Histomorphometric analysis of the rumen of sheep during development

A. Franco, DVM, PhD; S. Regodón, DVM, PhD; A. Robina, DVM, PhD; E. Redondo, DVM, PhD

SUMMARY

Histomorphometric and scanning electron microscopic analyses were carried out on 74 embryos and fetuses and 20 sheep (early postnatal to adult age). Histodifferentiation of the rumen took place at 33 days of fetal life. Ruminal pillars were observed at 42 days, and at 61 days, ruminal papillae appeared as evaginations of the epithelial stratum basale. Neutral mucopolysaccharides first appeared in epithelial cells at 46 days of fetal life; thereafter, numbers decreased gradually and subsequently stabilized in postnatal life. Acid mucopolysaccharides, mucins, and mucoid compounds were not detected. Age and diet were recognized as factors that determine the structure of the ruminal mucosa. Growth curves and formulas were set out for each tissue layer.

The notable ability of ruminants to convert fibrous foods into products of great nutritive value has focused scientific interest on the structure and function of the digestive tract in these animals. A great deal of research has been carried out concerning the histologic structure of the rumen during postnatal development,[1-4] though few studies deal specifically with prenatal development of the compartmentalized rumen.[5-7]

The purposes of the study reported here were to trace the morphologic development of the rumen from the early stages of fetal life until adult age and to assess the histochemical behavior of the epithelium, with particular reference to secretion of nitrogenated polysaccharides. Comparative studies were made of the ruminal mucosa at birth and at adult age.

Materials and Methods

Sheep—Embryos and fetuses (n = 74) and sheep from early postnatal to adult age (n = 20) were studied. Specimens from these sheep were arranged in 9 age groups, with reference to the most relevant histomorphogenic characteristics (Table 1). To obtain embryos and fetuses at various stages of development, cesarian section was performed after synchronization of estrus, using hormal techniques, followed by mating and uterine flushing.

Table 1—Experimental design—detail of age, crown-to-rump length (C-R), and number of specimens per group from which the study of rumen was completed

Group	Age (d)	C-R Length (cm)	No. of specimens
1	23 to 29	0.4 to 1.9	9
2	30 to 32	2.0 to 2.5	6
3	33 to 38	2.6 to 3.6	7
4	39 to 52	4.0 to 8.0	11
5	53 to 79	8.5 to 19.0	13
6	81 to 112	20.0 to 31.5	10
7	113 to 118	32.0 to 36.0	8
8	120 to 150	37.0 to 40.0	10
9	Postnatal life	2 d to 2 yr	20

Specimen acquisition—Square specimens measuring 1.5 × 0.5 cm were taken from the medial region of the dorsal sac and from the cranial sac of the rumen of each sheep. Tissues for histologic study were processed by usual paraffin-embedding methods, and sections 5 μm thick were cut and treated with H&E, Masson's trichrome, and van Gieson stains for morphologic studies. Samples also were stained with periodic acid-Schiff (PAS; pH 7.2) and PAS-alcian blue (pH 4.2) for specific differentiation of neutral and acid mucopolysaccharides. Specific staining for mucoproteins and glycoproteins was performed, using Mayer's mucicarmine.

Morphometric analysis—Specimens for morphometric analysis were embedded in paraffin, stained with H&E, and viewed through a microscope[a] equipped with a videocamera.[b] The image was reflected onto the screen of a semiautomatic image analyzer.[c] Variables studied were height of various tissue strata (epithelium, lamina propria and submucosa, tunica muscularis, and serosa) and total wall thickness. Eight specimens were selected from each group, and 30 measurements were made for each tissue stratum.

Using the same screen image, a reference area, 10 × 10 μm, was marked off; the portion of this area occupied by a positive histochemical reaction was measured. One hundred such measurements were made for each group studied.

Tissue growth models were created, using a personal computer and a statistics program.[d] Initial tests involved multiplicative ($y = a \times b$) and exponential [$y = \text{EXP}(a + bx)$] models. If the model obtained failed to yield an acceptable adjustment [coefficient of correlation (r^2) < 0.70], adjustment was performed by segment. A polynomial model ($y = a + bx + c^2 + dx^3 + ex^4$) was selected in cases where the process dynamics yielded a sigmoid

Received for publication May 10, 1991.

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Supported in part by DGICYT PB88-0429.

The authors thank Pilar Parra and Germán Fernández for technical assistance.

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[a] Optiphot, Nikon Inc, Tokyo, Japan.
[b] Kestrel 25 Videocamera, Rego & Cia, Madrid, Spain.
[c] Olivetti M-24, Rego & Cia, Madrid, Spain.
[d] Statgraphics V 2.1 (1986).

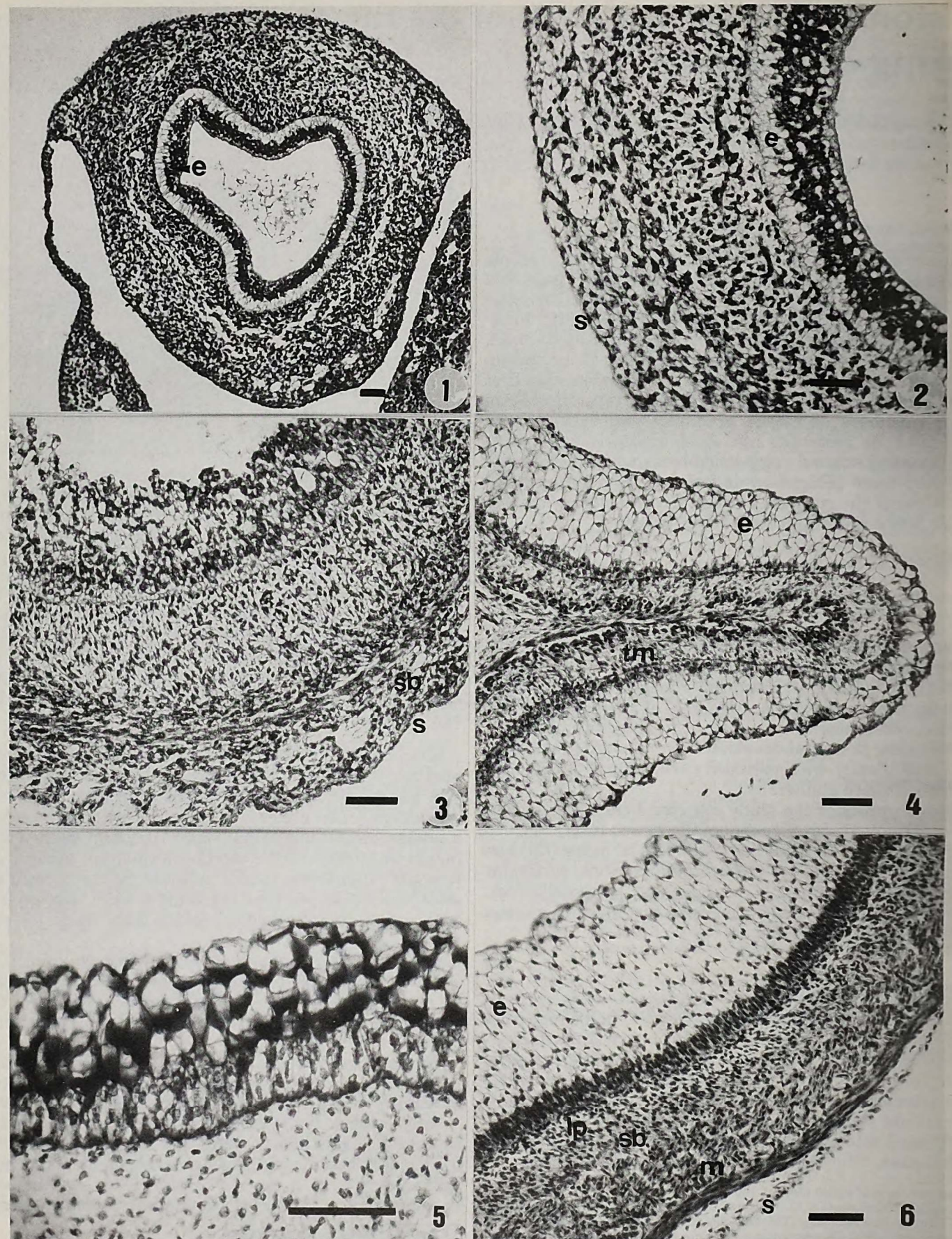

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Figure 1—Photomicrograph of a section of the *undifferentiated* rumen at 26 days of gestation (e = epithelium). H&E stain; bar = 100 μm.
Figure 2—Photomicrograph of a section of the ruminal wall at 30 days of gestation (s = serosa; e = epithelium). H&E stain; bar = 100 μm.
Figure 3—Photomicrograph of a section of the ruminal wall at 33 days of gestation (s = serosa; sb = submucosa). H&E stain; bar = 100 μm. *See* Figure 2 for key.
Figure 4—Photomicrograph of a section of a ruminal pillar at 46 days of gestation (e = epithelium; tm = tunica muscularis). H&E stain; bar = 100 μm.
Figure 5—Photomicrograph showing neutral mucopolysaccharides in epithelial cell cytoplasm at 46 days of gestation. Periodic acid-Schiff (PAS) stain; bar = 100 μm.
Figure 6—Photomicrograph of a section of the ruminal wall at 53 days of gestation. Five distinct layers—epithelium (e), lamina propria (lp), submucosa (sb), tunica muscularis (m), and serosa (s)— are visible. H&E stain; bar = 100 μm.

pattern. In all cases, embryo body length (crown to rump; C-R cm) was used as the independent variable; the thickness (in micrometers) of each tissue stratum served as the dependent variable.

Scanning electron microscopy (EM)—For scanning EM, specimens were taken from ten 2-day-old lambs and 10 adult sheep (up to 2 years old) to assess the influence of milk and fibrous diets on structural modifications of the rumen. Specimens were fixed in 2.5% glutaraldehyde, dehydrated through graded ethanols and amyl acetate, and dried in a critical-point dryer. Sections were attached to metal stubs with carbon and gold and were examined at various tilt angles and at magnification of 10 to 800×.

Results

Ruminal Histomorphogenesis

Days 23 to 29 of gestation (0.4 to 1.9 cm C-R)—Early in gestation, the rumen appeared as a fusiform tube enclosing a single cavity with a thin, though irregular, wall (mean ± SEM, 229 ± 13 μm). It consisted of distinct layers (Fig 1), one internal (epithelium) and the other external (pluripotential blastemic tissue).

The epithelium (54.1 ± 8 μm) was pseudostratified and was without secretory capacity. It was formed by cylindrical cells, the spherical nuclei of which were located in the middle and apical thirds of the epithelium, leaving a peripheral band of light cytoplasm.

The subepithelial and mesenchymal pluripotential blastemic tissue covered most of the wall. The tissue had thickness of 175.9 ± 28 μm and was formed by a blastema rich in undifferentiated stellate cells grouped in several irregularly distributed layers.

Days 30 to 32 (2 to 2.5 cm C-R)—The rumen still was a single cavity, but now had signs of the internal dilatation corresponding to the various compartments. The wall was composed of 3 layers (Fig 2).

The first layer was internal stratified epithelium (62.1 ± 8.1 μm) formed by 2 bands, a larger basal band (light and anuclear) and a smaller apical band (dark and rich in nuclei). The next layer was middle pluripotential blastemic tissue (190.3 ± 33.5 μm). A cluster of longitudinally arranged fusiform cells was detected in the middle zone. These cells were to form the tunica muscularis after transformation of mesenchymal cells into myoblasts. The final layer was the external serosa, formed by a single layer of flat cells (mesothelium), and a subserosa composed of mesenchymatous tissue rich in cells.

Days 33 to 38 (2.6 to 3.6 cm C-R)—Histodifferentiation of the rumen took place at 33 days (Fig 3). The wall, consisting of the light-staining peripheral band of the epithelium that is characteristic of previous stages of development was considerably smaller, even disappearing in certain zones, to be replaced by a dark-staining membrane as a result of a substantial degree of nuclear condensation. Increase in thickness, attributable to an increase in the number of cell elements (98.1 ± 18.4 μm) was noticeable.

Pluripotential blastemic tissue was highly vascularized. Signs of cell differentiation started to become evident. Two layers of myoblasts were observed within the serosa. These were arranged along the lumen in a circular formation. The serosa (117.7 ± 17.8 μm), composed of a highly cellular subserosa, was lined externally by a simple flat epithelium (Fig 3).

Days 39 to 52 (4 to 8 cm C-R)—At 42 days of development, the outline of the pillars, which later provide the internal division of the rumen into sacs, was evident in the form of papilliform projections toward the lumen (Fig 4). All tissue strata were involved in pillar formation.

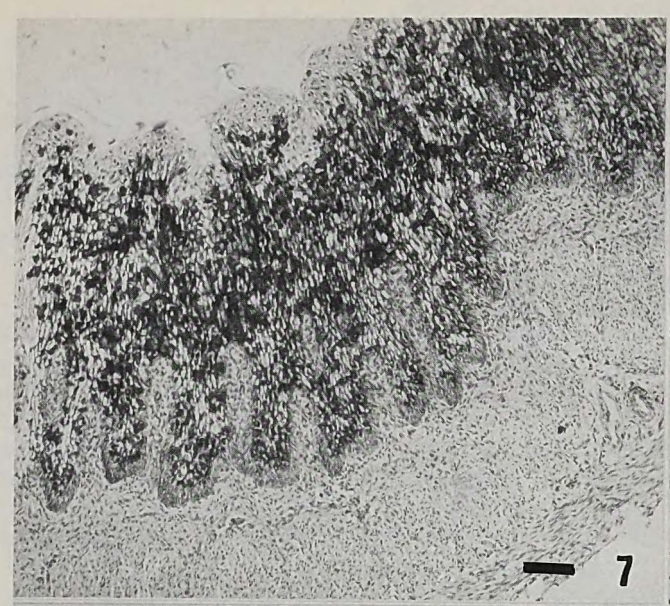
The epithelium (Fig 4, 114.7 ± 19.5 μm) represented the principal element in the formation of incipient ruminal pillars, growing actively toward the compartmental lumen and pulling with it some of the parietal structures. A basal layer of dark cells and a zone containing cells with light cytoplasm and pyknotic nuclei, the mosaiclike arrangement of which indicated incipient stratification, were clearly evident.

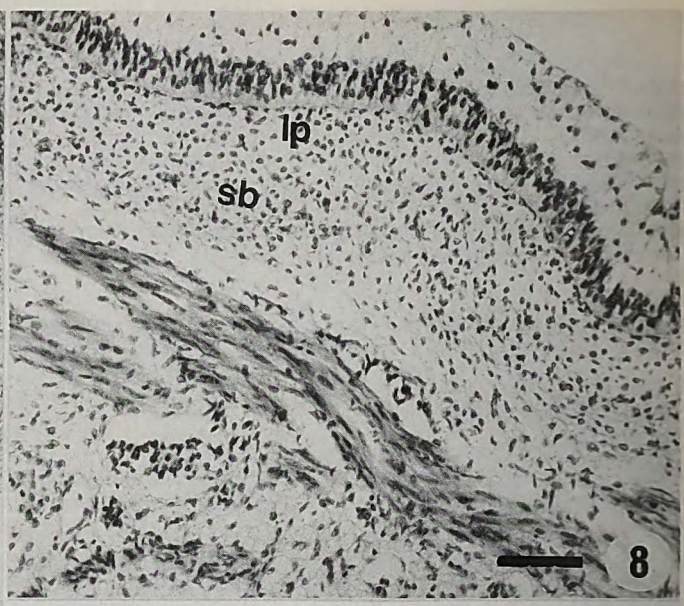
At 46 days of gestation, neutral mucopolysaccharides were observed for the first time in epithelial cell cytoplasm (Fig 5). Evidence was not detected, however, of acid mucosubstances, mucins, or mucoid compounds. Numerous capillaries were observed in subepithelial blastemic tissue, indicative of the imminent differentiation of the lamina propria and submucosa (Fig 4).

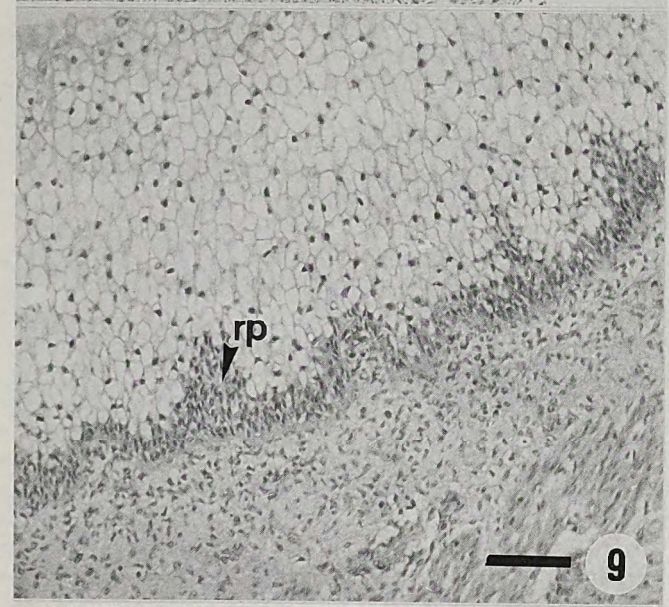
The tunica muscularis of early gestation had slight swelling as it entered the inner area of the pillar parenchyma (341.9 ± 2.5 μm) to participate in the formation and growth of the pillars (Fig 4). The highly vascularized serosa (44.6 ± 7.7 μm) protruded into the body of the pillar, although to a lesser degree than that for the other parietal strata.

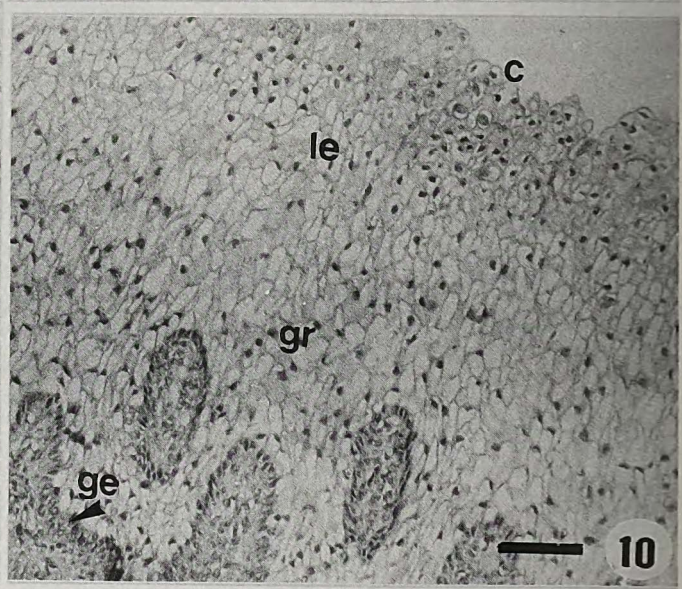
Days 53 to 79 (8.5 to 19 cm C-R)—At 53 days of development, the ruminal wall was formed by distinct layers (Fig 6): epithelium, lamina propria, submucosa, tunica muscularis, and serosa.

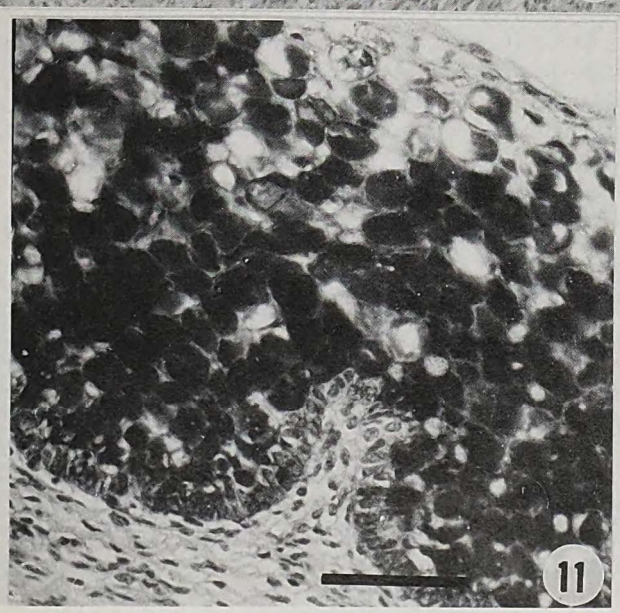
The mucosa was smooth, with a stratified epithelium that continued to swell (121.3 ± 10.5 μm) owing more to cell elongation than to increase in cell numbers. The ep-











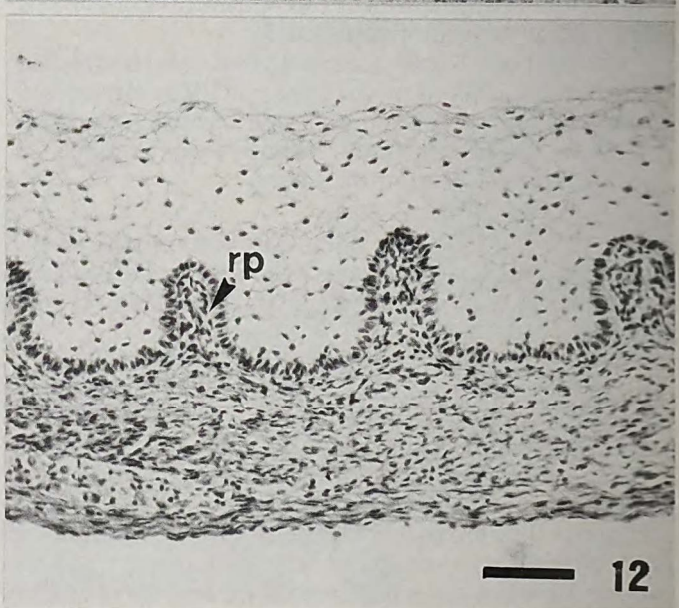


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Figure 7—Photomicrograph of a section of the ruminal wall at 57 days of gestation. Alternation of PAS-positive and PAS-negative cells in the apical two-thirds of the epithelium. PAS stain; bar = 100 μm.

Figure 8—Photomicrograph of a section of the ruminal wall at 57 days of gestation. Notice incipient differentiation of lamina propria and submucosa. Tunica muscularis is formed by 2 bundles of smooth muscle fiber. Van Gieson's (VG) stain; bar = 100 μm. *See* Figure 6 for key.

Figure 9—Photomicrograph of a section of the ruminal wall at 61 days of gestation. Evaginations of the epithelial stratum basale are forming rudimentary ruminal papillae (rp). Masson's trichrome (MT) stain; bar = 100 μm.

Figure 10—Photomicrograph of a section of the ruminal wall at 86 days of gestation. Notice stratification of epithelium (ge = stratum germinativum; gr = stratum granulosum; le = stratum lucidum-spinosum; c = stratum corneum). MT stain; bar = 100 μm.

Figure 11—Photomicrograph of a section of the ruminal wall at 86 days of gestation. Intense, uniformly-distributed PAS-positive reaction is evident throughout the epithelium except in the stratum corneum. PAS stain; bar = 100 μm.

Figure 12—Photomicrograph of a section of the ruminal wall at 81 days of gestation. Incipient ruminal papillae (rp) contain visible lamina propria. VG stain; bar = 100 μm.

ithelium had 2 clearly differentiated zones: a thin, dark-staining stratum basale and a broad, light-staining external zone corresponding to the most internal portion of the gastric wall. The uppermost zone of the epithelium (stratum corneum) was in contact with the lumen, and was formed by a single layer of flat anuclear cells. The stratum granulosum and the indistinct lucidum-spinosum layer lay between the germinativum and the corneum layers. In the apical two thirds of these intercalated strata, positive cells alternated with cells negative for neutral mucosubstances (Fig 7).

At 57 days, the pluripotential blastemic tissue developed into 2 distinct zones: the lamina propria and the submucosa (Fig 8). The lamina propria was composed of connective tissue rich in stellate cells. The submucosa, adjacent to the tunica muscularis, contained fewer cell elements and a larger amount of ground substance. These 2 zones together had a thickness of roughly 96.6 ± 12.8 μm.

The tunica muscularis increased in thickness (70.5 ± 7.2 μm) and, in its definitive form, was composed of 2 interwoven bundles of smooth muscle fiber. The internal bundle was circular and oblique, and the external bundle was long and thin, and was positioned perpendicular to the other (Fig 6).

The thin serosa (24 ± 4.4 μm) was lined by a flat epithelium (mesothelium) with an underlying connective tissue rich in fibers and ground substance. The serosa was highly vascularized, and some nerve endings were visible.

At 61 days of fetal development, a number of evaginations of the epithelial stratum basale were observed. These marked the origin of the ruminal papillae (Fig 9).

Days 81 to 112 (20 to 31.5 cm C-R)—The epithelium (Fig 10; 342 ± 49.9 μm) was composed of the following strata: basale (formed by a single layer of cells with intensely staining cytoplasm), granulosum (composed of polyhedral vesiculiform cells), lucidumspinosum (indistinct and composed of large cells), and corneum (formed by elongated anuclear cells arranged parallel to the surface). A uniformly distributed, intense PAS-positive reaction (Fig 11) was observed throughout the epithelium, except in the stratum corneum.

The ruminal papillae, of which signs had been observed in the previous phase, were now more evident (Fig 12). These appeared initially as simple evaginations of the stratum basals protruding toward the ruminal lumen and involving the basal membrane, the lamina propria, and to a lesser degree, the submucosa.

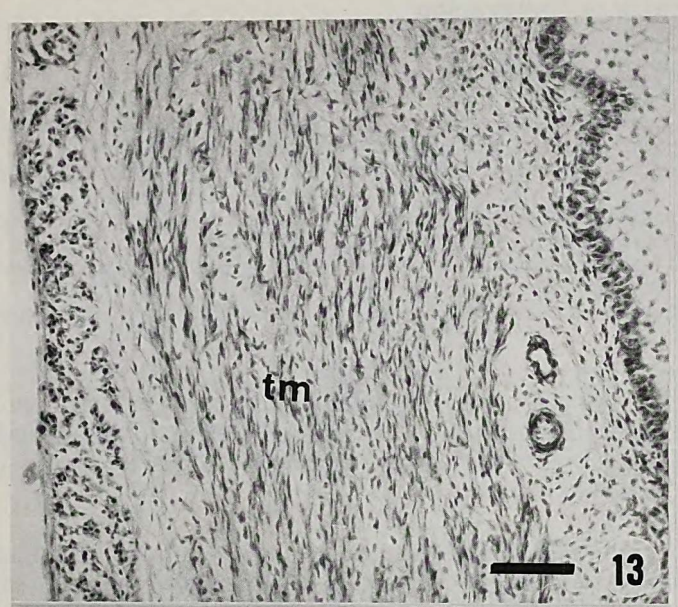
The tunica muscularis was considerably thicker (183.3 ± 9.8 μm), the increase being most pronounced in the internal bundle. Blood vessels (Fig 13) and bands of nerves were observed within intermuscular and perimuscular connective tissue.

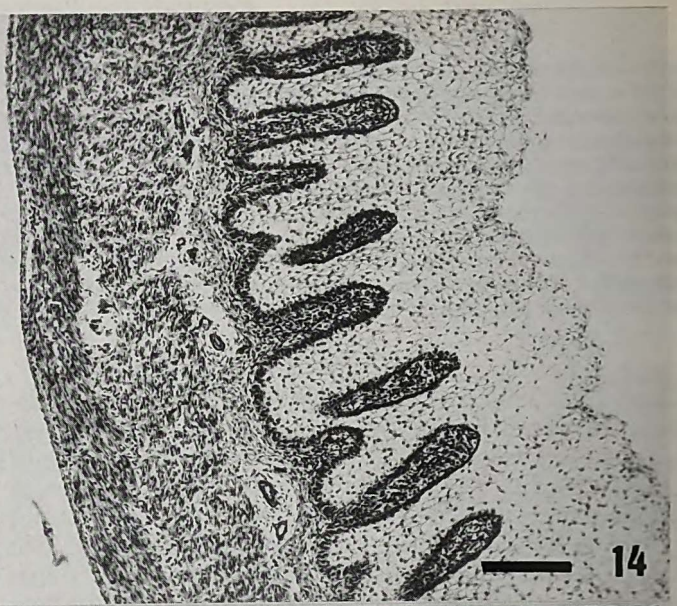
Days 113 to 118 (32 to 36 cm C-R)—Some undulation was now detected in the hitherto smooth mucosa. Small prominences were observed on the luminal surface, coinciding with the tips of the most developed papillae (Fig 14). Substantial growth of papillae within the epithelium was recorded. At 113 days, papillae had reached half the height of the epithelium, and 5 days later, they were close to the epithelial surface. The concentration of neutral mucopolysaccharides started to decrease at 120 days, leaving them scattered in the strata granulosum and lucidum-spinosum (Fig 15).

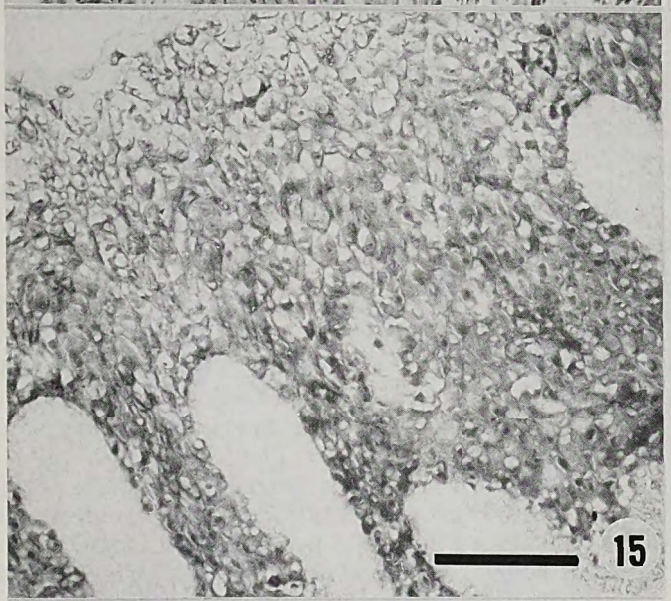
Days 120 to 150, fetuses at term (37 to 40 cm C-R)—Ruminal papillae were now fully differentiated, compared with birth (Fig 16), and were incorporated into the mucosa; incipient papillae protruded through the mucosal surface. The epithelial surface was not keratinized, and desquamation was not evident.

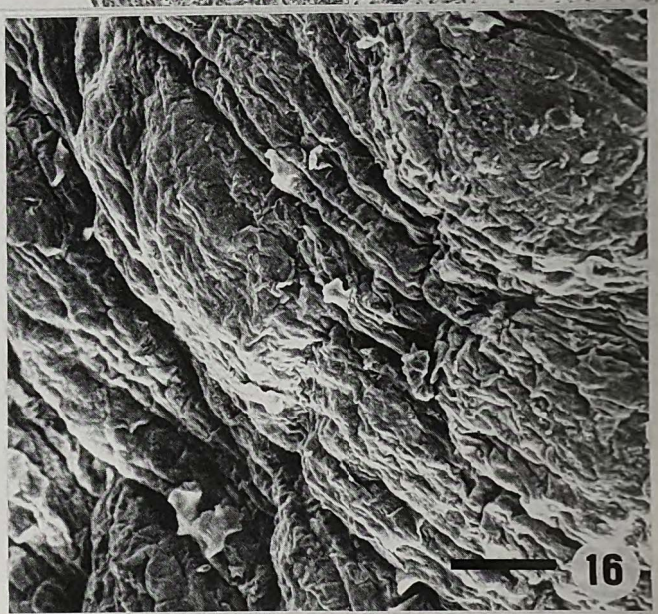
The decrease in neutral mucopolysaccharides observed in the previous phase reached its maximum at birth (Fig 17). The PAS-negative cells clearly predominated over PAS-positive cells. The latter were scattered over the apical two-thirds of the epithelium, but were lacking in the corneal and basal strata. Acid mucopolysaccharides, mucins, or mucoid compounds were not evident.

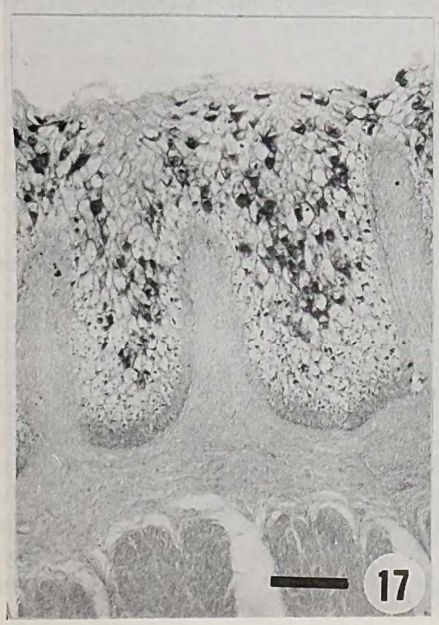
Lamina propria (Fig 18) had now entered into each pillar and was composed of a fibrous variety of molded dense connective tissue to form the papillar support. This tissue was highly vascularized. Neither glands nor mucosa muscle were observed. The submucosa (Fig 18) was composed of a loose arrangement of elastic and collagen fibers, and clear boundary between it and the lamina propria was not apparent. A large number of blood vessels and nerve bands were observed. The tunica muscularis (Fig 18) was composed of 2 layers of smooth muscle tissue. The fibers of the inner layer were arranged in a circular pattern, whereas those of the outer layer were arranged lengthwise. Both layers followed a mosaiclike arrangement, so that the broadest areas of some fibers came in contact with the narrowest areas of others. Nuclei were located in the bulk of the thickest areas. The serosa, composed of loose mesenchymal connective tissue with mesothelial lining, was thin, although variable amounts of adipocytes, blood vessels, and nerve tissue were observed at some points.











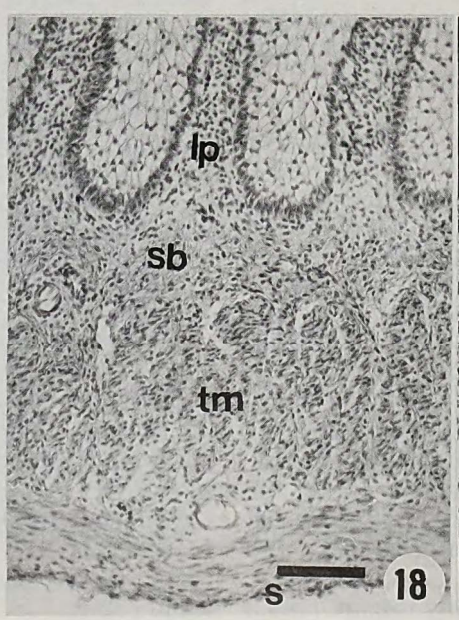


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Figure 13—Photomicrograph of a section of the tunica muscularis (tm) at 90 days of gestation. Notice differing bundle arrangement. VG stain; bar = 100 μm.
Figure 14—Photomicrograph of a section of the ruminal wall at 113 days of gestation. Prominences in epithelial surface coincide with tips of the most developed papillae. H&E stain; bar = 100 μm.
Figure 15—Photomicrograph of a section of the ruminal wall at 120 days of gestation. Notice decrease in neutral mucopolysaccharides, compared with previous groups. PAS-alcian blue stain; bar = 100 μm.
Figure 16—Scanning electron micrograph (EM) of the rumen surface at birth. Ruminal papillae appear as small undulations. Bar = 100 μm.
Figure 17—Photomicrograph of a section of the ruminal wall at birth. Notice reduced amount of neutral mucopolysaccharides in epithelium. PAS-alcian blue stain; bar = 100 μm.
Figure 18—Photomicrograph of a section of the ruminal wall at birth. MT stain; bar = 100 μm. *See* Figures 4 and 6 for key.
Figure 19—Scanning EM of ruminal papillae in adult sheep. Bar = 100 μm.

Postnatal development (2 days to 2 years)—The histologic characteristics of tissue strata had reached their definitive form in the previous phase. By scanning EM analysis of the rumen (Fig 19), however, long, foliate papillae were seen emerging on the surface. The surface itself was keratinized, with abundant cell desquamation. The scanning EM analysis was performed on only 2 groups: lambs consuming a milk diet and adult sheep consuming a fibrous diet.

Histochemical Behavior of the Epithelium

Neutral mucopolysaccharides appeared in large numbers from day 46 of fetal development. A positive reaction was noticed over 95 ± 6% of a reference surface area of 100 μm². The percentage of PAS-positive reactions remained high, although somewhat lower (73.2 ± 7%) at 57 days, reaching 64 ± 6% by day 88. This decrease continued in fetuses at 117 days of gestation (50 ± 5%), further decreasing to 16 ± 3% at birth, and stabilizing thereafter at 12 ± 6%. Acid mucopolysaccharides, mucins, and mucoid compounds were not found during development.

Histomorphometric Analysis

Each tissue stratum was fitted to mathematical growth models (Fig 20–24), using the corresponding growth equation.

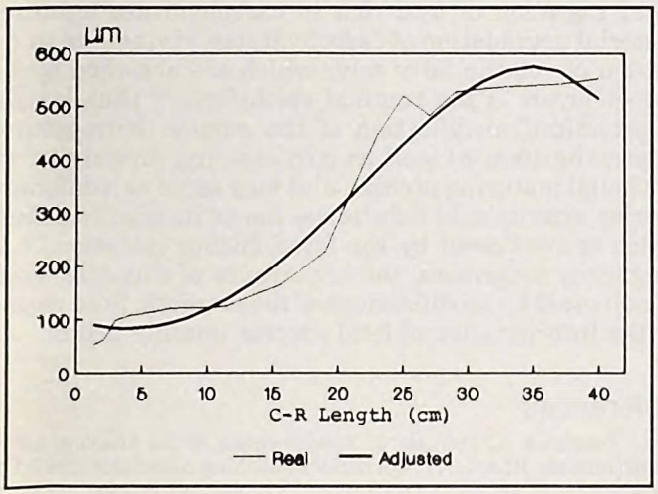

Figure 20—Mathematical model of rumen growth (epithelium). C-R = crown-to-rump length. $y = 99.77 - 8.05x + 0.94x^2 - 5.57E - 4x^4$.

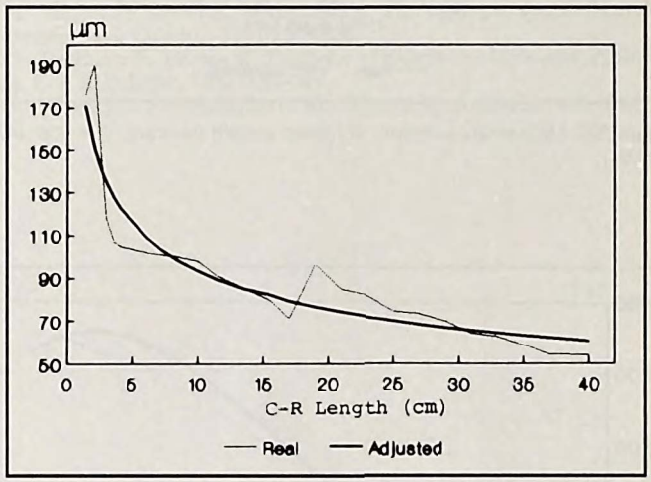

Figure 21—Mathematical model of rumen growth (lamina propria and submucosa). $y = 5.24x^{0.30}$.

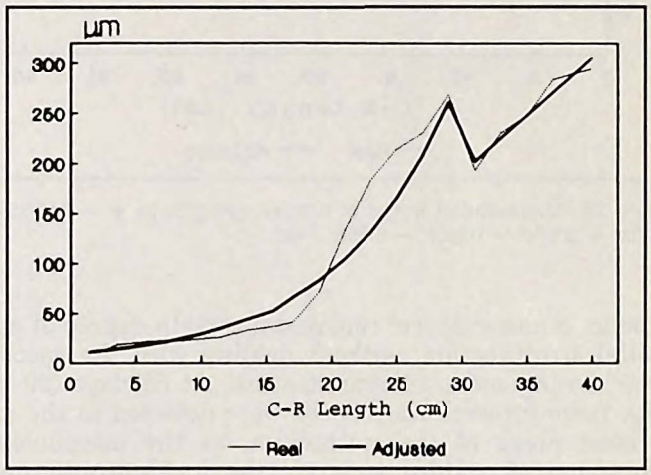

Figure 22—Mathematical model of rumen growth (tunica muscularis). Adjusted, y = EXP (2.23 + 0.11x); real y = 155.1 + 11.5x.

Discussion

At 23 days of fetal life (0.4 cm C-R), the ruminal wall was already structured in 2 layers: the epithelium and the pluripotential blastemic tissue.[8] These were primitive, indistinct structures with high blastic capacity.[5,8]

The rumen was first observed in certain samples at 33 days (2.6 cm C-R). The pseudostratified epithelium[5] lacked secretory capacity. At 53 days, uniform distribution was noticed in the stratum germinativum and stratum gran-

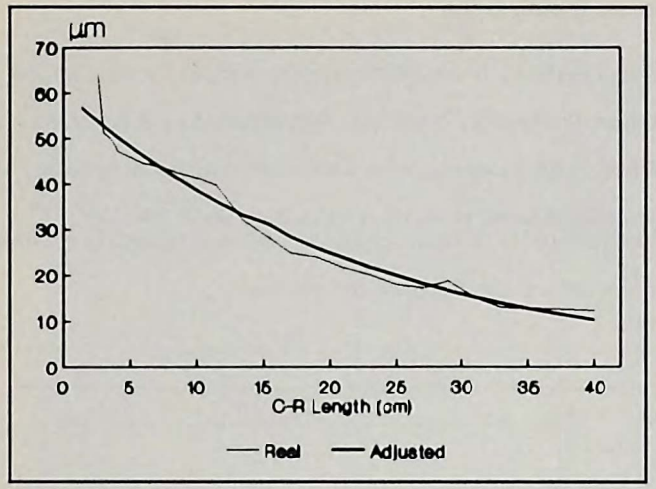

Figure 23—Mathematical model of rumen growth (serosa). y = EXP (4.09 − 0.04).

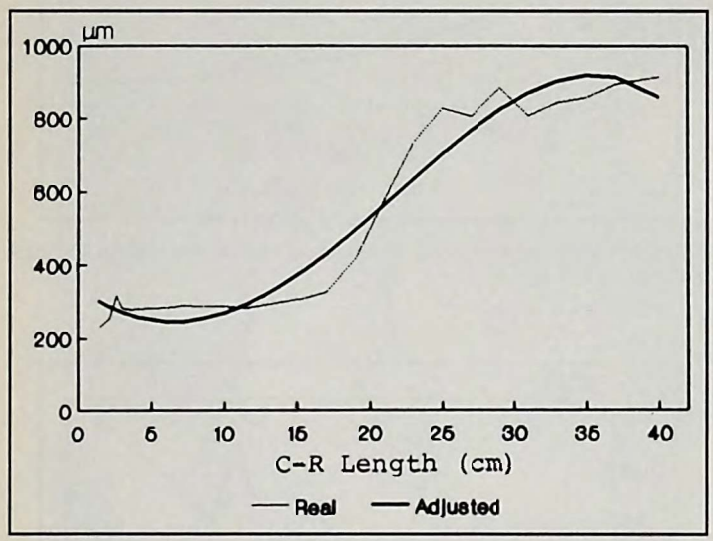

Figure 24—Mathematical model of ruminal wall growth. y = 340.35 − 30.70x + 2.61x² − 0.02x³ − 3.85E −4x⁴.

ulosum. Some authors[8] reported a certain degree of epithelial stratification, without dealing with the specific layer arrangement of each stratum. At 81 days (20 cm C-R), large intercellular spaces were detected in the uppermost areas of the epithelium, as the morphologic expression of a third stratum, the lucidum-spinosum. Opinions differ regarding the appearance of these epithelial strata. The first stratum has been reported as follows: at 10 cm C-R and at birth in sheep[9,e]; at 28.3 cm C-R in goats[10]; and at an advanced stage of development, close to birth in buffalo[11,12] and cattle.[5,8,13] Little research has been carried out on the stratum spinosum,[14] and that study reports only its appearance at an advanced stage. In our study, stratification of the epithelium was accompanied by a considerable increase in thickness and by structural modifications: the appearance of ruminal pillars and papillae. These appeared as simple evaginations of the stratum basale,[14,15] which, as gestation progressed, involved the lamina propria and submucosa. At 33 days of gestation (2.6 cm C-R), a primitive tunica muscularis was seen distinct from the pluripotential blastemic tissue from which it was derived.[8] This differentiation was most pronounced at 83 days (21 cm C-R), coinciding with the increased growth and development of the pillars.[8] For this reason, we believe that although the epithelium is certainly involved in pillar formation, the major role in formation is the development of the tunica muscularis. The first appearance of the lamina propria and submucosa, both derived from the pluripotential blastemic tissue,[10] was noticed at 57 days of gestation (8.5 cm C-R). A certain degree of continuity (in growth and in differentiation) was observed in the serosa, from the earliest stages of gestation onward.

Results of scanning EM analysis of the ruminal mucosa seemed to indicate that age and diet are determining factors in the structural development of ruminal papillae. As others[2,3,16] have observed, the transition from the milk diet of the newborn lamb to food with medium-to-high fiber concentration requires increased competence of the ruminal wall, giving rise to a twofold mechanism: strengthening of the mucosa, morphologically evident in epithelial expansion (growth recorded morphometrically); and muscle contraction as a direct result of contraction of the inner bundle. These phenomena govern the active growth of papillae that are initially rudimentary elements.

Results indicate that the epithelium lacked secretory capacity during initial embryo phases. Neutral mucopolysaccharides were first detected at 46 days, gradually decreased thereafter until birth, and subsequently became stable. We believe that the mechanical protection of the ruminal mucosa against aggressions, which may give rise to the first products of embryo metabolism, is a function located in the stratum corneum, where nitrogenated polysaccharides were not detected. We would suggest, in this respect, that neutral mucopolysaccharide content in the deeper epithelial layers is directly related to the gradual adaptation of the ruminal mucosa to its function of chemical protection in postnatal life, where it acts as a buffer in the neutralization of the acid compounds produced during ruminal fermentation.[17] Postnatal ingestion of food rich in carbohydrates enhances bacterial degradation of carbohydrates, giving rise to formation of volatile fatty acids which are absorbed by the deeper layers of the ruminal epithelium,[18] thus leading to structural modification of the rumen. Nitrogenated polysaccharides, as well as participating directly in the epithelial maturing process, also may serve as a defensive barrier against acid substances found in amniotic fluid, which is swallowed by the fetus during gestation.[18] As pregnancy progresses, the importance of this role would be enhanced by modifications of the amniotic fluid caused by the incorporation of fetal excreta (mainly urine).

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