Histomorphometric analysis of the omasum of sheep during development

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Summary

Histomorphometric and scanning electron microscopic analyses were performed on 74 embryos and fetuses and 20 sheep (early postnatal to adult age). Histologic differentiation of the omasum took place at 33 days of fetal life, with the appearance of first-order laminae. Second-, third-, and fourth-order laminae appeared at 39, 50, and 59 days, respectively. Neutral mucopolysaccharides first appeared in epithelial cells at 46 days of fetal life, decreasing quantitatively until birth, before subsequently stabilizing in postnatal life. Acid mucopolysaccharides, mucins, and mucoid compounds were not detected. Growth curves and formulas were constructed for each tissue layer. Initial tests involved multiplicative ($y = axb$), exponential ($y = EXP\ [a + bx]$), linear ($y = a + bx$), and polynomial models ($y = a + bx + cx^2 + dx^3$).

The known ability of ruminants to convert fibrous foods into usable nutrients has focused scientific interest on the structure and function of the digestive tract in these animals. Much research has been performed on the histologic structure of the omasum during postnatal development.[1–4] Few studies, however, have dealt specifically with prenatal development of the compartmentalized stomach.[5–7]

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

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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 histomorphogenetic characteristics (Table 1). To obtain embryos and fetuses at various stages of development, cesarean section was performed after synchronization of estrus, using hormonal techniques, effect of sire, and uterine flushing.

Fluorogestone acetate was administered 14 days before introduction of rams. Then 600 IU of pregnant mare serum globulin was inoculated. From day 26 on, cesarian section was performed after ewes were mated to rams. Ewes were tranquilized by IM injection of 0.5 mg of propionyl phenothiazine 100 kg of body weight; then anesthesia was induced by IV injection of thiopental sodium (4 g in a 20% aqueous solution).

Once embryos and fetuses were separated from maternal linking, they were euthanatized by umbilical vein administration of barbiturate. When fetuses were small, 2 transverse cuts, one in the caudal angle of the scapula and the other cranial to the tuber coxae, were made. A central section was selected and fixed in 10% formalin.

Specimen acquisition—Specimens, measuring 1.5 × 0.5 cm^2, were taken from the medial region of the dorsal sac and from the cranial sac of the omasum of each sheep—embryos and fetuses. Tissues for histologic study were processed by use of paraffin-embed-

Table 1—Experimental design: details of age, crown-to-rump (C-R) length, and number of specimens per group from which the omasal study 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

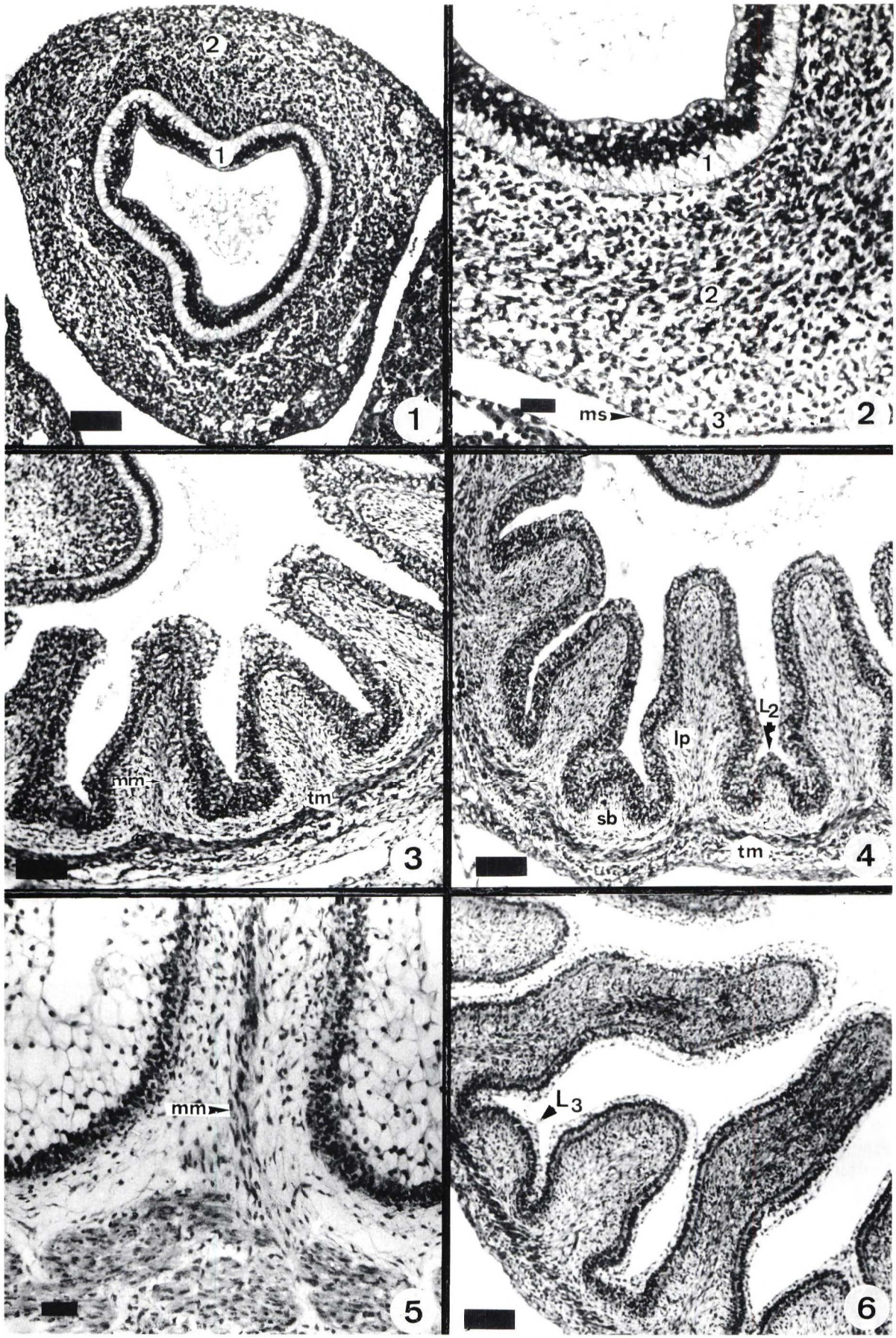

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Figure 1—Photomicrograph of a section of undifferentiated omasum at 26 days of gestational age, (1 = epithelium, 2 = pluripotential blastemic tissue). H&E stain; bar = 100 μm.
Figure 2—Photomicrograph of a section of the omasal wall of a fetus at 30 days of gestation (3 = serosa, ms = mesothelium). H&E stain; bar = 100 μm. *See* Figure 1 for key.
Figure 3—Photomicrograph of a section of the omasal wall of a fetus at 33 days of gestation, showing first-order laminae (mm = muscularis mucosae, tm = tunica muscularis). H&E stain; bar = 100 μm.
Figure 4—Photomicrograph of a section of the omasal wall of a fetus at 39 days of gestation, showing second-order laminae (L_2; lp = lamina propria, sb = submucosa, tm = tunica muscularis). H&E stain; bar = 100 μm.
Figure 5—Photomicrograph of a section of the omasal wall of a fetus at 46 days of gestation, showing muscularis mucosae (mm). Masson's trichrome (MT) stain; bar = 100 μm.
Figure 6—Photomicrograph of a section of the omasal wall of a fetus at 50 days of gestation, showing third-order laminae (L_3). H&E stain; bar = 100 μm.

ding methods.[8] Sections 5 μm thick were cut and treated with H&E, Masson's trichrome, and van Gieson's 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 by microscope[a] equipped with a video camera.[b] The image was projected on 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.

Scanning electron microscopy—Tissue specimens for scanning electron microscopy were taken from 2-day-old lambs (n = 10) and from adult sheep (up to 2 years old; n = 10) to assess the influence of milk and fibrous diets on structural modifications of the omasum. 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 magnifications of 10 to 800×.

Analysis of data—Tissue growth models were created, using a personal computer and a statistics program.[d] In each instance, the model used depended on the form of the real growth curve.[9] Initial tests involved multiplicative ($y = axb$) and exponential ($y = \text{EXP}[a + bx]$) models. A criterion for the models was correlation coefficient (r^2) > 0.70. A polynomial model ($y = a + bx + cx^2 + dx^3 + ex^4$) was selected in instances when the process dynamics yielded a sigmoid pattern. When the real model obtained was not a good adjuster, because of low r^2 (< 0.70) or because of its deviation from the real kinetics on some segments, an adjustment was performed by segments. The initial segment was adjusted as formerly explained, and the final segment was adjusted by a linear model ($y = a + bx$) or a new multiplicative or exponential equation with a different pendent. In all instances, embryo body length (crown to rump [C–R], in centimeters) was used as the independent variable; the thickness (in micrometers) of each tissue stratum served as the dependent variable.

[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, Rego & Cia, Madrid, Spain.

Results

Omasal Histomorphogenesis

Gestation days 23 to 29 (0.4 to 1.9 cm C–R)—Early in gestation, the stomach appeared as a fusiform tube enclosing a single cavity with a thin, irregular wall (mean ± SEM, 229 ± 13 μm). It consisted of 2 distinct layers (Fig 1): internal (epithelium) and external (pluripotential blastemic tissue).

The epithelium (54.1 ± 8 μm; n = 100) was pseudostratified and functionally secretory. 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 stomach wall. The tissue had thickness of 175.9 ± 28 μm (n = 100) and was formed by a blastema rich in undifferentiated stellate cells grouped in several irregularly distributed layers.

Gestation days 30 to 32 (2 to 2.5 cm C–R)—The stomach still was a single cavity, but now had signs of 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).

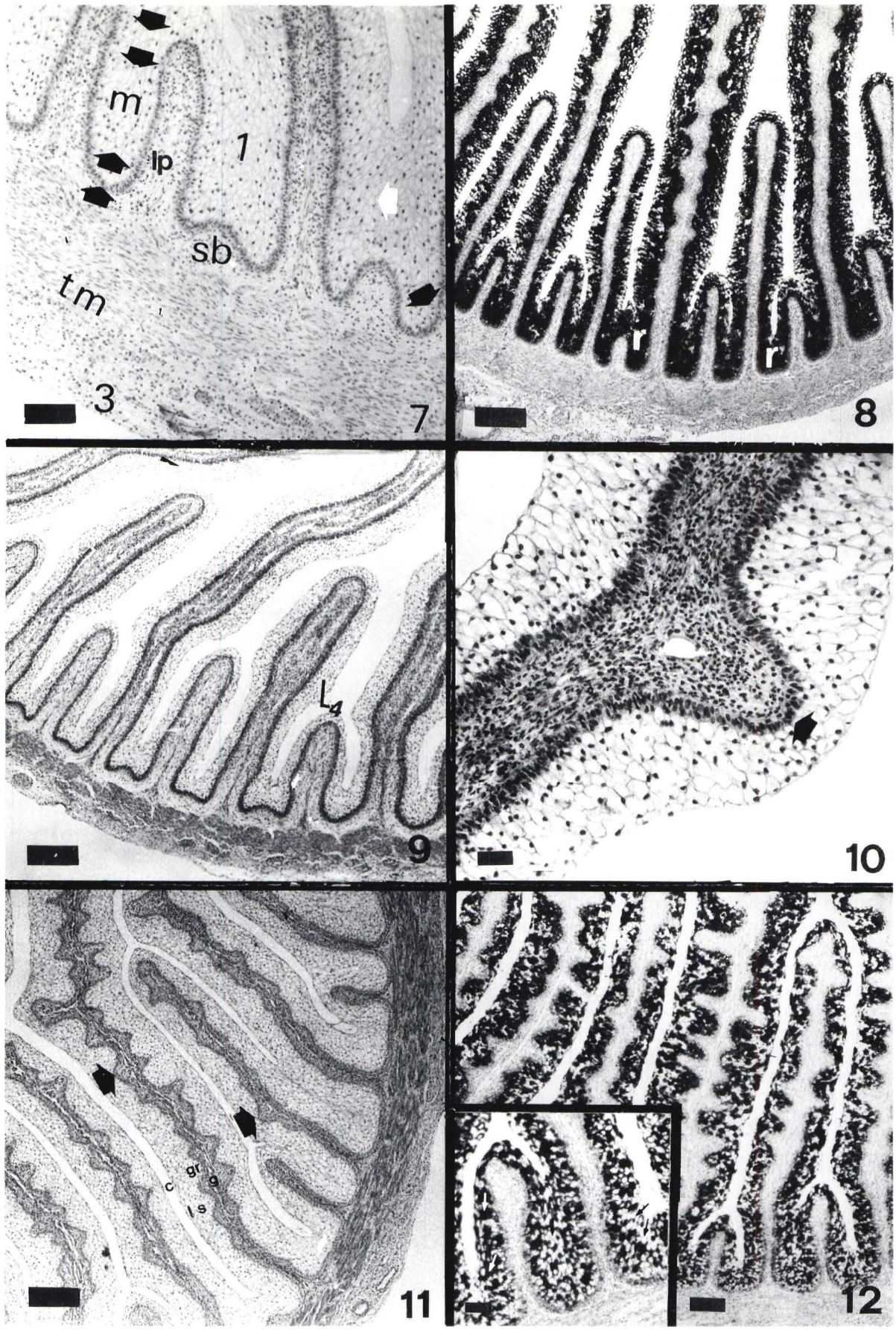

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Figure 7—Photomicrograph of a section of the omasal wall of an embryo at 53 days of gestation. Von Giessa (VG) stain; bar = 100 μm. (black arrows indicate mucosa [m]). *See* Figures 3 and 4 for key.
Figure 8—Photomicrograph of a section of the omasal wall of an embryo at 53 days of gestation. Notice epithelial periodic acid-Schiff (PAS)-positive reactions (r). PAS stain; bar = 100 μm.
Figure 9—Photomicrograph of a section of the omasal wall of an embryo at 59 days of gestation, showing fourth-order laminae (L_4) H&E stain; bar = 100 μm.
Figure 10—Photomicrograph of a section of the omasal wall of an embryo at 69 days of gestation. Papillae are visible (arrow). H&E stain; bar = 100 μm.
Figure 11—Photomicrograph of a section of the omasal wall of an embryo at 83 days of gestation. (arrows = papillae, g = stratum germinativum, gr = stratum granulosum, ls = stratum lucidum-spinosum, c = stratum corneum). H&E stain; bar = 100 μm.
Figure 12—Photomicrograph of a cross section of the omasal wall at 83 days of gestation. Bar = 100 μm. Notice PAS-positive (white arrow) and PAS-negative (black arrow) cells alternated with laminae and interlaminar spaces in inset. PAS stain; bar = 100 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 mesenchymal tissue rich in cells.

Gestation days 33 to 38 (2.6 to 3.6 cm C-R)—Histologic differentiation of the omasum took place at 33 days (Fig 3). Five or 6 undulations appeared in the previously smooth wall. These were interpreted as rudimentary or first-order laminae, all similar in height.

The wall was composed of 3 layers: epithelium, pluripotential blastemic tissue, and serosa. The stratified epithelium (45.9 ± 10.5 μm) consisted of 2 distinct zones: a dark-staining basal zone consisting of 4 to 5 layers of germinative cells with large centrally located nuclei, and an apical light-staining zone containing globular cells with small nuclei. Positive reaction to nitrogenated polysaccharides was not observed.

Pluripotential blastemic tissue, formed by mesenchymal cells and a large amount of ground substance, penetrated the epithelium, exerting pressure on the basal zone and, thus, participating in lamina formation. Within this tissue, a thin tunica muscularis could be distinguished; this was formed by 2 myoblast layers oriented circularly and longitudinally and had a series of undulations coinciding with the base of each lamina. Myoblast fibers had emigrated into the laminae from the inner bundle to form the muscularis mucosae. The serosa (77 ± 6.4 μm) was formed by a connective subserosa with an external mesothelial lining.

Gestation days 39 to 52 (4 to 8 cm C-R)—At 39 days (Fig 4), epithelial swellings in the interlaminar spaces gave rise to the appearance of second-order laminae; these were shorter than first-order laminae and were formed from pluripotential blastemic tissue, which initiated a division of lamina propria and submucosa.

Neutral mucopolysaccharides were detected in epithelial cell cytoplasm at 46 days. They were largely restricted to the middle third of the epithelium.

A thin layer of smooth muscle fibers was observed between the submucosa and the lamina propria of first-order laminae. These fibers, which formed the muscularis mucosae, originated in the inner bundle of the tunica muscularis (Fig 5).

By 50 days (Fig 6), first- and second-order laminae had grown considerably, and the initial stages of third-order laminae were visible between them. This is a sequential process, in that the growth of one order of laminae gives rise to the birth of a new order. All the structural components (mucosa, submucosa, and muscularis) are involved in this process, except the serosa, which serves as a lining.

Gestation days 53 to 79 (8.5 to 19 cm C-R)—At 53 days (Fig 7), the wall was formed by 4 distinct layers: mucosa (composed of epithelium and lamina propria), submucosa, muscularis, and serosa. The epithelium contained 2 zones: a thin, light-staining stratum basale or stratum germinativum and a broad, dark-staining external zone with numerous mitotic figures (nuclei of many germinative cells). An intense PAS-positive reaction (Fig 8) was detected in the epithelium of omasal folds with interlaminar spaces. Positive reaction was not obtained to alcian blue or Mayer's mucicarmine.

The lamina propia and the submucosa were composed of highly cellular mesenchymal connective tissue. The thickness of the tunica muscularis was increased (193.2 ± 21.5 μm), particularly in the inner bundle, from which the muscularis mucosae projected into the center of the laminae.

Fourth-order laminae appeared at 59 days (Fig 9). At 69 days, lateral connective tissue evaginations of the stratum basale of first-order laminae in the direction of the epithelial surface gave rise to the formation of rudimentary omasal papillae (Fig 10). These papillae were visible in second-order laminae by 79 days. The serosa (52.4 ± 7.6 nm) was formed by a loose subserosa lined by a flat epithelium.

Gestation days 81 to 112 (20 to 31 cm C-P)—The epithelium (199.9 ± 19.6 μm) consisted of the following strata: basale or germinativum (formed by a single layer of cells with intensely staining cytoplasm), granulosum (polyhedral vesicular cells), lu-

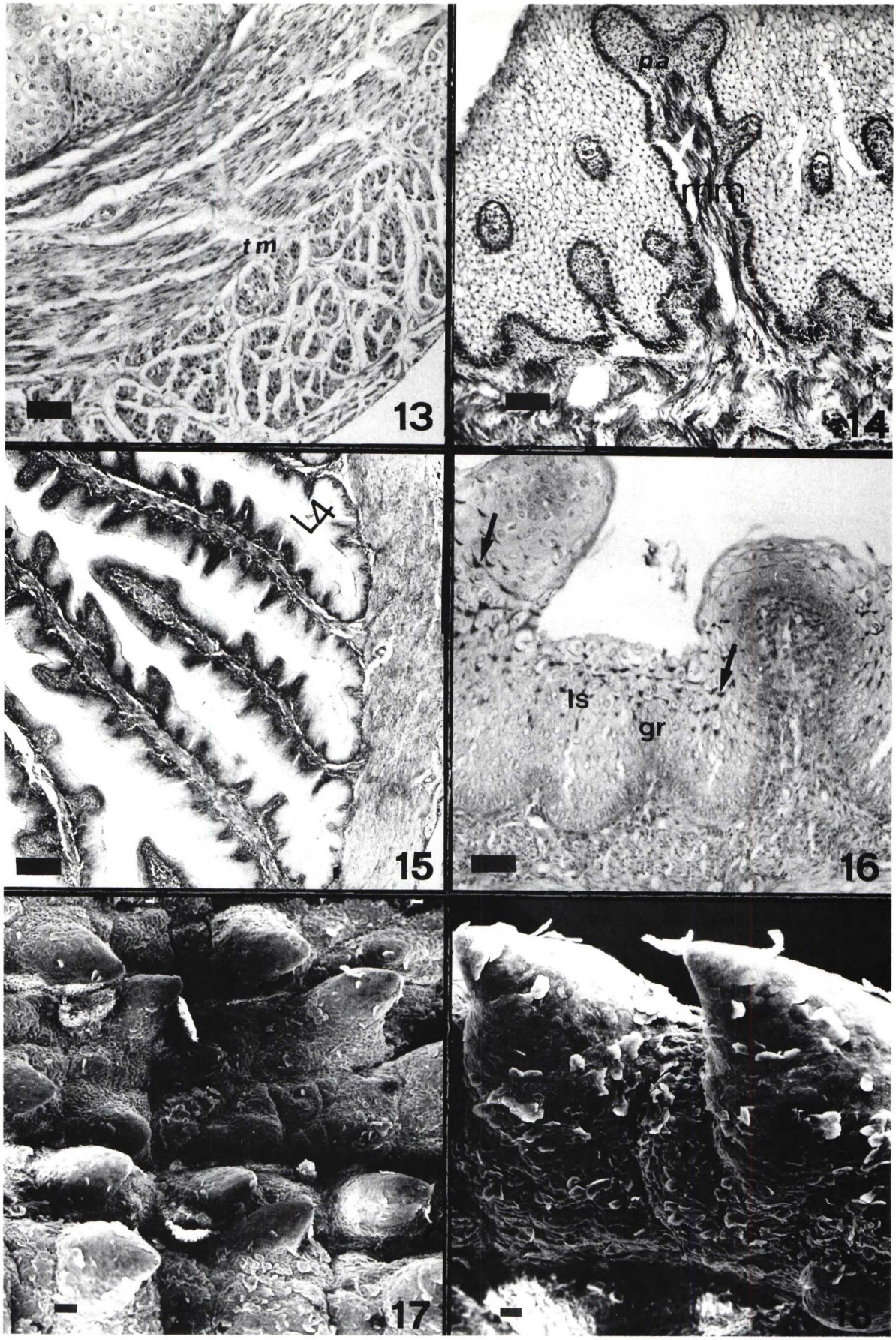

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Figure 13—Photomicrograph of a section of the omasal wall at 100 days of gestation, showing tunica muscularis (tm). Notice inner layer of circular smooth muscle and interlayer of longitudinal smooth muscle. VG stain; bar = 100 μm.
Figure 14—Photomicrograph of a section of the omasal wall at 118 days of gestation; muscle fibers (white arrow and mm) are visible within some conical papillae (pa). MT stain; bar = 100 μm.
Figure 15—Photomicrograph of a section of the omasal wall at birth (L_4 = fourth-order laminae). MT stain; bar = 100 μm.
Figure 16—Photomicrograph of a section of the omasal wall at birth. Notice isolated PAS-positive cells (arrows). PAS stain; bar = 100 μm. *See* Figure 11 for key.
Figure 17—Scanning electron micrograph of a section of the omasal surface at birth. Bar = 100 μm.
Figure 18—Scanning electron micrograph of a section of the omasal laminar surface in adult sheep (compare with Fig 17). Bar = 100 μm.

cidum-spinosum (indistinct, large cells), and corneum (elongated anuclear cells arranged parallel to the surface). Papillar formations were abundant in first- and second-order laminae, but were not found in lower order laminae (Fig 11). The PAS-positive and PAS-negative cells alternated in laminae and in interlaminar spaces (Fig 12).

A substantial increase was observed in the thickness of the tunica muscularis (222.7 ± 14.4 μm). Blood vessels and bands of nerve were visible within peri-and intermuscular connective tissue (Fig 13).

Gestation days 113 to 118 (32 to 36 cm C-R) and 120 to 150 days, and fetuses at term (37 to 40 cm C-R)—The histogenetic structure of the omasal wall had not changed substantially with respect to the previous stage, except that papillae had increased in size and number. Smooth muscle fibers from the muscularis mucosae were clearly visible within some papillae (Fig 14). Numerous laminae or longitudinal folds were observed in the omasal mucosa (Fig 15); 4 orders of length could be distinguished clearly. Omasal laminae contained numerous papillae.

At birth, histochemical reactions during omasal histogenesis were inverted: the slight, moderate, or intense PAS-positive reactions detected during prenatal development gave way to the isolated PAS-positive cells in the stratum granulosum and stratum lucidum-spinosum of embryos at term (Fig 16). Evidence of acid mucopolysaccharides, mucins, or mucoid compounds was not detected.

Scanning electron microscopic analysis of the mucosal surface revealed a series of longitudinal folds or omasal laminae, above which smaller folds or papillae emerged in the direction of the omasal lumen. The length of these papillae varied according to the time elapsed since their formation. They were round and conical and did not have signs of keratinization or cell desquamation (Fig 17).

Postnatal development (2 days to 2 years)—The histologic characteristics of tissue strata had reached their definitive form in the previous phase. Scanning electron microscopic analysis of the omasal mucosa (Fig 18) indicated a large number of laminae in the form of longitudinal extensions of variable length and considerable degree of keratinization. Papillae protruding laterally from the mucosal surface of these laminae toward the interlaminar lumen were classified into 3 types, according to their degree of development, which varied as a function of the time elapsed since their formation.

Histochemical Reaction of the Epithelium

Neutral mucopolysaccharides appeared from day 46 of fetal development. A positive reaction was observed over 91.8 ± 5% of a reference surface of 100 μm^2. The percentage of PAS-positive reactions increased to reach maximal values (93.3 ± 4%) at 79 days. Thereafter, values started to decrease (75 ± 4% at 96 days and 66.3 ± 5% at 117 days). A PAS-positive reaction was not observed at birth. Acid mucopolysaccharides, mucins, and mucoid compounds were not found during development.

Histomorphometric Analysis

Each tissue stratum was fitted to mathematical growth models (Fig 19–23), using the corresponding growth equation.

Discussion

At 23 days of fetal life (0.4 cm C-R), the stomach wall was already structured in 2 layers: the epithelium and the pluripotential blastemic tissue.[10] These were indistinct structures, with high blastemic capacity.[5]

The omasum was first observed in certain samples at 33 days (2.6 cm C-R). The pseudostratified epithelium[5] lacked secretory capacity. Evidence of stratification was not detected until 53 days (8.5 cm C-R). By 83 days, stratum corneum and stratum lucidum = spinosum had been added to the 2 initial layers (stratum germinativum and stratum granulosum). The stratum germinativum and stratum granulosum have been observed in sheep at 10 cm C-R and at birth[11], in goats at 28.3 cm C-R[12], and in buffalo and cattle at an advanced stage of development, close to birth.[5,10,13,14] Few authors actually define the stratum lucidum/spinosum; indeed, some make no reference to these strata.[13,15]

First-order omasal laminae were detected in the form of small undulations of the compartment wall at 2.6 cm (33 days). Zietschann and Krölling[15] reported initial appearance of laminae at 2 cm, Del Río

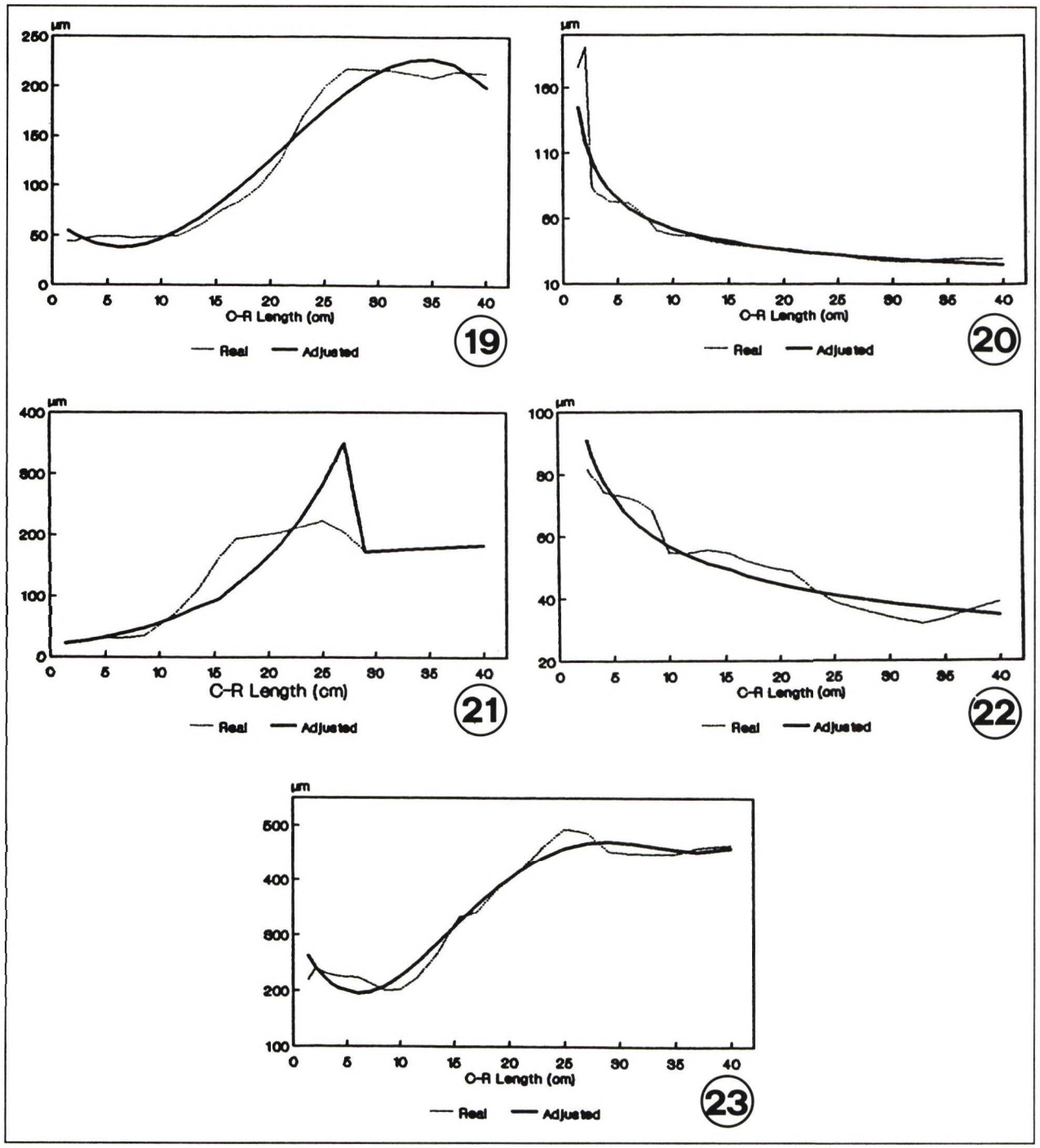

Figure 19—Mathematical model of omasum growth (epithelium). C-R = crown-to-rump length. $y = 66.56 - 9.50x + 0.86x^2 - 0.01x^3 - 8.6E - 5x^4$.
Figure 20—Mathematical model of omasum growth (lamina propia + submucosa) $y = 5.15x^{0.52}$.
Figure 21—Mathematical model of omasum growth (tunica muscularis). Adjusted, $y = EXP(2.93 + 0.11x)$; Real, $y = 143.16 + 0.96x$.
Figure 22—Mathematical model of omasum growth (serosa). $y = 4.84x - 0.35$.
Figure 23—Mathematical model of omasum growth (wall). $y = 316.59 - 45.31x + 5.11x^2 - 0.17x^3 + 1.72E - 3x^4$.

Ortega observed it at 3.2 cm,[c] and Lubis and O'Shea observed it at 2.5 cm C-R.[16] All other available reports start at stages of development when first-order laminae and lower orders are already present.

[c] Del Río Ortega S. *Desarrollo prenatal del estómago de la oveja—ovis aries.* MS Thesis, Department of Anatomy and Embriology, Facultad de Veterinaria de Zaragoza, University of Zaragoza, 1973;36–57.

Differentiation of the tunica muscularis also takes place at this early stage of development, taking the form of undulations coinciding with the base of each lamina. Myoblast fibers project from here into the laminae to form the primitive muscularis mucosae. This was reported by Del Río Ortega at 13.5 cm[c] and by Fath El-Bab et al at 19 cm[6], when, according to

our evidence, all 4 orders of omasal laminae are present. Osman and Berg[17] and Vivo et al[10] reported interlaminar fibers at 32 cm and 27 cm C-R, respectivfely, in cattle.

Like those 2 investigators, we found that the 4 orders of laminae differ from each other only in order of appearance and, consequently, in the height attained. Reports concerning the chronology of appearance vary widely: Del Río Ortega reported first-, second-, third-, and fourth-order laminae at 3.2, 5, 6.5, and 7 cm C-R, respectively[c]; Lubis and O'Shea observed them at 2.5, 3.5, 5.5, and 11 cm.[16] Fath El-Bab et al reported the first 3 orders at 52 days (8 cm) and the fourth as late as 78 days (19 cm).[6] The 4 orders appeared in this study at 2.6, 4, 7.2, and 11 cm.

Most authors report 4 laminae. In studies of goats,[18] sheep,[11] and cattle,[10,15] the existence of a fifth order at birth has been reported. Osman and Berg[17] also reported a fifth order in cattle at 32 cm C-R.

Omasal papillae appeared at 15 cm C-R (69 days) in the form of lateral evaginations of connective tissue from the stratum basale of first-order laminae, oriented toward the epithelial surface. At 19 cm (79 days), papillae were observed in second-order laminae, but it was not until 33 cm (113 days) that they became visible in the remaining orders. Some papillae contained smooth muscle fiber originating in the muscularis mucosae, a finding not reported by others, to our knowledge. These conical papillae were observed by Del Río Ortega at 13.5 cm,[c] by Lubis and O'Shea at 15 cm,[16] and by Fath El-Bab at 39.3 cm.[6] Fath El-Bab did not observe papillae in fourth-order laminae at birth.

Scanning electron microscopic analysis of the omasal mucosa at birth revealed a series of longitudinal folds or laminae, the lateral surfaces of which were lined with papillae. Papillae were perfectly arranged in accordance with their histophysiologic function (ie, in such a way that with the movement of folds they acted on the food, channeling it into interlaminar spaces).

We believe that mechanical protection of the omasal mucosa against damage that may give rise to the first products of embryo metabolism is a function located in the stratum corneum, where nitrogenated polysaccharides were not detected. Nitrogenated polysaccharides in deeper epithelial layers from 46 days of gestation until birth, 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 routinely swallowed during gestation.[19,20]

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