17–19 weeks of age, but consisted mainly of small morphological types. Micro-organism population of all lambs at 31–33 weeks of age was consistent with that normally found in healthy adult sheep (Hoover & Miller 1991;

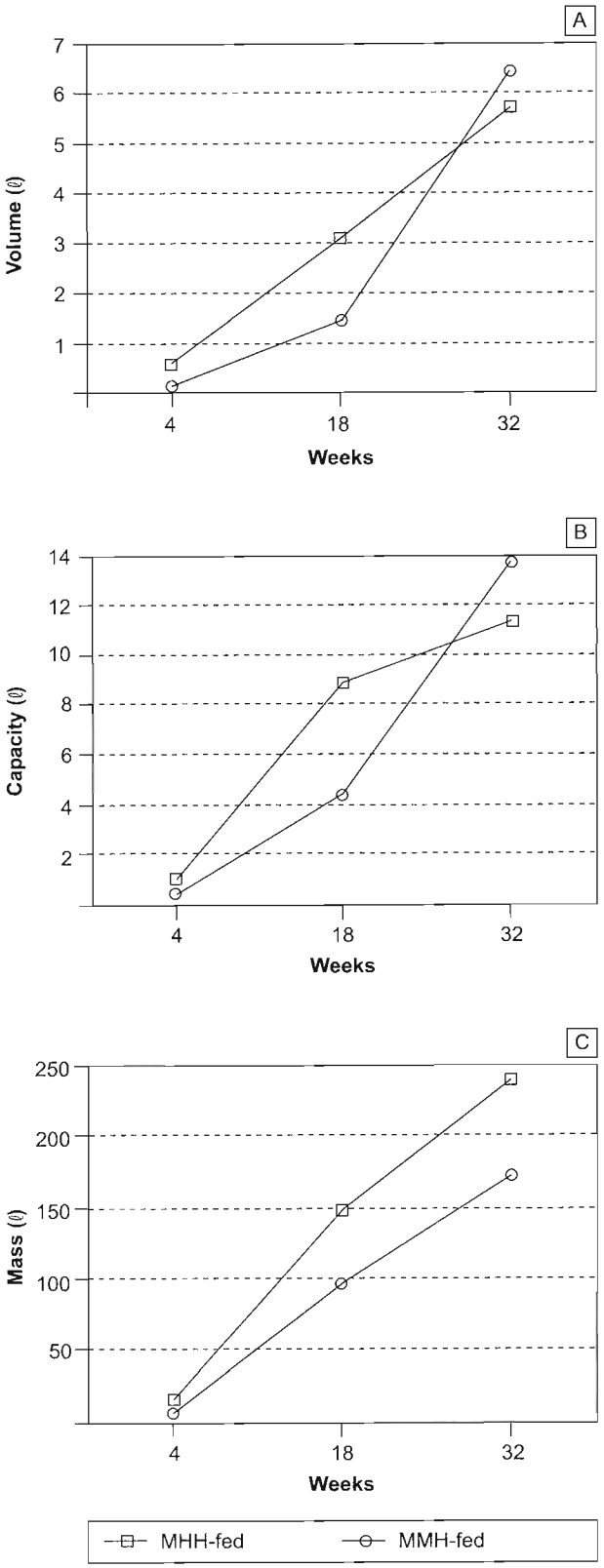

FIG. 1 Changes in the mean (n = 2) volume of contents (A), capacity (B) and wall mass (C) of the rumino-reticulum of MMH- and MHH-fed lambs at 3–5 (4) weeks, 17–19 (18) weeks and 31–33 (32) weeks of age

Orskow & Ryle 1990). Except for one lamb at 3–5 weeks of age, the rumen pH of the rumino-reticulum content was between 6,6–7,1 in all lambs and increased slightly in the lambs 31–33 weeks of age

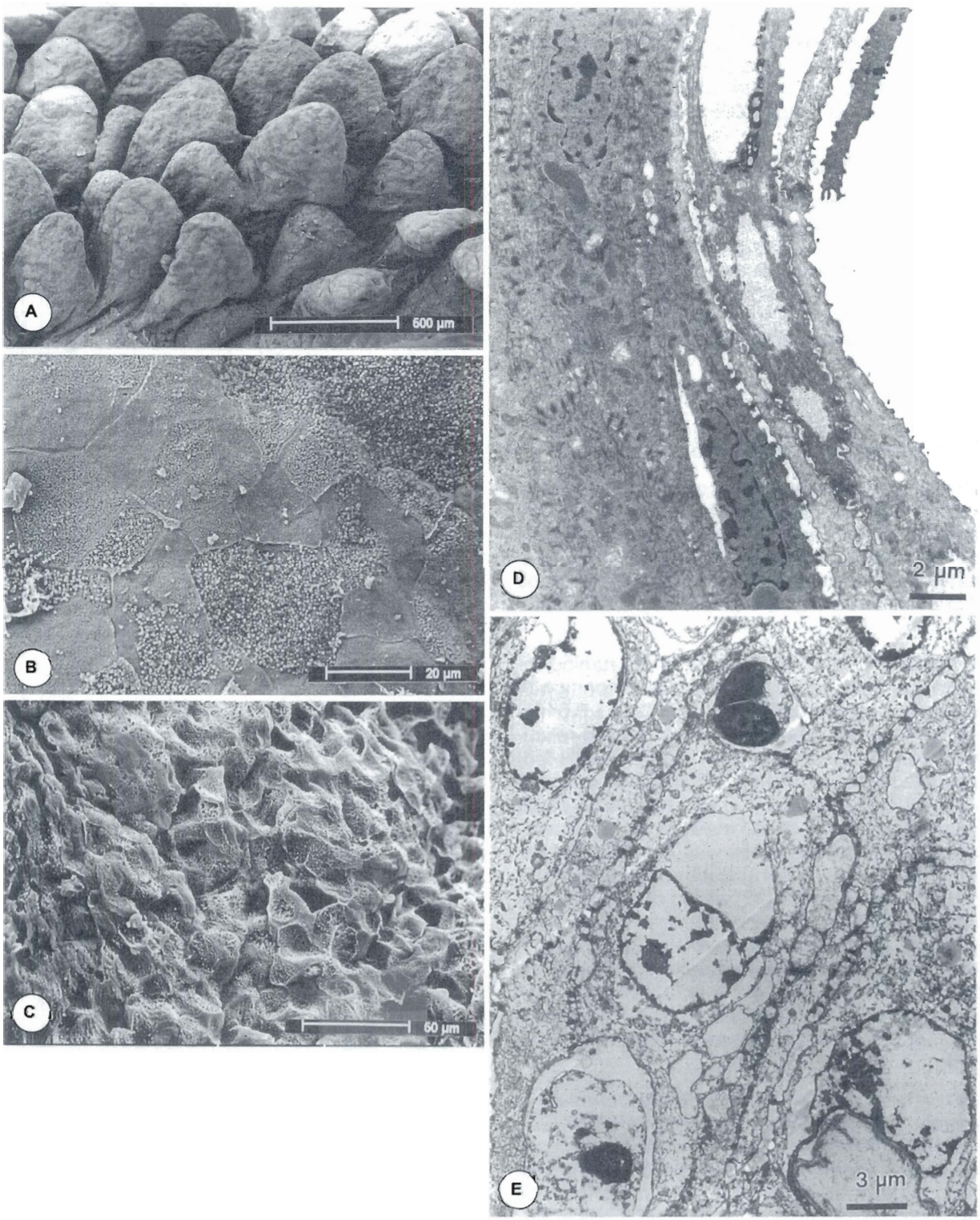

FIG. 2 Ultrastructural of ruminal wall collected from indicator MMH- and MHH-fed lambs at 3–5 weeks of age (A, B, C, D, E)

(7,05–7,1), while that of the 3–5 week-old lamb with the milk-filled rumen was 4,3. Rumen proteolytic activity, as measured by methylene reduction, was greater in the older lambs.

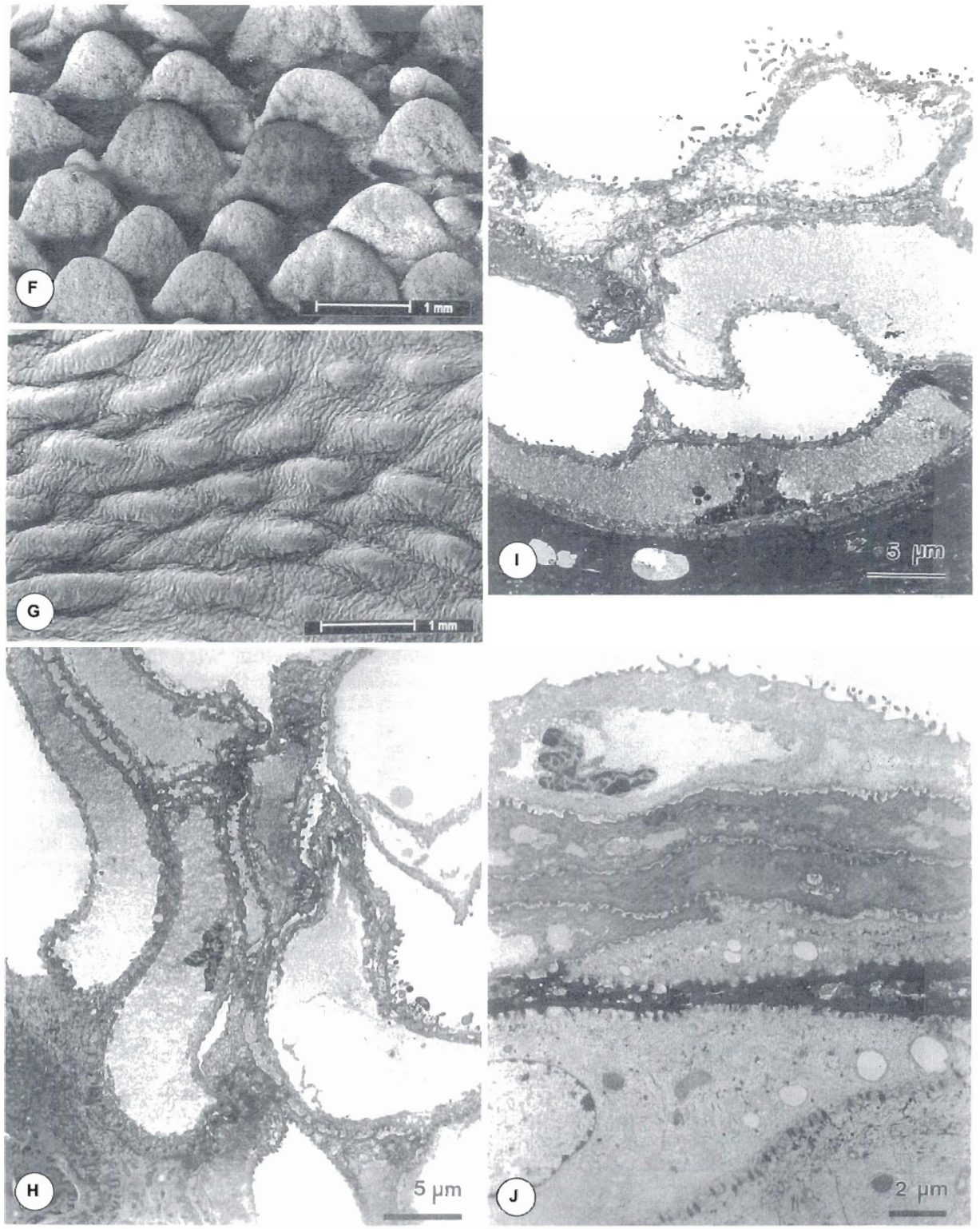

FIG. 3 Ultrastructural of ruminal wall collected from indicator MMH- and MHH-fed lambs at 17–19 weeks of age (F, G, H, I, J)

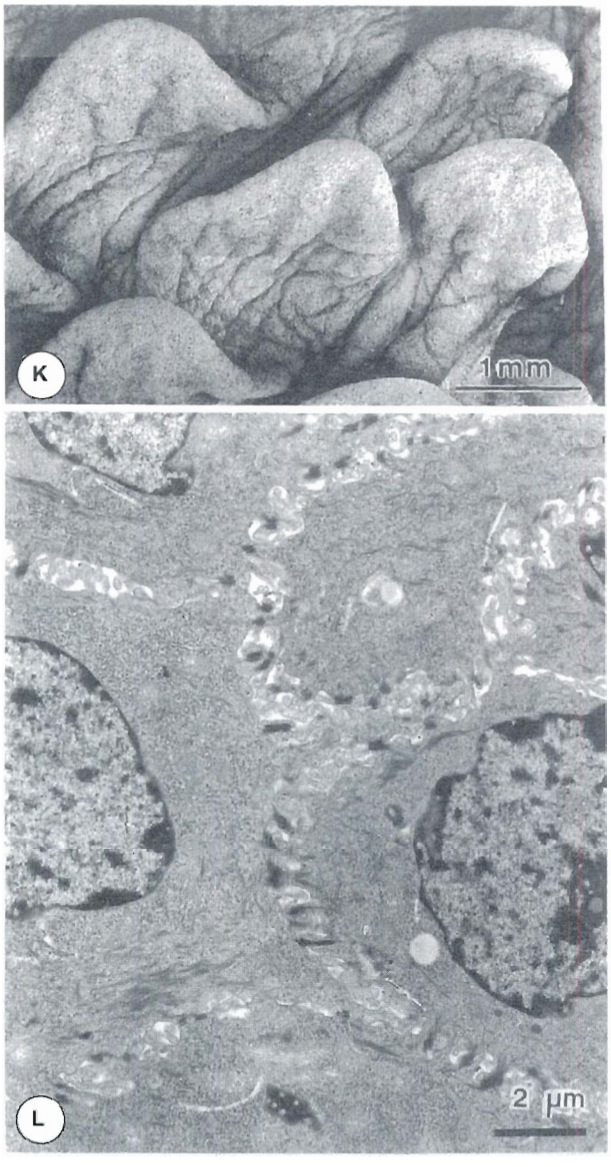

FIG. 4 Ultrastructural of ruminal wall collected from indicator MMH- and MHH-fed lambs at 31–33 weeks of age (K, L)

Ultrastructure of the ruminal wall

Scanning electron microscopy revealed that the rumen papillae in lambs slaughtered at 3–5 weeks of age were relatively well developed (Fig. 2A). There was little or no difference between those of the dorsal and ventral rumen. Some sloughing of surface cells was evident in all lambs (Fig. 2B). Severe sloughing of surface cells was evident in one lamb and the surface of the papillae had an eroded appearance (Fig. 2C). Limited sloughing was evident in the other three lambs by TEM (Fig. 2D). The stratum corneum of the rumen (dorsal and ventral) consisted of dark, electron dense cells. No organelles were present in the superficial layer (Fig. 2D). However, a few mitochondria were observed in the deeper layers of the stratum corneum. Extensive sloughing of the superficial layers of the stratum corneum was evident in the above-mentioned lamb. The cells of the stratum corneum were swollen and showed many vacuoles (Fig. 2E). No organelles were found, but most of the cells contained degenerating nuclei.

At 17–19 weeks of age the rumen papillae of lambs that were MHH-fed were well developed (Fig. 3F). Lambs which had been milk-fed (MMH-fed) from birth showed poorly developed papillae (Fig. 3G). Sloughing of surface cells was evident in all four lambs. The superficial cells of the stratum corneum of the MHH lambs were non-nucleated and swollen. The cells were light-coloured and contained flocculent material, but no organelles (Fig. 3H). Some cells appeared empty with coarse material arranged along the cell borders. Micro-organisms were apparent along the cell borders (Fig. 3I). The surface of cells of the MMH lambs were dark-coloured with no nuclei or organelles present. A limited number of cells contained remnants of nuclear material (Fig. 3J). The appearance of the cells was much the same as that of the lambs at 3–5 weeks of age.

The rumen papillae of all four lambs slaughtered at the end of Phase III from both the MHH and MMH fed groups were well-developed in comparison to those of the other age-groups (Fig. 4K). The papillae in the ventral rumen were further developed than those in the dorsal rumen. The papillae of one lamb from the MMH-fed group were less developed in comparison to the other three lambs. The papillae in one lamb each from the MMH-fed and MHH-fed groups were the best developed. Sloughing of surface cells was evident. The appearance of the cells of the stratum corneum in all four lambs was identical to that in MHH-fed lambs slaughtered at 17–19 weeks of age. In the lamb in which the rumen papillae were less developed, a number of the surface cells appeared large and swollen and had empty interiors.

Cells of the stratum basale and stratum spinosum in all lambs displayed the typical features described for sheep (Hyden & Sperber 1965; Lavker *et al.* 1969) (Fig. 4L).The basal cells were cuboidal and contained a large, round nucleus, numerous mitochondria, poliribosomes, a rough endoplasmic reticulum and a Golgi apparatus. The apical and lateral cell membranes were wavy as result of cytoplasmic processes and contained intermittent desmosomes. Cells of the stratum spinosum were polygonal in shape with many finger-like processes. Desmosomes were evident where the processes of adjacent cells made contact. Mitochondria, polyribosomes, a rough endoplasmic reticulum and a Golgi apparatus were present in the cells.

DISCUSSION

The development in the size and function of the rumino-reticulum observed in the lambs of various ages in this study is consistent with that previously reported (Church 1988). At 3–5 weeks of age, the lambs were typically in the non-ruminant phase, although some of the older lambs in the group may have already reached the transitional phase. The development of the rumino-reticulum in lambs maintained on a full-milk replacement diet until 17–19 weeks of age was markedly inhibited and remained in the transitional stage in contrast to that of the lambs of the same age fed hay from 5–6 weeks of age. The latter had achieved the morphological features of the adult phase. A similar period of delay in the development of the forestomach in calves fed exclusively on milk has been described (Church 1988).

The morphology of the papillae in the reticulum and rumen as described by Scott & Gardner (1973) were confirmed in this study. The current study clearly indicated an increase in the size of the papillae in lambs fed with hay, whereas little growth of papillae were observed in those fed milk. Similar observations were made by McGavin & Morrill (1976) and Church (1979) in calves. The results of this study on the ultrastructure of the rumen are similar to those described by Hyden & Sperber (1965) and Lavker *et al.* (1969). One of the lambs slaughtered at 3–5 weeks of age had a pot-bellied appearance. Its rumen was enlarged when compared to those of the other lambs, and was filled with milk. Electron microscopy showed severe sloughing of the surface cells of the stratum corneum. Transmission electron microscopy also revealed swollen and vacuolated cells in the stratum corneum. These findings were not observed in the other lambs, but were similar to those described by Groenewald & Booth (1992) and Groenewald (1992, 1993) in grey Karakul lambs carrying a lethal factor in which there was also a decrease in the number of myenteric neurons and ganglia. This led to defective oesophageal groove function and fluid-filled rumens. The same defect could have been responsible for the observations made in this specific lamb.

The confirmation of inhibition of the morphological and ultrastructural features in the milk-fed lambs at 17–19 weeks of age in this study allowed a comparison to be made of the pharmacokinetics of rafoxanide in poorly developed rumino-reticulums of older lambs relative to that in lambs at the same age in which the rumino-reticulum development had achieved adult proportions. Rafoxanide had a significantly more rapid distribution and shorter elimination half-life in hay-fed compared to milk-fed lambs at 17–19 weeks of age (Swan 1997). The differences in the pharmacokinetics observed were mainly the result of an overestimation of dose rate of rafoxanide administered to the hay-fed lambs, due to the larger size and volume of the rumino-reticulum, compared to those of the milk-fed lambs.

ACKNOWLEDGEMENTS

The authors thank Prof. C.J. Botha of the Department of Pharmacology and Toxicology for assisting in the collection of samples, Drr. P. Thompson and A. Shakespear of the Department of Medicine for the evaluation of ruminal microbial activity, Mr H.J. Els of Electron Microscope Unit for the transmission and scanning electron microscopy preparations, and the Faculty of Veterinary Science, University of Pretoria for supplying most of the funds which enabled this research project to be undertaken.

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