EPITHELIAL DEVELOPMENT IN THE FORESTOMACH OF PASTURE-FED LAMBS (BIRTH TO 8 WEEKS)

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ABSTRACT

The histology, mitotic index, ultrastructure and Na+-K+-ATPase cytochemistry of lambs reared on pasture were studied during the period of weaning.

Two embalmed mature non-pregnant Romney cross-bred ewes and thirty Romney-cross lambs reared on Massey University sheep farms pastures were used. Five lambs (three in 1981 and two in 1982) were taken from their dams on pasture at each of the following respective ages: within 24 hours of birth, and at 12, 23, 34, 45 and 56 days. Stomach-tissue samples from 1 adult and from the lambs reared during the 1981 lambing season were prepared for histology using Haematoxylin and Eosin, Masson's green trichrome, Periodic-acid-Schiff and Toluidine Blue stains, and for conventional transmission electron microscopy. Tissue samples from the rumens of lambs reared during the 1982 season were used for strontium-capture technique Na+-K+-ATPase cytochemistry.

Gross dissection of the stomach of one-day-old lambs confirmed that the largest compartment at birth is the abomasum, followed, in decreasing order of size, by the rumen, reticulum and omasum. Progressive development resulted in the forestomach compartments assuming their adult proportions by 56 days of age.

Preliminary histological studies of epithelium taken from the rumen, reticulum, omasum and reticular groove of the adult sheep confirmed it to be a stratified keratinizing epithelium. Five general cell layers were clearly seen: stratum basale, stratum spinosum, stratum granulosum, stratum transitionale and stratum corneum. (In previous studies, the stratum granulosum and the stratum transitionale have been considered as one layer.) Mucopolysaccharides were located in the intercellular spaces in the stratum corneum.

Examination of the forestomach mucosa revealed a number of changes between birth and 56 days of age: (1) an increase in papillary length; (2) starting from 23 days, the development of extensive papillary process-epithelial bulb interactions accompanied by proliferation of blood vessels in the papillary processes; (3) a decrease in epithelial thickness for the first 45 days of age; (4) the appearance of a complete layer of transitional cells by 45 days; (5) the disappearance of glycogen from the epithelium; (6) an increase in the amount of mucopolysaccharide in the intercellular spaces in the stratum corneum; (7) the increase in the number of non-keratinocytes in the basal layer; and (8) the appearance of apoptotic bodies due to single cell death in the basal layer of the epithelium from 45 days of age.

The mitotic index of the epithelium in developing lambs decreased from birth until 23 days of age, had increased at 34 days, but decreased again between 45 and 56 days.

Examination of the ultrastructure of the adult epithelium provided general results consistent with previous studies. However, gap junctions were found in the stratum basale, stratum spinosum and were extensive in the stratum granulosum. Tight junctions (zonulae occludentes) were seen between the cells of the stratum corneum.

Langerhans cells and mast cells/globule leukocytes were classified as non-keratinizing cells. Other non-keratinocytes recognised were the 'indeterminate cells', lymphocyte-like cells and cells similar to Merkel cells.

Completely keratinized cells appeared in the epithelium at about 12 days of age, in association with the increased production of tonofilaments, keratohyalin granules, endoplasmic reticulum protein and membrane-coating granules. Proliferation of mitochondria in the basal layers started at about 12 days of age, and glycogen deposits in the intermediate layers were not found after this age.

Increased folding of the basolateral membrane surfaces of basal cells and progressive thinning of the endothelium of sub-epithelial blood vessels were also observed. Gap junctions in the stratum granulosum became progressively more obvious between birth and 56 days of age. Annular gap junctions were also found.

Na^+-K^+-ATPase enzymatic sites were identified from 12 days of age, on the cytoplasmic membranes of lower granular, spinous and basal cells: the reaction products being localised on both the cytoplasmic and intercellular-space surfaces of the plasma membranes. Ouabain inhibited the formation of deposits only on the cytoplasmic side. Alkaline phosphatase activity was localised in the stratum corneum. Mg^{++}-ATPase was demonstrated in the stratum corneum, the intercellular spaces in the stratum granulosum and stratum transitionale, and in mitochondria.

It is concluded that the ultrastructural features and Na^+-K^+-ATPase cytochemistry of the epithelium at 12 days of age appeared to be similar to those found in older animals. However, structural (and presumably functional) maturity did not appear to be complete until after 45 days of age at which stage the stratum transitionale had become complete and the mitotic index and the thickness of the epithelium had become stable. The increase of non-keratinocytes suggests the increasing immunocompetence of the epithelium.

Tight junctions and extruded contents of membrane-coating granules in the intercellular spaces of the stratum corneum could provide a barrier to the diffusion of solutes across the epithelium. The development of gap junctions and the presence of Na^+-K^+-ATPase enzymatic sites in the membranes are consistent with the absorptive and transport functions of the epithelium, particularly the active transepithelial movement of sodium ions. Future studies could well show hormones, hormone-like substances and antibiotics to be important in the development of the forestomach epithelium in ruminants.

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INTRODUCTION

Herbivorous animals are different from all other animals in that they thrive on large amounts of plant roughage as the main part of their diet. The use of such indigestible material is entirely dependent on the microbial breakdown of cellulose and hemicellulose, the high energy containing compounds of plant fibres (Church, 1976). Herbivores have developed modifications of their digestive tracts so that they can play host to a wide variety of cellulolytic microorganisms. In horses and rabbits the large caecum provides the site where microbial activity occurs; in camelids and ruminants the main site is the modified stomach. Of all the herbivores, the ruminants are considered to be the most successful and the most economically important (Ruckebusch and Thivend, 1980). It is their stomach which is the dominant feature of their digestive system and provides the machinery by which they are able to convert fibrous forage as well as other materials such as crop residues and other organic waste-products into bankable sources of human food.

The ruminant stomach is a large muscular organ composed of four compartments, namely, the rumen, reticulum, omasum and abomasum, the first three being collectively known as the forestomach. The forestomach, particularly the reticulo-rumen (reticulum and rumen combined)* serves as a capacious pH and temperature controlled chamber for the fermentation of plant polysaccharides and other substrates by a mixed population of bacteria and protozoa into metabolisable products, chiefly volatile fatty acids. This fermentation precedes digestion in the abomasum and the intestines.

Considerable interest has been devoted to the study of the structure of the stratified keratinizing epithelium

*The term reticulo-rumen (or rumino-reticulum) reflects the fact that the rumen and reticulum freely communicate with each other through the reticulo-ruminal orifice (Habel, 1965; Hofmann, 1973).

that lines the ruminant forestomach. This interest has been justified in view of the discoveries that volatile fatty acids are absorbed (Barcroft, McAnally and Phillipson, 1944 a and b) and metabolised (Pennington, 1952) in the epithelium, that important electrolytes such as sodium and chloride are actively transported across the epithelial layers from the rumen to blood direction (Sperber and Hyden, 1952), that volatile fatty acids are stimulatory to the growth of the rumen mucosa (Brownlee, 1956; Warner, Flatt and Loosli, 1956; Flatt, Warner and Loosli, 1958) and that a potential difference is normally maintained across the epithelium (Dobson and Phillipson, 1958).

Extensive structural studies on both developing and mature ruminant forestomach have corroborated and expanded these original discoveries. The refinements of techniques in both light and electron microscopy and histochemistry, particularly in enzyme histochemistry and cytochemistry, and their rapid application to the research on the epithelium of the ruminant forestomach have made possible more detailed interpretation of the functional importance of various structures. For example, with the use of histological methods, the stimulatory effect of volatile fatty acids on the maturation of papillae and the epithelium of the rumen has been confirmed (Tamate, McGilliard, Jacobson and Getty, 1963; Tamate and Sakata, 1978 b and 1979).

Histochemical observations on the adult ruminal epithelium have included various carbohydrates (Habel, 1963; Lavker, Chalupa and Opliger, 1969; Schnörr and Hild, 1974; Filotto, 1976), lipids (Habel, 1959; Lavker et al., 1969; Cerny, 1974), protein components (Henriksson and Habel, 1961; Schnorr and Hild, 1974) and enzymes such as dehydrogenases (de la Hunta, 1965; Ruiz and Molina, 1976; Galfi, Neogrady and Kutas, 1982), acid and alkaline phosphatases (Habel, 1963; Gardner and Scott, 1972; Lauwers, Sebruyns and de Vos, 1974; Filotto and Chiericato, 1977; Galfi et al., 1982); non-specific esterases (Habel, 1963; Lauwers et al., 1974) and Mg^{++}, Ca^{++} and non-specific ATPases (Henrikson, 1971; Schnorr, 1971; Ruiz and Molina,

1976; Gray and Habel, 1979) all of which have been associated with the absorptive, metabolic and transport functions of the epithelium.

With the use of the electron microscope (Steven and Marshall, 1970; Gemmel and Stacy, 1973) and in conjunction with electron-dense tracers and other cytochemical markers (Henrikson, 1970c; Henrikson and Stacy, 1971; Schnorr and Wille, 1972 a), it has been possible to rationalise the transport pathways taken by solutes as they are absorbed across the epithelium. Furthermore, also with the use of the electron microscope, the presence of Langerhans cells in the epithelium has been demonstrated (Gemmel, 1973; Nagatani, Kikuchi, Sakata and Tamate, 1974; Gerneke, 1977) thus posing a possible immunologic function of the epithelium.

The present study was undertaken initially to survey the ultrastructural changes occurring in the epithelium of the forestomach of lambs reared on pasture during the period of weaning - i.e., the transition from the pre-ruminant (monogastric) to the ruminant phase of digestion. Ultrastructural studies on the epithelium taken from the developing forestomach of the ruminants are few and concerned mainly with its pre-natal (Steven and Marshall, 1970; Arias, Fernandez and Cabrera, 1979 and 1980) to its early post-natal development (Henrikson, 1970 a) and particularly emphasising on its glycogen content (Sheynaova, Demidova, Davletova and Kruglyakov, 1978; Arias et al., 1979). So far as the present author is aware, no studies have been reported in the literature on the ultrastructure of the epithelium during weaning.

In order to give a better perspective to the present ultrastructural study, particular attention was given the functional anatomy of the ruminant stomach and the development of the ruminant state in the review of literature. Also, since most of the ultrastructural studies on the forestomach epithelium were conducted in the late 1950's to early 1970's (Lindhe and Sperber, 1959; Hyden and Sperber, 1965; Schnorr and Vollmerhaus, 1967; Lavker, Chalupa and Dickey, 1969; Marshall and Steven, 1969; Henrikson, 1970 b and c; Steven and Marshall, 1970;

Henrikson and Stacy, 1971; Schnorr and Wille, 1972 a and b; Scott, Gardner, Fulton and McInroy, 1972; Gemmel, 1973; Gemmel and Stacy, 1973; Hofmann, 1973; Scott and Gardner, 1973 a; Nagatani et al., 1974; Sakata and Tamate, 1974; Tamate, Kikuchi and Sakata, 1974), the ultrastructure of the adult epithelium was re-examined in view of the more recent findings, particularly those on the structure of the intercellular junctions and the features of the permeability barrier (Friend and Gilula, 1972; Elias and Friend, 1975; Lavker, 1976; Elias, McNutt and Friend, 1977; Elias, Goerke and Friend, 1977; Hayward, 1979) and on the presence of non-keratinizing cells besides Langerhans cells (Breathnach, 1980) in other keratinizing epithelia.

The rate of mitosis in the basal layers of the rumen epithelium was also examined in different ages to give an idea on some of the mechanisms responsible for the maintenance of the epithelial cell populations.

Finally, the Na^+-K^+-ATPase enzyme complex that is generally accepted as the enzymatic expression of the Na^+ pump which mediates cellular ionic homeostasis and transepithelial movement of electrolytes and water (see reviews by Bonting, 1970; Albers, 1976; Ernst, Riddle and Karnaky, 1980) was localised in the rumen epithelium. Previous attempts on the histochemical (Ruiz and Molina, 1976) and cytochemical (Henrikson, 1971; Schnorr, 1971; Gray and Habel, 1979) localisation of this enzyme complex in the epithelium using variants of the original Wachstein and Meisel (1957) technique for ATPase provided uncertain results because of the non-specificity of the techniques used (Firth, 1980). The present localisation employed the strontium capture technique developed by Ernst (1972). This histochemical method has been found to be more specific and more reliable for Na^+-K^+-ATPase localisation (Ernst and Mills, 1980; Ernst et al., 1980; Firth, 1980).

This thesis is the result of a series of structural, histochemical and kinetic studies on the forestomach epithelium of the sheep. These studies were undertaken with a view of describing and explaining in accordance with current physiological concepts, the structural events

taking place in the epithelium lining the forestomach in lambs reared on pasture when they are undergoing the greatest modification in their digestive physiology - that is, the transformation from the monogastric to the ruminant state of digestion.

CHAPTER ONE

REVIEW OF LITERATURE

After a brief survey of the history of studies on the structure and function of the ruminant stomach, the rest of this chapter will be devoted to the functional anatomy and development of this organ.

1.1 Early Studies on the Ruminant Stomach

The complex structure of the stomach of the ruminants (already outlined in the Introduction of this thesis) has been well recognised since Aristotle noted its four chambers in his Historia Animalium which appeared in 400 B.C. The anatomy of the digestive tracts of ruminants is well described in surgical textbooks in the 18th and 19th centuries (Chaubert, 1787; Leblanc and Trousseau, 1823). However, little was known of the function of the ruminant stomach until studies on rumination in the 19th century, particularly those of Flourens (1854), Toussaint (1874) and Colin (1886) and the development of the rumen fistula by these workers, sparked interest in the propulsive movements of the ruminant stomach.

It was also in the 19th century that Tappeiner (1884) established the importance of the forestomach microflora as digesting agents and, together with Zuntz (1885), demonstrated that fermentation of cellulose in the rumen could give rise to acetic, propionic and butyric acids, as well as carbon dioxide, methane and hydrogen gases. Zuntz (loc. cit.) also proposed that propionic and butyric acids could serve as energy sources for cattle. These findings were confirmed by Henneberg and Stohmann (1885), who also postulated that fermentation products can be absorbed by the ruminants. However, in spite of this information and the fact that the ruminant apparently subsists to a large extent upon the products of carbohydrate

breakdown, investigators were loathedto admit the value of these products to the animal and searched instead for a more conventional explanation in line with metabolic processes in non-ruminants. By the end of the 19th century, it was still widely believed that the forestomach merely served to prepare food for digestion in the lower parts of the digestive tract (Strangeway, 1895).

Although Ellenberger (1911) was the first to describe the histology of the forestomach, Aggazzotti (1910) had already produced the first experimental evidence that water is absorbed from any part of the forestomach. This was followed by the observation of Trautmann (1933) that pilocarpine and atropine in solution are absorbed from the forestomach of goats. It was more than 10 years later however, before absorption of volatile fatty acids (VFAs) from the forestomach was established and the relationship between absorption and forestomach histology shown. By measuring and comparing VFA concentrations in the blood supply and drainage of various parts of the digestive tracts from different animals, Barcroft, McAnally and Phillipson (1944 a) were able to conclude that considerable VFA absorption occurs in the rumen, reticulum, omasum and caecum. They also showed (Barcroft, McAnally and Phillipson, 1944 b) that the stratified keratinizing epithelium lining the forestomach was the site concerned.

The classical studies of Wester (1926) and Schalk and Amadon (1928) provided the basis for much of the present information on the motor function of the forestomach. In both studies, the contractions of the reticulum and the rumen were described by means of direct observation, palpation and the use of balloons to record pressure changes in the various compartments. Clear sequences of the contractions of the reticulum and the rumen were demonstrated later by many authors including Czepa and Stigler (1926), Phillipson (1939), Dougherty and Meredith (1955), Benzie and Phillipson (1957), Reid and Titchen (1959), Reid (1963), Sellers and Steven (1956), Akester and Titchen (1969) and Ruckebusch (1970) using

strain-gauge-catheter systems, radiology, open fistulas, chronic partial exteriorisations and electromyographic techniques (see review by Wyburn, 1980). These approaches made valuable contributions to the understanding of the function of the ruminant stomach.

In the past two decades, there has been an upsurge in the interest in the anatomical and physiological development of the ruminant stomach (see reviews by McGilliard, Jacobson and Sutton, 1965; Warner and Flatt, 1965; Thivend, Toullec and Guilloteau, 1980). This is especially so because of the emerging role of the young ruminant as an alternative food source. Presently, studies on the ruminant stomach are directed towards finding more ways to harness the versatility of the forestomach particularly its microflora to utilize alternative feed stuffs, especially in the control of protein degradability and digestibility of crop residues (Ruckebusch, 1980). There also has been an upsurge of interest on the CNS control mechanisms dictating stomach motility and rumination (see reviews by Carr, McLeay and Titchen, 1970 and Bueno and Fioramonti, 1980). This expansion of ruminant stomach research into many directions could help in the detailed understanding of the factors regulating food intake in the ruminant which in turn, could improve both animal production and human well-being (Ruckebusch and Thivend, 1980).

1.2 Functional Anatomy of the Ruminant Stomach

The principal feature of ruminant digestion is the massive fermentation in the forestomach which occurs ahead of enzymatic digestion in the abomasum and the intestines. The forestomach of the ruminant delays the passage of food through the digestive tract. In grazing sheep, food which enters the forestomach stays in the reticulo-rumen for an average of eight hours (Phillipson, 1977). Bacteria and protozoa live on the food that the animal eats and cause extensive chemical changes. This is aided by the wetting, mixing and maceration of stomach contents simultaneously accomplished by salivary secretion, by the regurgitation and remastication and swallowing of food

during rumination and by the complex series of contractions during this process and during mixing and eructation cycles. Selective retention of larger food particles which ensures prolonged exposure of slowly digesting roughage to fermentation is also accomplished by muscular contraction (Sellers and Stevens, 1966). In addition, pH is maintained at optimum values between 5.8 and 7.0 (Phillipson, 1977) for cellulolytic microorganisms to be viable. Regulation of pH depends largely on the structure of fibre and the buffering effect of saliva and the structure of the absorptive surface of the stomach (Kroman and Meyer, 1972). Normal temperatures in the forestomach vary between 39 - 41^{o}C (Church, 1976).

Volatile fatty acids (chiefly acetic, propionic and butyric), methane, carbon dioxide, ammonia and protein in the form of microbial cells are the main products of fermentation. The gases are removed from the forestomach by eructation, the VFA's are absorbed through the forestomach wall and material leaving the reticulo-rumen consists of a mixture of food residues, bacteria and protozoa and some soluble fermentation products dissolved in buffered fluid (Leng, 1970; Phillipson, 1977). About nine-tenth's of the total daily production of VFAs is absorbed in the reticulo-rumen and omasum (Keynes, 1969). Part of this is metabolised in the epithelium into ketone bodies with butyrate being metabolised at a greater rate than other straight- and branched- chain VFAs (Pennington, 1952). In sheep given a wide range of forage rations, VFAs provide 60 - 80 % of the daily metabolisable energy intake (Seeley, Armstrong and McCrae, 1969).

In addition, bacteria and protozoa also synthesise almost all of the B complex vitamins and vitamin K and while protein is also hydrolysed into ammonia, syntesis of protein from non-protein nitrogenous sources such as urea also occurs. Transfer of water, urea, ammonia, sodium, chloride, bicarbonate and other inorganic ions occurs across the epithelium.

Ruminant stomach function is not limited to fermentation.

Digestion by tissue-elaborated enzymes also takes place in the stomach. The fourth compartment, the abomasum, is a glandular organ that secretes digestive juices as in the stomachs of monogastric animals. In contrast to simple-stomached animals, however, production of abomasal juice is continuous (Ash, 1961). This is associated with a continuous flow of digesta from the omasum and reticulo-rumen. This continuous nature of the digestive process in the ruminant stomach is thus an important feature which distinguishes the ruminant from most monogastric animals (Hill, 1961).

1.2.1 Gross anatomy

The anatomy of the stomach of the sheep has been described extensively by many authors, including Strangeway (1895); Sisson and Grossman (1953); May (1964); Nickel, Schummer and Seiferle (1973); Habel (1975); Church (1976) and Bressou (1978). The following descriptions are largely based on the accounts of May (1964); Nickel et al. (1973); Habel (1975); and Church (1976).

The stomach of the sheep occupies nearly three-fourth's of the abdomen. Its long axis extends from the costal arch to the pelvis. The rumen, reticulum, omasum and abomasum do not strictly lie in series and are bunched together so that the rumen lies on the left, the reticulum cranially and the omasum on the right. The abomasum lies ventrally with its proximal portion below the rumen, reticulum and omasum (see Figure 1.1).

The external surface of the stomach is demarcated by grooves and constrictions which are synonymous with the internal ridges separating the organ into cavities. These ridges can be prominent and muscular or just mere thickenings of the stomach wall. Food from the esophagus enters the stomach through the cardia located in the dorsal wall of the reticulum and leaves the abomasum through the pylorus to the duodenum.

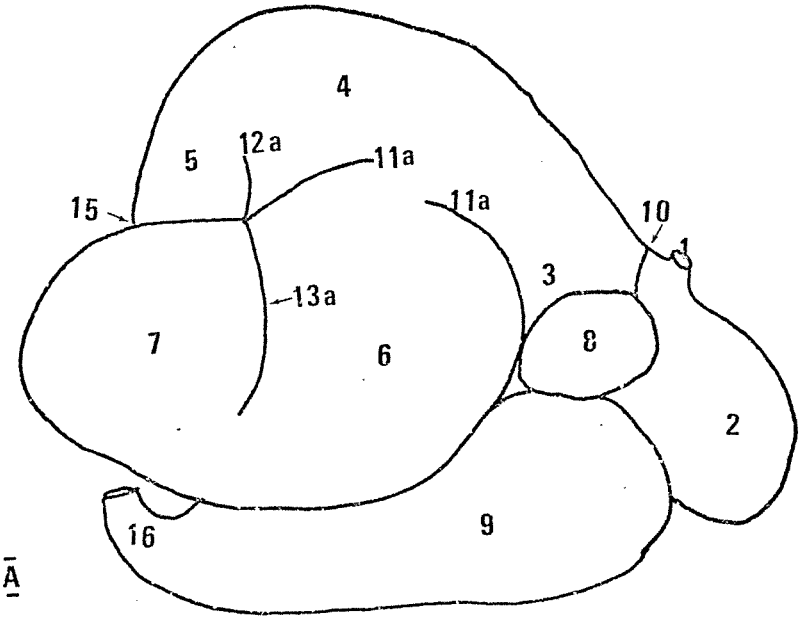
1.2.1.1 The rumen and reticulum

The reticulo-rumen, where most of the microbial activity takes place, has six chambers partially separated by

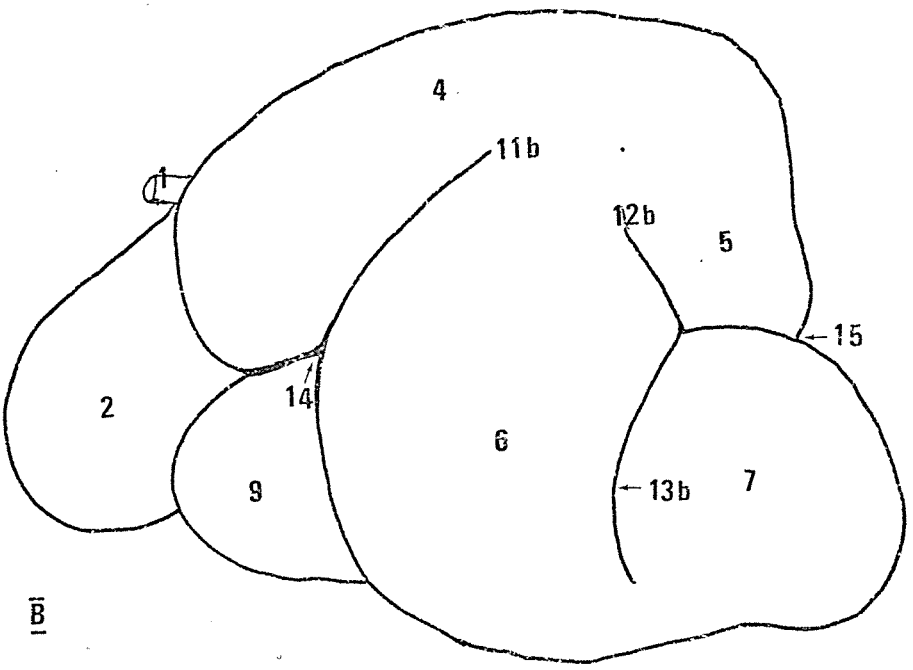
Figure 1.1 Diagrammatic representation of the stomach of the sheep. (A = right side; B = left side).

Structures of major importance are numbered as follows:

1 - esophagus
2 - reticulum
3 - cranial sac of rumen
4 - dorsal sac of rumen
5 - caudodorsal blind sac
6 - ventral sac of rumen
7 - caudoventral blind sac
8 - omasum
9 - abomasum
10 - reticulo-ruminal groove (not so distinct as indicated here)
11a - right longitudinal groove
b - left longitudinal groove
12, 13a - right dorsal and ventral coronary grooves
b - left dorsal and ventral coronary grooves
14 - cranial groove
15 - caudal groove
16 - pylorus



A



B

grooves externally and corresponding muscular pillars and folds internally. The reticulum, named after its "honeycomb" mucosal appearance is the most cranial part of the stomach lying directly caudal to the diaphragm. It is demarcated on the outside from the rumen by the reticulo-ruminal groove which corresponds internally to the reticulo-ruminal fold. This fold separates the reticulum from the cranial sac of the rumen and extends from the right (visceral) wall round up the parietal wall to fade out just caudal to the cardia. The reticulo-ruminal fold forms the margin of the large reticulo-ruminal orifice through which food flows freely between the reticulum and rumen. Within the reticulum on its medial (right) wall is the reticular groove which is formed by two muscular ridges or lips running from the cardia to the reticulo-omasal orifice. The groove is spirally twisted so that the edges of the lips are twisted mainly to the left. Apposition of the two lips, a reflex triggered by the passage of liquids through the pharynx and the proximal part of the esophagus, especially in young animals, forms a tube which bypasses the reticulo-rumen to ensure the direct passage of milk to the omasal canal and abomasum. The reticulo-omasal orifice which is located in the medial wall of the reticulum is said to be involved in the mechanism for the selective retention of longer fermenting food particles (Stevens, Sellers and Spurrell, 1960; Hume and Warner, 1980).

The rumen is a large laterally compressed sac occupying most of the left portions of the abdominal cavity. Its middle is constricted by the confluence of four grooves which divides it into a dorsal and ventral sac: two longitudinal grooves, one on each side (parietal and visceral) and cranial and caudal transverse grooves in the cranial and caudal extremities, respectively. Arising from the cranial transverse groove, the visceral (right) longitudinal groove splits into two limbs enclosing a narrow area before rejoining caudally and connecting with the caudal groove. The parietal (left) longitudinal groove also arises from the cranial groove on its left side, runs upwards and caudally but does not meet the caudal

groove. From the caudal groove, on each side, coronary grooves pass dorsally and ventrally to mark off the dorsal and ventral caudal blind sacs from the main cavity. The venral coronary grooves almost completely meet at the ventral surface of the rumen but the dorsal grooves are short and do not form a complete ring in the dorsal sac.

Internally, and in positions corresponding to the external grooves are muscular pillars partially separating the chambers. The cranial pillar is a shelf-like muscular projection directed dorso-caudally into the cavity. The small cranial sac lies between this pillar and the reticulo-ruminal fold. This sac communicates with the reticulum thorugh the reticulo-ruminal orifice and with the dorsal sac above the cranial pillar. The dorsal sac is separated from the caudodorsal blind sac by the dorsal (parietal and visceral) coronary pillars and from the ventral sac by the confluence of the longitudinal pillars on each side and the cranial and caudal pillars in corresponding extremities. The ventral sac is separated from the caudoventral blind sac by the ventral coronary pillars which form a complete ring. Both dorsal and ventral coronary pillars branch off from the caudal pillar which projects cranially between the dorsal and ventral caudal blind sacs. The caudoventral blind sac in the sheep extends farther caudally than the dorsal one.

The cyclic motility of the reticulo-rumen has been reviewed by Church (1976) and by Wyburn (1980). The series of complex contractions in the reticulo-rumen result in mixing newly ingested food with that already in the stomach, in regurgitation, in eructation of gas and in the movement of food into the omasum. Mixing of stomach contents also aids in absorption (Sellers and Stevens, 1966).

1.2.1.2 The omasum

The omasum is somewhat oval and lies between the ventral sac of the rumen on the left, the liver on the right and the abomasum ventrally so that it does not come

in contact with the abdominal wall. It is connected to the reticulum by a narrow neck and to the abomasum by a wider constriction. Internally, it is filled for the most part, with omasal laminae. These are thin muscular sheets numbering about 100 in varying orders of length, covered by epithelium and studded with papillae. The laminae arise from the omasal wall except from the lesser curvature with their free edges projecting towards the interior.

The omasal groove continues the reticular groove in the omasum and runs on the lesser curvature of the organ. This groove connects the reticulo-omasal orifice with the omaso-abomasal orifice and together with the free ends of the laminae, surrounds and forms the omasal canal. Before reaching the omaso-abomasal orifice, the omasal groove is crossed by the omasal pillar. The reticulo-omasal orifice is surrounded by a sphincter that allows its complete closure (Sellers and Stevens, 1966).

The omaso-abomasal orifice is the entrance to the abomasum. It is protected by folds of the abomasal mucous membrane, the velae which are thought to play a role in the closure of the opening. The omaso-abomasal orifice lacks a sphincter as in the reticulo-omasal orifice.

Omasal function is not well understood, probably because its relatively concealed position makes it difficult to observe. Even radiographic examination of this compartment is unrewarding (Wyburn, 1980). The old idea of the grinding or fractioning function of the omasum has been questioned, although ingesta trapped in interlaminar spaces seen in post-mortem resembles dehydrated cakes of fine fibrous material. It is certain that the omasum provides the passage the reticulo-ruminal contents must take to reach the abomasum, and its motility contributes to this passage. Bost (1970), in reviewing omasal physiology, has suggested that the reticulo-omasal orifice, whose motility is correlated with the cyclic movements of the reticulo-rumen (Stevens, Sellers and Spurrell, 1960), actively regulates the reticular outflow of digesta and

that the laminae with their papillae passively sieve the material. He also speculates that the movement of the omasal body prevents overfilling and clogging as well as squeezes the contents. Water and VFA absorption from the omasal contents may just be an accessory function.

1.2.1.3 The abomasum

The abomasum is an elongated tubular organ which tapers from the omasal end towards the pylorus. The cranial blind end, the fundus, is attached to the reticulum by a broad fibrous band. The fundus and the body of the abomasum lies on the abdominal floor between the omasum and ventral rumen. It crosses the midline obliquely from left cranial to right caudal direction just behind the xiphoid cartilage. It then inclines up the lateral wall of the abdominal cavity behind the omasum to end at the pylorus.

Internally, the mucosa of the fundus and body is arranged into spiral folds. The abomasal groove, the continuation of the omasal groove into the abomasum, is represented by a band-like area devoid of folds along the lesser curvature. The pyloric gland region is small and constricted at the distal end to form the pylorus. Except for the absence of a sphincter at its cranial pole and the presence of a potent milk-curdling enzyme, rennin in young animals, the structure and secretory functions of the abomasum is considered to be analogous to the simple stomach of non-ruminants since food received in here is attacked and digested under acid conditions by the enzymes of gastric juice. The characteristic continuous nature of abomasal function developsat the onset of fermentative digestion (Hill, 1968).

1.2.2 Forestomach Mucosal Form and Surface Architecture

Because of its importance in absorption, the structure of the mucosa lining the forestomach is now discussed in detail. The entire surface of the various sacs and compartments of the ruminant stomach is lined by stratified keratinizing epithelium which is the site of VFA, water

and electrolyte absorption. Its surface area is increased considerably by the presence of numerous papillae. The surface is further expanded in the reticulum by the presence of thick-walled crests or ridges forming four-, six-, or eight-sided cells (hence the "honeycomb" pattern) and in the omasum, by laminae of varying lengths which themselves are furnished with papillae respectively.

1.2.2.1 Rumen

Hofmann (1969) classified papillae found in the rumen into two basic types: (1) flattened, tongue- or leaf-shaped papilla and (2) filliform or finger-shaped papilla. The latter he considered to be the primitive type as found in fetal stomachs and gives rise to other shapes. In the sheep, these 'other' shapes could be any of the following described in the literature: hair-like and triangular spades (Harrison, Warner, Sander and Loosli, 1960); wart-, keel-, tongue-, rod-, lance-, and leaf-like shapes (Schnorr and Vollmerhaus, 1967); low knob-like protrusions and pendulous extensions (Henrikson, 1970 c); flattened blunt blocks and foliate structures (Scott and Gardner, 1973 b), small nodules and branched cauliflowers (McGavin and Morrill, 1976 b) and many others.

Typical tongue- or leaf-shaped papillae are found throughout the ventral parts of the forestomach sacs and are most dense in the blind sacs, especially in the atrium ruminis of the cranial sac, the main absorptive area of the reticulo-rumen (Habel, 1975; Church, 1976; Stinson and Calhoun, 1976 a). Papillae found in the dorsal sac tend to be sparse, small, flap-like and heavily keratinized while those found in the ruminal pillars are closely packed giving them a distinctly ridged appearance. Foliate papillae are found in the transitional zone between the rumen and reticulum (Scott and Gardner, 1973 b).

These wide variations in papillary size and length are so marked in one given location in the rumen that evaluation of their length by histologic methods can be unreliable (Scott and Gardner, 1973 b; McGavin and Morrill, 1976 b). Instead, the use of scanning electron microscopy

(SEM) is advocated by these workers. These variations are further influenced by the age of the animal and by the nature of the diet of the animal, particularly by the products of rumen fermentation (Brownlee, 1956; Warner, Flatt and Loosli, 1956; Wardrop, 1961 a and b; Tamate, McGilliard, Jacobson and Getty, 1962) and degree of stratification of stomach contents (Henrikson, 1970 c).

Species variations in papillary form and density also occur, as seen by the absence of papillae in the dorsal sac of cattle and in the diversity of papillar forms in African game ruminants. In the latter, Hofmann (1973) has shown that the mucosal relief is directly linked to feeding preferences of the species and also to seasonal availability of preferred food or to an adaptation to a special diet (e.g., in captivity).

Ruminal papillae are structures which increase the absorptive surfaces of the rumen and are specialised for exchange between tissue and rumen contents. Schnorr and Vollmerhaus (1967) have calculated for cattle and goats a surface enlargement of up to seven times. It is also thought that papillae have a mechanical function by increasing friction between ingesta and rumen wall thereby facilitating mixing during contraction (Nickel et al. 1973). However, it is now generally agreed that this mechanical function of papillae is insignificant (Hofmann, 1973).

Another function attributed to the papillae is a role in pH regulation (Kroman and Meyer, 1972). VFAs are stimulatory to papillary growth and development (Brownlee, 1956; Warner et al., 1956; Flatt et al., 1958). Papillary enlargement occurs when large quantities of VFAs are produced especially from high-concentrate rations. While decreasing pH values increases the rate of absorption of VFAs in itself (Kaufmann, Hagemeister and Dirksen, 1980), enlargement of the absorptive area of papillae aids in the quick removal of rapidly produced fermentation products, thus further stabilizing pH. In addition, papillae provide, especially in denser papillated areas sufficient narrow interspaces for the extensive

populations of bacteria and protozoa and may also have an anti-froth function in legume-eating ruminants (Hofmann, 1973). In practice, papillary lengths could serve as an indirect basis for estimating energy intake and weight gains in weaned lambs (Clarke, 1982).

1.2.2.2 Reticulum

The crests of the reticulum are studded laterally with small pointed conical papillae (Habel, 1975). Larger conical papillae occur in the edges of crests and smaller ones, in some cases with bifid tips are found in the secondary and tertiary crests and in the floor of reticular cells (Scott and Gardner, 1973 a). These conical papillae are heavily keratinized and this is correlated with the physiological activities of this chamber. The crests and cells of the reticulum, upon contraction, are thought to act like a sieve which hold onto coarse materials while finely chewed particles pass through to the omasum, and, the papillae aid in this sieve-like action (Hofmann, 1973). Epithelial sensory receptors have also been located in the conical papillae surmounting the reticular crests through reflex observation after the application of specific stimuli and through recording the activity as detected by applying single fibre technique to peripheral nerves or by inserting recording micro-electrodes in the brain stem (Leek and Harding, 1975). According to these authors, these receptors are sensitive to mechanical stimulation especially reticular distension and to certain kinds of chemical stimulation like acids, alkalis, hyperosmotic and hypoosmotic solutions.

1.2.2.3 Omasum

The mucosal form and structure of the omasum has been studied using SEM techniques by Gardner and Scott (1971). Papillae found in the omasum vary in form, size and density within the organ. The most common one is conical in shape with sharply pointed and heavily keratinized tips as those found on the lateral surfaces of laminae. Smaller conical forms with more rounded tips

and peg-like papillae with distinct multifid tips are also found. Unguliform papillae (papillae with distinctly curved tips) are found in the omasal groove near the reticulo-omasal orifice.

The omasal papillae are thought to act more as a sieve in regulating the flow of digesta; their absorptive function may be secondary (Bost, 1970). Hair-like structures found in the omasum between the papillae have been considered to represent foreign materials (Gardner and Scott, 1971).

1.2.3 Blood supply and drainage, lymphatics and innervation

The descriptions in this section are largely based on the accounts of Comline, Silver and Steven, 1968; Ghoshal, 1975; and Ghoshal, Koch and Popesko, 1981.

1.2.3.1 Arterial supply

The major blood supply of the ruminant stomach is derived from the celiac artery. Anastomoses between the peripheral branches of this artery and branches from the broncho-esophageal trunk, mediastinal arteries and the cranial mesenteric arteries provide alternative blood supply. Individual variations occur, but the celiac artery usually gives off to the forestomach: (1) the right ruminal artery which passes cadually on the right longitudinal groove and supplies the pancreas, greater omentum and the left and right aspects of the caudal part of the rumen; (2) the left ruminal artery which courses on the left longitudinal groove and gives off numerous branches to the left rumen wall; (3) a main branch supplying the left wall of the reticulum, cardia and neighbouring areas and which is found on the left reticulo-ruminal groove; (4) the terminal branch of the celiac artery which divides into a dorsal branch supplying the omasum and a ventral omaso-abomasal branch supplying the right wall of the reticulum, omasum and abomasum.

The small terminal branches of the arteries penetrate the stomach wall and join a vascular plexus between the muscular layers and a very extensive subepithelial network

(Comline et al., 1968). The presence of butyrate, of osmotic gradients across the rumen epithelium and of high concentrations of carbon dioxide in the rumen can stimulate blood flow to the rumen (Dobson, Sellers and Gatewood, 1976 a and b).

1.2.3.2 Venous drainage

The veins draining blood from the stomach of the ruminant are satellites of corresponding arteries. Again, individual variations occur which in the sheep could be significant (Goshal et al., 1981). However, the usual pattern is the following: The right ruminal vein is formed by veins from the right side of the rumen together with a tributary from the spleen. The left ruminal vein unites the veins draining the reticulum, omasum and abomasum. The gastric vein is the result of the confluence of the right and left ruminal veins and which is joined by the gastro-duodenal vein. Before entering the porta of the liver, the gastric vein joins the cranial mesenteric vein.

1.2.3.3 Lymphatics

The ruminant stomach is drained by extensive network of lymph channels which pass through several groups of lymph nodes belonging to the celiac lymphocentre (Saar and Getty, 1975). The lymph nodes and vessels lie superficially on the walls of the stomach with their efferent trunks ultimately terminating in the cisterna chyli.

1.2.3.4 Innervation

The major components of the innervation of the stomach are derived from the esophageal vagus nerves which contain both efferent and afferent fibres. The greater part of the rumen, the left sides of the omasum and abomasum are innervated by the dorsal vagus, while the reticulum, the right sides of the omasum and abomasum are innervated by the ventral vagus. In addition, the sympathetic system contributes to the innervation via the celiacomesenteric plexus and celiac and cranial mesenteric

ganglia. The nerves end in a rich plexus of ganglia and post-ganglionic fibres located between the two muscle layers (Comline et al., 1968).

Sectioning of both vagus nerves results to the abolition of contraction of the first three compartments of the stomach, stagnation of food within these organs, loss of rumination and loss of reticular groove reflex (Duncan, 1953; Phillipson, 1977). Stimulation of the various branches of the abdominal vagus causes contraction of the parts they supply; movements of the reticulum and rumen are suppressed by the injection of atropine (Phillipson, 1977). Sectioning of the splanchnic nerves is not followed by any apparent abnormality in the movements of the stomach (Duncan, 1953).

1.3 Histology

The histology of the mature ruminant forestomach is well presented in the literature since Ellenberger (1911) first described it and Barcroft et al., (1944 b) showed the relation of the epithelium lining the forestomach to absorption. Dobson, Brown, Dobson and Phillipson (1956) published a detailed account of the histology of the rumen epithelium of sheep with particular reference to the distribution and orientation of mitochondria in the epithelial cells. Recent reviews include those of Comline et al. (1968), Hofmann (1973), Fell and Weekes (1975) and Stinson and Calhoun (1976 a).

There are minor differences in the basic histology of the different regions of the forestomach, in spite of the diversity of their mucosal and papillary forms. However, Hofmann (1973) on comparing a wide range of ruminants with different diets, has shown structural variations in the histological appearance of the mucosa especially in the arrangement of blood vessels in the papillary core and in the number of cell layers in different types of papillae. The following descriptions, unless otherwise stated, are largely based on the accounts of Comline et al. (1968) and Stinson and Calhoun (1976 a).

1.3.1 Rumen

The rumen papilla consists of a highly vascularised connective tissue core composed of densely packed

collagen, elastic and reticular fibres surrounded by stratified keratinizing epithelium. Papillary processes similar to papillary bodies in the skin extend from the connective tissue core into the epithelium towards the cavity of the rumen (Dobson et al., 1956). Scanning electron microscopic studies of these processes (Tamate, Shiomura and Sakata, 1979) revealed that they are arranged in ridges along the long axis of the papilla and become individual finger-like projections at the tips. The basal surface of the epithelium on the other hand, is characterised by epithelial pegs or bulbs which interdigitate with the papillary processes and extend into the connective tissue core (Dobson et al., 1956). The connective tissue-epithelial interface provides the territory where active interactions played by epithelial and connective tissue cell populations occur (Klein-Szanto and Schroeder, 1977).

The stratified keratinizing epithelium lining the rumen is composed of four or five cellular layers which appear to merge into one another without distinct boundaries (Dobson et al., 1956): stratum basale, composed of a layer of columnar cells located on a basement membrane; stratum spinosum, consisting of one to 10 layers of polyhedral cells which show conspicuous intercellular bridges; stratum granulosum, composed of 2 - 3 layers of flattened cells with keratohyalin granules; and stratum corneum consisting of 2 - 20 layers of flattened and/or swollen keratinized cells. A stratum transitionale, composed of swollen, nucleated cells with a non-stainable cytoplasm has been described beneath the stratum corneum (Henriksson and Habel, 1961; Habel, 1965; Janacek, Borik and Holman, 1972). The structure and function of the rumen epithelium is discussed in detail in Section 1.5.

Large blood and lymphatic vessels are found in the central connective tissue core (Dobson et al., 1956). A dense network of fenestrated capillaries penetrate into the papillary processes (Dobson et al., 1956; Stinson and Calhoun, 1976 a) which come into intimate contact with the basal layer of the epithelium. A sub-epithelial network of nerve fibres has also been described in the connective tissue core in papillae of young animals from which free nerve endings originate and enter the surrounding epithelium (Hill, 1957;

1958 a and b).

The papillary core (lamina propria) blends with the tunica submucosa without any distinct boundaries. The tunica submucosa is composed of loose collagen and elastic fibres where a network of blood vessels and nerves courses through. The tumica muscularis has two smooth muscle layers - the inner circular layer and the outer longitudinal layer; autonomic nerve plexuses with ganglia are located between the muscular layers.

The tunica serosa is composed of collagen and elastic tissue covered by mesothelium. Fat, blood and lymph vessels and nerves are also found in the serosal tunic.

1.3.2 Reticulum, reticular groove and omasum

The basic histology of the reticulum, reticular groove and omasum is similar to that of the rumen although the muscularis mucosae are present in the tunicae mucosa and the smooth muscle layers are arranged differently in these organs. Their epithelial linings are also stratified keratinizing.

The muscularis mucosae in the reticulum and the reticular groove are extension of the esophageal muscularis mucosae. The muscularis mucosa in the reticulum forms a well-developed continuous network of smooth muscle following the reticular crests. The tunica muscularis in this compartment is composed of two layers of smooth muscle orientated obliquely and crossing at right angles to each other.

In the reticular groove, the internal layer of the tunica muscularis is arranged transversely and blends with the external layer of the reticular tunica muscularis. The outer longitudinal layer contains both smooth and skeletal muscle fibres, the latter being a continuation of the esophageal musculature.

The omasal tunica muscularis is composed of an outer thin lingitudinal layer and inner thicker circular layer of smooth muscle. The inner most fibres of the inner layer send extensions into the omasal laminae. The muscularis mucosae lie on either side of these extensions with the effect that three layers of smooth muscles are seen in cross sections of the omasal laminae.

The tunicae serosa of the reticulum, reticular groove and omasum are similar to that found in the rumen.

1.3.3 Abomasum

The transition from the omasum to the abomasum is sharply defined by the change from stratified keratinizing to simple columnar epithelium on the omasal side of the lip separating these chambers, and by the occurrence of typical glands containing zymogen- and acid-secreting cells (Stinson and Calhoun, 1976 a). The fundic part of the abomasum is lined by mucosa with gastric glands which extend into the body. A small region of cardiac type glands (mucus secreting) is found close to the omaso-abomasal orifice. The pyloric region, composed of typical mucus-secreting glands, is similar to the pyloric gland region of the simple stomach in other animals. There is evidence that volatile fatty acids could penetrate the abomasal epithelium (Ash, 1961).

1.4 Embryology

The embryology of the ruminant stomach (bovine) has been extensively studied (Pernkopf, 1931; Warner, 1958) and reviewed (Comline et al., 1968; Moir, 1968) and was shown that it originated from the primitive stomach spindle as any other vertebrate stomach, and that the esophagus has nothing to do with its development. The idea that the forestomach compartments are esophageal dilatations has however, persisted in the literature including a recent veterinary anatomy outline-textbook (Stokoe, 1968) and a meat inspection manual (MacGregor, 1980). The following accounts are primarily based on the descriptions of Warner (1958) and Comline et al. (1968).

1.4.1 Organogenesis

The early development of the ruminant stomach is similar to any other mammalian stomach until the 7 mm stage when a prominence develops at the dorsocranial aspect of the spindle. This enlargement extends cephalad, dorsad and to the left and is later identified to give rise to the rumen and reticulum. At the 9 mm stage, an indication of the positiuon of the gastric canal appears ventrally as an expanison along the lesser curvature of the stomach

spindle. Later, a bulb-like swelling also appears at the lesser curvature caudal to the gastric canal to constitute the omasum. The tapering caudal stomach region is destined to become the abomasum. By the 12 - 15 mm stage, all the parts of the stomach are recognised with the rumen being the largest cranially. The gastric canal has appeared connecting the esophagus with the omasum marking the developing reticular groove. The omasal groove can also be recognised at the dorsal aspect of the omasum.

The next series of development between the 20 - 50 mm stage involves expansion, migration and regional differentiation resulting in marked division of the compartments and with the greatest increase in size occurring in the rumen. The reticulum is marked off from the rumen as a swelling caudal and ventral to it. The first folds in the omasum are distinguishable and the abomasum also shows folds similar to that in the adult. A shelf of muscular tissue which forms the longitudinal pillars appears at the blind end of the rumen and divides it into a dorsal and ventral parts. The reticular groove becomes markedly enlarged with its right lip developing first.

Starting at the 50 mm stage, a period of external and internal differentiation takes place. The typical honeycomb mucosal pattern in the reticulum becomes established at this stage and the secondary folds appear in the omasum. The most marked event however, is the series of complex rotational movements occurring in the whole stomach resulting in the adult conformation. The rumen rotates 180^{o} so that the primitive left dorsal aspects of the omasum and abomasum become right ventral and the esophageal groove which was originally in a left ventral position becomes right dorsal. When the rotation is complete, the mesenteries are also changed in position and a strong adhesion between the dorsal sac and the crura of the diaphragm develops to support the stomach from the dorsal body wall. Later, differential growth of the various compartments occur with the abomasum becoming the largest part at birth. This differential growth may be due to the developing swallowing reflexes

and the redirection of fluid by the esophageal groove into the abomasum (Duncan and Phillipson, 1951; Comline et al., 1968).

1.4.2 Histogenesis

The histological changes occurring in the sheep forestomach during the prenatal period of development has been studied by many workers including Wardrop (1961 b), Henrikson (1970 a) and in the omasum in particular, by Lubis and O'Shea (1978). There appears to be no data reported in the literature on the histogenesis of the sheep abomasum although comparable information on the bovine abomasum can be found (Warner, 1958). The following descriptions summarises the histological development of the forestomach during the pre-natal period.

The forestomach wall in early fetal specimens (46 days) is clearly differentiated into mucosal, muscular and serosal coats. The mucosal surfaces of the rumen, reticulum and omasum are smooth; their epithelia consist of two types of cells - a single layer of densely packed basal columnar cells and multiple layers of swollen hexagonal cells extending to the free surface of the epithelium. These swollen cells contain large amounts of glycogen in their cytoplasm as demonstrated by P.A.S. staining (Henrikson, 1970 c; Ramkrishna and Tiwari, 1979; Arias et al., 1980).

Minor changes occur in the mucosa of the rumen before 100 days. The development of mucosal ridges in the reticulum is seen at 70 days with the basal layer of the epithelium folded to form the ridges. In the omasum, the four orders of laminae are found at 70 days with the first order having developed at 35 days. The appearance of papillary projections from the connective tissue manifesting the formation of conical papillae in the omasum has occurred at 70 days although its mucosal surface is still smooth.

The lamina propria and submucosa of the forestomach in the early fetus consists of loose network of cells lacking in collagen or muscularis mucosae. At 70 days however, collagen fibres are found in the submucosa.

In the omasum, thin strands of smooth muscle fibres coming from the tunica muscularis appear at the centre of each laminae at 70 days to constitute the muscularis mucosa. The tunica muscularis is relatively thin in early specimens and increasesin width, in size of muscle bundles and in the number of connective tissue fibres between the layers as the fetus grows.

The most marked changes in the prenatal histology of the forestomach take place between 100 days of fetal life and birth, especially in the rumen. The basal layer of the epithelium becomes folded to accommodate the advancing papillary cores formed by the submucosa. In the reticulum, further folding of the basal layer and submucosa produces the developing reticular ridges and papillary cores. Further lengthening of the omasal laminae occurs with the conical papillae developing in the first and second order laminae. The superficial cells of the epithelium in the reticulum and omasum becomes flattened as they are pushed towards the surface.

At birth, the rumen mucosal surface is studded with papillae which are joined for the first third of their length. In the reticulum, the reticular ridges are well-developed and conical papillae are found between the ridges although not on them. In the omasum, a fifth order of laminae is seen to develop (Wardrop, 1961 b) although this is disputed by Lubis and O'Shea (1978) who found no evidence of the 5th order even in the adult.

The transformation of the epithelium from stratified polyhedral to stratified keratinizing occurs during the last 50 days in utero. The epithelium at birth consists of: (a) a distinct basal layer of densely packed cells; (b) an intermediate layer of less densely packed cells with glycogen in the cytoplasm and equivalent to the stratum spinosum; (c) a discontinuous and indistinct stratum granulosum; and (d) an outer layer of flattened keratinized cells (Wardrop, 1961 b). Henrikson (1970) however, believes that the forestomach epithelium is still non-keratinized at birth and becomes keratinized during the first three days after birth.

1.5 Functional Organisation of the Forestomach Epithelium

A wide range of physiological experiments have been carried out to investigate the absorptive and transporting functions of the forestomach epithelium (for a general review of work in these fields, see Keynes, 1969; Keynes and Harrison, 1970).

Although it has been shown that a significant net movement of water occurs across the epithelium from rumen to blood direction (Keynes, 1969), according to Engelhardt (1970) this is not large under physiological conditions. It is suggested that a net flux of water due to osmotic gradients normally present between the rumen contents and blood is prevented by a zone of high osmotic pressure in the deeper layers of the epithelium (Engelhardt, 1970).

As far as the transport of volatile fatty acids is concerned, in a review by Stevens (1970), it was shown that absorption of VFAs from rumen to blood can be explained by diffusion alone and could be modified by their metabolism in the epithelium, and by pH and electrical gradients between epithelial contents and blood, due to asymmetry in the relative permeabilities of the tissue membranes facing the lumen and blood. Many studies including those of Pennington (1952); Walker and Simmonds (1962) and Sutton, McCilliard and Jacobson (1963) have shown that VFAs are metabolised in the epithelium with butyrate used at a greater rate than other straight- and branched-chain VFAs resulting in the production of considerable amounts of ketone bodies.

With regard to the transport of inorganic ions, the discovery that the contents of the rumen in sheep are electrically negative by a potential at about 30 mV with respect to blood (Dobson and Phillipson, 1958) suggested that Na^+ was being actively transported from rumen to blood thus generating a potential difference (Keynes and Harrison, 1970). It is now generally accepted that most ions are absorbed across the epithelium against a

net electrochemical gradient.

Active transport of ions in any epithelium may either directly use a high-energy compound such as ATP or indirectly use energy through coupling of the transported species to the electrochemical gradient of another ion (Lewis and Wills, 1982). The first type is exemplified in the rumen epithelium by the finding that Na^+ net flux from rumen to blood is completely abolished by ouabain which could indicate a complete dependence on ouabain sensitive ATPase (Harrison, Keynes, Rankin and Zurich, 1975) which has been biochemically demonstrated in basal membrane fractions of epithelial cells (Hegner and Tellhelm, 1974). The second mechanism is illustrated by the finding that a part of the Cl^- flux across the epithelium is coupled to Na^+ transport.

A separate K^+ pump has been postulated to operate in the epithelium since a part of the K^+ flux was found to be insensitive to ouabain (Harrison et al., 1975).

Urea has also been found to diffuse freely across the epithelium from blood to rumen direction, a part of which is probably hydrolysed into ammonia in the cornified layer (Houpt, 1970).

The structural interpretation of these physiological data is difficult because of the complexity and stratification of the epithelium (Keynes, 1969).

1.5.1 The ultrastructure of the forestomach epithelium

Electron microscopic studies of the epithelium taken from different species of ruminants have shown that the ultrastructural organisation appears to be similar in different locations in the forestomach (Steven and Marshall, 1970). The following descriptions summarise what has been reported in the literature on the ultrastructure of the cells of the forestomach epithelium.

The columnar cells of the stratum basale contain many mitochondria situated above and below the ovoid nuclei. Free ribosomes and rough endoplasmic reticulum are scattered between the mitochondria. A few small bundles of tonofilaments are sometimes arranged parallel to the cell membranes. Cytoplasmic microvilli extend from the

basal border of the cells to the basement membrane.

Irregularly interspersed between the basal cells are branching mononuclear cells which are smaller in size, have sinuous irregular outlines, have no points of attachments to adjacent cells and have cytoplasmic processes which thread their way through the intercellular spaces (Marshall and Steven, 1969; Steven and Marshall, 1970; 1972). Further ultrastructural works on these cells have identified some of them as Langerhans cells (Gemmel, 1973; Nagatani et al., 1974; Cerneke, 1977) which in certain conditions, appear to have phagocytic properties (Gemmel, 1973; Tamate and Fell, 1978). Other cells are structurally similar to lymphocytes (Steven and Marshall, 1970; Nagatani et al., 1974) and macrophages (Steven and Marshall, 1972).

The cytologic features of the cells in the stratum spinosum include numerous mitochondria and ribosomes distributed throughout the cytoplasm. Thick bundles of tonofilaments occur around the periphery of the cell; the cisternae of the rough endoplasmic reticulum are dilated with fine granular material, the so-called Er-protein (Lavker and Matoltsky, 1970) which further accumulates as the cells become flattened as they pass towards the lumen. Keratohyalin granules may also be found in the cells of this layer.

The keratohyalin granules in the stratum granulosum appear in large aggregated masses. The nuclei of the cells in this layer begin to show signs of degeneration; the endoplasmic reticulum continue to be dilated and filled with Er-protein. Tonofilament bundles further accumulate in the peripheral cytoplasm; membrane-coating granules are also seen in great numbers in the periphery of the cell (Lavker, 1969; Lavker and Matoltsky, 1970).

The most prominent ultrastructural features of the transitional cells are, the presence of lysosomal bodies in the cytoplasm, the degraded nuclei and cellular organelles, dispersed keratohyalin granules and highly dilated endoplasmic reticulum with Er-protein which are released in the cytoplasm upon degradation of endoplasmic reticulum and later dispersed (Lavker and Matoltsky, 1970). The degraded cell organelles are discharged into the

intercellular spaces (Lavker and Matoltsky, 1970).

Swollen cells in the stratum corneum appear to be those with a predominance of centrally placed Er-protein, while flattened cells resulted from granular cells with less developed endoplasmic reticula (Steven and Marshall, 1970).

From the accounts of Hyden and Sperber (1965); Lavker, Chalupa and Dickey (1969); Henrikson (1970 c); Steven and Marshall (1970); Henrikson and Stacy (1971); Schnorr and Wille (1972 b) and Scott et al. (1972), a pattern of distribution of intercellular junctions in the epithelium emerges.

In basal cells, the microvillus-like array of processes from the basal surface of basal cells are attached by hemidesmosomes to the basement membrane. The lateral and apical borders of the basal cells often appear smooth and closely apposed to neighbouring cells; they may also show a more wavy course with wide intercellular spaces (from 0.1 to 2 μm wide) between small areas of contacts - e.g., desmosomes, macula occludentes (Henrikson and Stacy, 1971) and tight junctions (Scott et al., 1972).

In the stratum spinosum and stratum granulosum, desmosomes increase in number and appear well-developed. Macula occludentes (Henrikson and Stacy, 1971) and tight junctions (Steven and Marshall, 1970) occur in great numbers. The latter are usually situated in close proximity to desmosomes and appear as short strands. The intercellular spaces in the mid-epithelium are usually wide, from 0.1 - 0.5 μm.

At the outer limits of the stratum granulosum, the intercellular spaces are obliterated by the formation of long strands of tight junctions (zonulae occludentes) and desmosomes; there are virtually no spaces between apposing cells (Steven and Marshall, 1970). Before the transition to the stratum corneum, desmosomes lose their tonofibrils and eventually disappear although a few recognisable elements sometimes persist at the lateral

extremities of keratinized cells. The cells of the stratum corneum are held together by loosely interlocking processes and by thin strands of amorphous material (glycocalyx) which bridge the intercellular spaces (Steven and Marshall, 1970). Schnorr and Wille (1972 b) have, however, shown the presence of tight junctions in the stratum corneum.

1.5.2 Proposed model for transport pathways in the epithelium

The pathways for the transport of inorganic ions, particularly for the active transport of sodium in the forestomach epithelium have been envisaged as similar to the model proposed for the frog skin (Koefoed-Johnsen and Ussing, 1958; Farquhar and Palade, 1966) because of the structural and functional similarities of the two epithelia.

According to this model, sodium diffuses inwardly through the cells of the stratum corneum. The passage of solutes through the typically durable, insoluble and unreactive keratin in the epithelium has been rationalised by Fell and Weekes (1975) in the following manner:

> There are no water-proofing sebaceous glands in the epithelium; sorption of water and other liquids by non-fibrillary ground-substance or matrix of animal keratins is possible especially under saturation conditions. In soft keratins such as those of the forestomach epithelium, it is possible that part of the water uptake is attributable to the presence of water-soluble materials.

From the cells in the stratum corneum, sodium diffuses through the deeper layers via intracellular routes - i.e., via desmosomes and limited points of contacts (Steven and Marshall, 1970). After reaching a certain level in the epithelium, possibly the middle layers (Henriksson, 1971), sodium is pumped out of the cells into the intercellular spaces. The extracellular accumulation of sodium leads to a localised increase in osmolality and water is therefore drawn into the intercellular spaces. In accordance with the theory of Diamond and Bossert (1967),

the hydraulic forces generated within the interstices sweep the sodium and water down the channels to the basal lamina where there is no selective resistance to the movements of ions and water (Gemmel and Stacy, 1973). Sodium then crosses the basal lamina and enters the blood capillaries.

For this model to work, both a Na^+-K^+-pump in the cells of the middle layer to pump Na^+ into the intercellular spaces, and a barrier to the backflow of diffusion of sodium into the lumen must exist. Although the enzyme Na^+-K^+-ATPase which is thought to be identical to the Na^+-pump has been demonstrated biochemically in the epithelium (Currell and Munn, 1970; Schnorr, 1971; Hegner and Tellhelm, 1974), attempts at its histochemical localisation yielded equivocal results (Schnorr, 1971; Henrikson, 1971; Gray and Habel, 1979). (The localisation of Na^+ pump sites is discussed in Section 4.7.)

The existence of a diffusion barrier in the ruminal epithelium has been demonstrated physiologically and postulated to be in the keratinized layer (Houpt, 1970) however, its structural location is subject to conflicting reports.

In an ultrastructural study of the rumen epithelium taken from the ox, sheep and goat, Steven and Marshall (1970) referred to the presence of extensive zonula occludentes or tight junctions in the stratum granulosum in the cells just below the junction with the stratum corneum. Because of these structures, they postulated that the barrier to diffusion is located in this layer.

From tracers studies using horseradish peroxidase, lanthanum and ferritin, Henrikson and Stacy (1971) proved the existence of a diffusion barrier in the epithelium: they found that the intercellular spaces in non-keratinized layers were readily permeable to horseradish peroxidase and lanthanum, indicating that the barrier to free diffusion is not found in these levels. The keratinized layer however, appeared much less permeable and marker substances were rarely encountered. As with an earlier study by Henrikson (1970 b), they noted the absence of zonulae occludentes that in the frog skin (Farquhar and

Palade, 1965) constitute the permeability barrier, and postulated that the barrier is in the keratinized layer and is related to the complex intercellular channels, the promient glycocalyx and the nature of the keratinized cell itself.

Schnorr and Wille (1972 b) also postulated that the barrier is located in the keratinized layer, however, they pointed out that this was due to the presence of zonulae occludentes between the cornified cells next to the stratum granulosum.

Gemmel and Stacy (1973) also consistently found zonulae occludentes which were unaffected by changes in ruminal osmolality (hyperosmolality), this time, in the outermost layer of the stratum granulosum. They stressed that these were different from tight junctions described by Steven and Marshall (1970) and that these junctional structures in this layer constitute the permeability barrier in the epithelium.

1.5.3 Histochemistry

Histochemical studies on the rumen epithelium have demonstrated various chemical components and enzymes in the different layers of the epithelium.

The epithelium is rich in neutral and both sulphated and non-sulphated mucosubstances, particularly in the stratum corneum (Habel, 1963; Lavker, Chalupa and Opliger, 1969; Henrikson, 1970 a; Schnorr and Hild, 1974) which have been associated with the transport function of the epithelium (Schnorr and Hild, 1974). However, Henrikson (1970 a) believes that the prominent mucopolysaccharide coating of the keratinized cells is an integral part of the permeability barrier in the epithelium.

Neutral fats (Habel, 1959; Lavker, Chalupa and Opliger, 1969; Cerny, 1977) and phospholipids (Cerny, 1977) have been located in the cells of the stratum corneum and in the intercellular spaces (Cerny, 1977). Phospholipids have also been demonstrated in the stratum

granulosum (Habel, 1959; Cerny, 1977). The function of lipids in the epithelium remains obscure. Habel (1959) does not consider them as absorption products but as products of normal keratinization.

Glycogen has been demonstrated in the epithelium of the forestomach in pre-natal and neonatal animals (Habel, 1963; Henrikson, 1970 a; Lubis and O'Shea, 1978; Arias et al., 1980). It is particularly abundant in the cells of the intermediate layer of the epithelium and has been considered as energy source of epithelial cells for local metabolism (Lubis and O'Shea, 1978).

Positive sulphydryl and disulphide group and histidine reactions in the rumen epithelium are correlated with keratinization (Henriksson and Habel, 1961; Lavker, Chalupa and Opliger, 1969). They are especially abundant in keratohyalin granules and tonofilaments.

Among the various enzymes localised in the epithelium, alkaline phosphatase has been consistently located in the stratum corneum (Habel, 1963; Lauwers et al., 1974; Filotto and Chiericato, 1977; Galfi et al., 1982). The function of alkaline phosphatase is not known as it is present in a wide variety of tissues and locations and composed of several isozymes (Doty, 1980). It has been implicated, however, that in the rumen epithelium, it may play a role in the transport process (Galfi et al., 1982).

Acid phosphatase has been found to be distributed throughout the epithelium (Habel, 1959) but is more intense in the transitional layer (Gardner and Scott, 1972). Its presence has been correlated with keratinization particularly in the degradation of cell organelles during the transformation phase (Lavker and Matoltsky, 1970). Its presence in the stratum corneum has led Gardner and Scott (1972) to believe that cornified cells may have some phagocytic properties.

A systematic study on dehydrogenase histochemistry of the bovine ruminal epithelium has demonstrated

9 dehydrogenases which are all part of the intermediary metabolic pathways. Lactic dehydrogenase was particularly found active in all ages including the newborn, while beta-hydroxy butyric dehydrogenase gave strong reaction in the epithelium taken from the adult. Ruiz and Molina (1976) have shown the increase activity of lactic dehydrogenase in concentrate-fed animals.

Various ATPases have been demonstrated in the epithelium. Henrikson (1971), in an attempt to localise the Na+-K+-ATPase transport enzyme, has localised instead, Mg++-ATPase in the intercellular spaces in the middle layers of the epithelium. Schnorr (1971) did not find any differences in the distribution of Mg++-, Ca++- and Na+-K+-ATPases in the deeper layers of the epithelium in goats. Gray and Habel (1979) demonstrated the preponderance of Mg++-ATPase on the cytoplasmic membranes of granular cells and in the stratum corneum and mitochondria.

1.5.4 Epithelial cell differentiation

In its broadest sense, cell differentiation in a keratinizing epithelium involves the transformation of committed basal and spinous cells into keratinized cells which eventually exfoliate. This also involves complex structural, metabolic and kinetic changes in all levels of the epithelium. In order to maintain a steady state, cells lost from sloughing are replaced by cells produced from basal cell division.

1.5.4.1 Keratinization

The formation of keratinized cells occurs in two phases (Matoltsky, 1975): (1) synthetic phase, when the cell is manufacturing specific products that are necessary for keratinization and (2) transformation phase, when a fully developed granular cell is transformed into a cornified cell.

Differentiation products in the rumen epithelium include tonofilaments, membrane-coating granules, endoplasmic reticulum-protein (Er-protein) and keratohyalin granules. The Er-protein in particular was first observed in the rumen epithelium (Lavker and

Matoltsky, 1970) and provided the evidence that besides keratohyalin, other proteins are also involved in the formation of the keratin matrix.

The sequential events that lead to the formation of horny cells in the epithelium is particularly observed in the transitional cell and have been characterised by Lavker and Matoltsky (1970). They observed that transformation is initiated by the release of hydrolytic enzymes as evidenced by the appearance of lysosomal bodies. As a result, the nucleus and other cell organelles including the membrane-coating granules are degraded; lysed material passes through the cell membrane and accumulates in the intercellular space as an amorphous substance. Keratohyalin granules coalesce and then spread in the cytoplasm mixing with the released Er-protein from the endoplasmic reticulum. These components infiltrate the filament network in the periphery, and after consolidation, a fibrous-amorphous complex is formed, contained in a thickened plasma membrane. Eventually, keratinized cells undergo desquamation.

1.5.4.2 Mitotic Index

Mitosis has long been recognised as the principal mechanism by which the cell population of an epithelium is maintained. DNA microphotometric study on the ruminal epithelium in the sheep has confirmed that basal cells are the proliferative fractions of the epithelium (Ohwada and Tamate, 1979). The cell kinetics of the ovine ruminal epithelium in normal as well as in experimental conditions have been extensively studied using the mitotic index as the marker of activity.

It is well-known that volatile fatty acids are stimulatory to the growth and maturation of the ruminal epithelium. Sakata and Tamate (1976 a; 1976 b; 1978 b; 1979) have elucidated some of the mechanisms by which VFAs mediate epithelial maturation in a series of experiments involving the intraruminal administration of salts of butyrate, propionate and acetate. They demonstrated that the increase in the intraruminal

production of the acids accelerated rumen epithelial proliferation as shown in the rise of mitotic indices after administration of the acids. It was also shown that the mitogenic effect of acetate and propionate is weaker than that of butyrate and that slowly administered acids did not elicit any significant change in the mitotic indices.

Other experiments have also shown that the mitotic index is modified by the pattern of feeding. Tamate, Kikuchi and Sakata (1974) have shown that fasting significantly lowered the value of mitotic index and that recovery to normal values occurred after refeeding. Sakata and Tamate (1974) have also shown that the mitotic index was extremely high in intermittently-fed sheep.

Sakata and Tamate (1978 a) reported the presence of circadian rhythm in the rate of cell division, being low in the morning, high in the afternoon and in the evening, and declined at about midnight. These fluctuations however, may have been influenced by food intake because mitotic indices became higher several hours after the meal (Sakata and Tamate, 1978 a).

Tamate and Fell (1978) demonstrated that changes in the composition of the diet also markedly altered the rate of mitosis. When feed was changed from hay to barley, there was an increase in the rate of mitosis; a subsequent abrupt change of diet from barley to hay resulted in the fall in the rate of mitosis.

Sakata and Tamate (1979) have determined that the normal values for the mitotic index in conventionally-fed sheep do not exceed 1 % (Table I).

All these experiments showed that the single-most important factor that affects rumen epithelial cell kinetics is food intake. However, other factors which affect cell proliferation in general could also affect cell division in the rumen epithelium. Thivend, Toullec and Guilloteau (1980) has suggested that insulin could also mediate cell proliferation.

Table I. Normal Values of Mitotic Index of the Ovine Ruminal Epithelium from Different Studies

Authors	Mitotic Index (%)
Sakata and Tamate (1974)	0.81
Tamate, Kikuchi and Sakata (1974)	0.81
Sakata and Tamate (1976 b)	0.48
Sakata and Tamate (1978)	0.50
Tamate and Fell (1978)	0.66/0.72
Sakata and Tamate (1979)	0.53

1.6 Establishment of the Ruminant State

The shift of the young ruminant from an essentially non-ruminant state at birth to the ruminant state during its adult life is characterised by major changes in its anatomy and physiology.

At birth, there is an abrupt transition from the sheltered intra-uterine to an exposed extra-uterine environment that imposes numerous stimuli upon the newborn. Although pre-natal maturation (genetically determined and irreversible physical growth and functional differentiation, e.g. lung and kidney tissue maturation) has prepared the newborn for its life outside the uterus, the young animal is actively engaged in postnatal adaptation, adjusting its vital functions to its new environment to achieve survival with minimal risks (Stave, 1970). The physical process of birth itself is a stressful event which subjects the young animal to various trauma such as hypoxia, physical impacts and sudden cooling. However, it appears that the newborn has more tolerance to endure these birth hazards as such stresses do not produce the typical alarm reaction as they would do in the adult (Jilek, Travnickova and Trojan, 1970; Bruck, 1970) thus protecting it from premature exhaustion of its energy reserves (Stave, 1970).

Postnatal adaptations are usually directed towards long term adjustments to the extra-uterine environment and associated with numerous circulatory and metabolic realignments in the young animal arising from the loss of placental routes for gaseous exchange, transference of nutrients and other humoral substances and exretion, and their substitution by the pulmonary, gastro-intestinal and renal routes respectively (Edwards, 1970). Metabolic adjustments for the generation of heat are also characteristic in the young animal especially so in the young ruminant as the dam makes no obvious attempt to provide it with shelter from the environment (Alexander, 1970). Furthermore, there arise different

requirements for the neurohumoral regulatory systems which have to direct these adaptations towards homeostasis and internal independence from the environment (Stave, 1970).

In the young ruminant, development of digestion in neonatal life is similar to the simple stomached ruminants and involves evolution of secretions in the abomasum, pancreas and intestines, development of digestive motility, microbial contamination of the gut, absorption of immunoglobulins and other nutrients, digestibility and the physical development of the digestive tract. The mechanism for their development seems to be mostly predetermined and may be evoked by exogenous factors like food intake and colostrum absorption and/or by an endogenous factor such as hormonal stimulation (Koldovsky, 1970). Typical behavioural patterns in the location and harvesting of food such as teat seeking, suckling and learning to graze and eat solid food accompany digestive development (Matthews and Kilgour, 1980).

Artificial maintenance at the preruminant stage for a long period of time (up to six months) requires further adjustments (Thivend et al., 1980). To many, it is debatable whether an animal whose alimentary tract has evolved for the utilization of fibrous foods should be exploited to the extent of restricting it to the pre-ruminant form (Roy and Stobo, 1975), but this is done in certain European countries in intensive veal production to meet the demand by a more sophisticated palate (Thivend et al., 1980). In this system, the animal is maintained on milk or milk replacers until its slaughter at 150 - 250 kg live weight and receives no solid food. It grows rapidly and continuously at a rate of more than one kg a day and produces a well formed white meat carcass. Its forestomach is non-functional and digestion is maintained at the monogastric stage.

Digestion in preruminant-maintained animals has

not been fully studied. It is known that development of the digestive tract is different from the ruminant. Thivend and Robelin (1980) studied the weight distribution of the digestive compartments in preruminant-maintained and ruminant calves at different liveweights. Their results show that little relative development takes place in the digestive tract as a whole in preruminant-maintained calves and that the total weight of the digestive tract is always lower than that in the normal ruminant animal for any given age.

At weaning, the preruminant is transformed into the ruminant animal and at this time, it undergoes the greatest modification in its digestive physiology. The most notable change which takes place here of course, is the drastic change in the relative sizes of the stomach compartments which results in the rumen becoming the largest. Under normal feeding regimen (milk and roughage), accompanying this increase in size of the rumen are normal rumen movements and rumination, papillary and epithelial development, absorption, saliva production and microbial population which all develop in concert in the establishment of fermentative digestion.

1.6.1 Anatomical development

In the normal lamb, the transformation from the pre-ruminant to the ruminant phase of digestion occurs in three stages: (a) a non-ruminant phase, from birth to three weeks of age; (b) a transition phase, from three to eight weeks of age; and (c) adult phase from eight weeks of age onwards (Wardrop and Coombe, 1961; Leat, 1970). During the first two weeks of life, although the young may nibble solid food - e.g. grass - little is actually eaten before the third week. The animal is entirely dependent on the mother's milk, and its forestomach, although present, is not well-developed and is non-functional. Milk bypasses the rumen through the reticular groove and it is assumed that digestion is similar to that in simple stomached neonates. During the process of weaning at the second stage, the young animal relies progressively less on the

mother's milk and increases its uptake of solids. Subsequently, milk is replaced by the adult type of diet and by the eighth week, the rumen becomes fully functional; the young ruminant can be weaned without serious setbacks to growth. During this period, there is a concomitant rapid growth of the rumen followed in order, by the reticulum, omasum and abomasum. By the eighth week, the relative adult proportions of the different compartments are reached suggesting that from the anatomical viewpoint, comparatively adult rumens in lambs are attained at this age (Leat, 1970).

The changes in the capacities of the stomach compartments during the post-natal development of the lamb are shown in Table II.

The normal growth pattern of the forestomach however, can be manipulated by feeding the animal with different kinds of diets - e.g. as in pre-ruminant maintained veal calves. The development of the rumen in similar lambs proceeds until the third week after which it becomes arrested. However, rumen development proceeds in the usual way when solid food is made available (Wardrop, 1961 b).

1.6.2 Histological development

Post-natal histological development of the forestomach involves growth and maturation of papillae into discrete entities, epithelial keratinization, development of epithelial pegs or bulbs, decrease in the number of cell layers in the epithelium, disappearance of glycogen from the cytoplasm of the stratum spinosum and thickening of the muscular walls (Wardrop, 1961 a; Arias et al., 1978; Henrikson, 1970 a).

The pattern of development seems to be age dependent, and according to Arias et al. (1978), corresponds to the completion of the differentiation pattern developed during the prenatal stage. Detailed structure however, is affected by the diet, particularly by the end products of fermentation (Wardrop, 1961 b).

Numerous experiments involving dietary manipulation

Table II Proportions of the Compartments of the Stomach of Grazing Lambs as Percentages of Weight of the Whole Stomach*

Age (days)	Reticulum and Rumen		Omasum	Abomasum
1		31 (combined)	8	61
14	(6-8)	36 (rumen)	5	59
20-21	(9-10)	44-51 (rumen)	5-6	39-40
49		71 (combined)	5	24
56-57	(10-11)	64-66 (rumen)	5-6	18-20
112		73 (combined)	6	21
Adult		69 (combined)	8	23

*Adapted from Church (1976) and Phillipson (1977)

in calves and lambs have suggested that end products of rumen fermentation, the volatile fatty acids, are chemical stimulants to papillary and epithelial development and inert bulk materials are physical stimulants to muscular development. These were suggested first by Brownlee (1956) and Warner, Flatt and Loosli (1956) from the results of their independent feeding trials undertaken to examine the effect of various dietaries on the development of the ruminant stomach. They showed that: (a) calves fed with an all milk diet showed little or no papillary development; (b) calves fed with low fibre-high concentrate (hay and grain) rations showed high degree of papillary development; (c) calves fed with all concentrate rations showed little difference in general papillary appearance from those of calves receiving hay or hay-grain rations although Brownlee (loc. cit.) noted degenerative changes in the mucosa such as superficial erosions; and (d) calves fed with milk and peat moss or nylon bristles to act as bulk showed little or no papillary development but with accompanying rumen tissue weight increase due to muscle development. These studies indicated that the chemical nature of the diet - i.e., the rapidity by which the diet is fermented into absorbable fractions - rather than its physical nature - i.e., roughage content - is largely responsible for papillary development.

Later, it was shown that salts of volatile fatty acids administered into the rumen via a cannula, caused marked papillary growth (Flatt, Warner and Loosli, 1958); that butyrate was more effective than propionate or acetate in eliciting papillary growth (Sander, Warner, Harrison and Loosli, 1959) and that papillae which developed in response to the infusions of these salts were histologically similar to those resulting from natural feedstuffs (Tamate, McGilliard, Jacobson and Getty, 1962). For VFAs to be effective however, a critical concentration in the rumen must be present (Tamate, McGilliard, Jacobson and Getty, 1963). These findings

provided strong evidence that VFAs are the main stimulants for papillary growth.

It was also later shown that these acids are also necessary for the maintenance of rumen papillae as calves reversed to milk diet showed disappearance of papillae from their rumen (Harrison, Warner, Sander and Loosli, 1960).

It was postulated that growth of rumen papillae is the result of the metabolism of VFA by the rumen wall and/or their effect on blood flow (Warner et al., 1956; Sander et al., 1959; Fell and Weekes, 1975) and that VFAs may provide for the energy needs of the tissue (Annison and Lewis, 1959). It is known that these acids are actively metabolised in the epithelium (Pennington, 1952). There is also usually an increase in blood flow through the stomach, intestines and pancreas after a meal (Grim, 1963) and the VFAs are active in creasing the rate of blood flow from the rumen (Phillipson, 1959). It is not known whether or not hyperemia promotes epithelial hyperplasia (Fell and Weekes, 1975). Tamate et al. (1962) and Arias et al. (1978) reported the regression in papillary height at birth and during the monogastric stage of digestion which they attributed to the withdrawal of maternal VFAs in circulation.

1.6.3 Physiological development

The post-natal digestive development in the ruminant animal has been reviewed in detail by McGilliard, Jacobson and Sutton (1965) and by Thivend, Toullec and Guilloteau (1980). In addition, the physiological aspects of suckling and milk passage through the ruminant stomach (Titchen and Newhook, 1975), the development of the rumen microorganisms (Eadie and Mann, 1970) and the feeding behaviour during post-natal development (Matthews and Kilgour, 1980) have also been reviewed. The reader is referred to these excellent articles for detailed discussion on these aspects. This section will mainly be concerned with the development of functions attributed to the forestomach epithelium - i.e., absorption, transport

and metabolism.

1.6.3.1 Absorption and transport of solutes

Since absorption from the rumen is an essential factor in rumen function, it is probable that the development of the rumen in the young animal is accompanied by the gradual establishment of the absorptive process. Absorptive ability, if related to mucosal structure, would be expected to change as the structure matures.

Sutton, McGilliard and Jacobson (1963 a) showed that the ability to absorb large quantities of acetic acid is not inherent in the rumen and does not develop in calves reared on milk diets but is stimulated upon the ingestion of solid feeds. They also showed that the intake of solid feeds also stimulated structural development as determined by the extent of papillary growth and this may account for some increase in absorptive ability. They also obtained similar results with butyric and propionic acids.

However, Khouri (1969) has reported that in fact, young calves (three to five days old) are capable of absorbing VFAs from the rumen, and that this ability is inherent in the rumen epithelium and does not depend on the intake of solid food or rumen fermentation. It has also been shown that utilisation of butyrate by the rumen wall of the new-born lamb is low, but by three weeks of age, it exceeds adult rates (Walker and Simmonds, 1962). Also, the calf has the enzymes necessary to metabolise VFA before fermentation begins (Young, Tove and Ramsey, 1965) and is equipped to meet a significant amount of its maintenance energy requirements from volatile fatty acids (Thivend et al., 1980). Thus, it appears that the young animal could absorb VFAs at any age although this would depend on the presence of VFAs in the rumen. The physical capacity of the reticulo-rumen, however, is not sufficiently advanced for the animal to ingest solid feeds to satisfy its requirements although at eight weeks it can (Leat, 1970). It has been shown that maximal concen-

trations of VFAs in the reticulo-rumen is attained at eight weeks and then decreased to adult values, suggesting that a more efficient absorption of VFAs from the rumen develops after eight weeks of age (Boda, Riley and Wegner, 1962; Leat, 1970).

The development of transport of ions has received less attention than that of VFAs. So far as this writer is aware, only the study of Scharrer, Medl and Liebich (1981) on the active transfer of Na^+ and Cl^- in isolated short-circuited immature epithelium taken from one-week-old and three week-old lambs has been reported. From this study, it was calculated that net Na transport in immature epithelium is of similar magnitude to those obtained from the rumen epithelium of the adult sheep and net Cl transpoft in 3 week-old lambs is also as high as in the adult although three times higher than in one-week-old. It was concluded that, in spite of their structural immaturity, epithelium taken from one-week- and three-week old lambs compares favourably with mature epithelium as far as the transport of Na^+ and Cl^- is concerned. Also, the development of mechanisms for the active transport of these ions can occur independently.

1.6.3.2 Metabolism

The transition of the young animal to the adult ruminant is accompanied by changes in carbohydrate metabolism. During weaning, the young ruminant is transferred from a high-carbohydrate to a high-fat diet and volatile fatty acids become the major source of energy (Leat, 1970).

Many studies, including those of Pennington, 1952; Walker and Simmonds, 1962; Sutton, McGilliard and Jacobson, 1963 b) have shown the preferential metabolism of individual fatty acids by the mature rumen epithelium, with butyrate being metabolised more readily than propionate and acetate. The same studies have shown that butyrate is metabolised extensively into ketone bodies (acetoacetate and D(-)beta-hydroxybutyrate) during its absorption by the epithelium, while acetate

and propionate are absorbed as such to be metabolised elsewhere in the body (Weigand, Young and McGilliard, 1972).

In a study of the metabolic activity of the rumen wall during the early development of the lamb, Walker and Simmonds (1962) have demonstrated that utilisation of butyrate in newborn lambs was lower than that in the adults, but exceeded adult levels at 3 weeks of age or longer. It was also shown in the same study that utilisation of acetate and propionate did not change markedly with age. Ketone production was found to be negligible at birth, but closely followed butyrate utilisation during development. The utilisation of butyrate was found to decrease in milk-maintained calves.

Sutton, McGilliard and Jacobson (1963 b), in a similar study have shown the low metabolism of VFA in the epithelium in milk-fed calves and the increased uptake of VFA and higher production of ketones in calves fed with milk, hay and grain. They indicated that epithelial metabolism is quite inactive before growth of papillae has commenced and that VFA metabolism is associated with intake of solid feed.

Giesecke, Beck, Wiesmayr and Stangassinger (1979) have shown that epithelial metabolism, at least as far as respiration is concerned is active even before development of papillae has begun. They have shown the decreased O_2 uptake (both endogenous and in the presence of glucose or butyrate) in developing epithelium. They have also demonstrated that the decrease in O_2 uptake was age-dependent and not dependent on epithelial development as respiration was still reduced in milk-maintained calves. The alterations in respiration were accompanied by a change in the dominant substrate of oxidation: the uptake of O_2 where glucose is the primary substrate decreased over the endogenous uptake during epithelial maturation, while that associated with butyrate increased over endogenous respiration. These authors also reported that endogenous production of ketones in undeveloped

and mature epithelia was similar and increased in both tissues in the presence of butyrate. They took this to mean that ketogenesis was functional in undeveloped epithelium and independent of ruminal fermentation.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Animals

A total of thirty-two animals were used in the study:

(a) Two mature non-pregnant Romney cross-bred ewes of unknown ages were embalmed in buffered formalin and used for gross dissection of the stomach.

(b) Thirty Romney-cross lambs, eighteen reared on Massey University No. 1 Sheep Farm pastures during the 1981 lambing season and twelve reared on Best's Farm (Massey University) pastures during the 1982 season were killed by pentobarbitone overdose (Section 2.3). Stomach tissue samples from lambs killed in 1981 were used for histologic and conventional electron microscopic study of the forestomach epithelium while samples from lambs killed in 1982 were used for Na^+-K^+-ATPase cytochemistry of the ruminal epithelium.

Five lambs (three in 1981 and two in 1982) per age group were taken from their dams on pasture at the following ages: (i) within 24 hours of birth; (ii) 12 days; (iii) 23 days; (iv) 34 days; (v) 45 days and (vi) 56 days. These selected ages were considered to cover sufficiently the stages of development of lambs as outlined by Wardrop and Coombe (1961) and Leat (1970) (see Section 1.6.1). The birth dates of these lambs ranged from 22nd July to 28th August in 1981 and 10th to 23rd August in 1982 (Appendix I). The lambs had been individually tagged with numbered ear tags and spray-painted with numbers on each flank within 12 hours of birth for identification purposes. Up to the time of removal of the lambs, all the ewes and lambs available for that year were run as one flock. On the day of collection, the lambs were removed from pasture between 8 and 9 a.m. and tissue samples were collected (Section 2.3) between noon and 2 to 3 p.m. the same day.

2.2 Gross Dissection

The stomachs of the two embalmed sheep were removed

from the carcasses and the reticulo-rumens dissected following the method suggested by McGavin and Morrill (1976 a) for the stomach of the ox.

Dissection Technique for Examination of the Bovine Reticulo-rumen (McGavin and Morrill, 1976 a)

1. Cut the esophagus several centimetres cranial to the cardia and free the reticulo-rumen from the omasum and abomasum by cutting through the reticulo-omasal junction.

2. Manually detach serosal, fibrous and smooth muscle attachments in all grooves and then lay the reticulo-rumen on its right side and make three incisions.

3. Start the first incision on the cranial surface of the reticulum and cut dorso-caudally in a sagittal plane to a point where an extension of the reticulo-ruminal groove would cross the dorsal greater curvature.

4. Make a second incision from the cranial to caudal groove just dorsal to the left longitudinal groove and then join the end of the first incision with the cranial end of the second.

5. Examine the mucosa and dissect the caudodorsal, caudoventral blind sacs and ventral sac free so that their mucosa may be examined carefully.

The omasum was opened along the omasal groove and the abomasum along its dorsal length and their mucosae were also examined.

The technique provided optimal examination and evaluation of the mucosa of the forestomach. Stomachs from 56 day-old lambs and from one-day-old lambs were also dissected.

2.3 Tissue Sampling

All animals were anesthetised by intraperitoneal injection of a 60 mg/ml solution of pentobarbitone sodium (South Island Chemicals) at a dose rate of 0.5 to 1.0 ml/kg body weight. Induction of anesthesia by this route, although not as rapid as by the intravenous route, was

quite short and accompanied by minimal movement by the animal. In some cases, especially in older animals, the higher dose rate had to be administered for the anesthetic to have sufficient effect. Anesthesia developed progressively, with the palpebral reflexes disappearing after 7 - 10 minutes. Once a lamb was deeply anesthetised, as shown by the presence of thoraco-abdominal respiration, absence of palpebral reflexes and all but sluggish withdrawal reflexes, its stomach was exposed through a left flank incision and 4 cm^2 whole thickness pieces of the stomach wall were quickly excised with scissors from representative stomach sites as shown in Figure 2.1.

For light and electron microscopic studies done in 1981, samples were taken from:

A. All ages, including one adult:
   1. Rumen
      a. left wall of caudodorsal blind sac
      b. left wall of caudoventral blind sac
      c. atrium ruminis, cranial sac
   2. Reticulum (cranial wall)
   3. Omasum (laminae)

B. Lambs up to 34 days old only and one adult:
   1. Lips and floor of the reticular groove.

For the cytochemical experiment (Na^+-K^+-ATPase cytochemistry) undertaken in 1982, tissue samples were taken from the caudoventral blind sac only.

After the removal of tissues from the stomach, the animal was immediately killed by pentobarbitone overdose injected directly into the heart. The tissue samples were then trimmed into 3 mm x 6 mm strips and fixed by immersion in different fixatives for light and electron microscopic and cytochemical procedures as outlined below.

2.4 Histological Techniques

Whole thickness samples of the stomach wall (3 mm x 6 mm strips) for histology were fixed in Bouin's fluid for 24 to 48 hours (Culling, 1974). In some cases, when it was not convenient to proceed with dehydration immediately after fixation, the tissues were stored in either distilled water or 70 % alcohol as recommended by

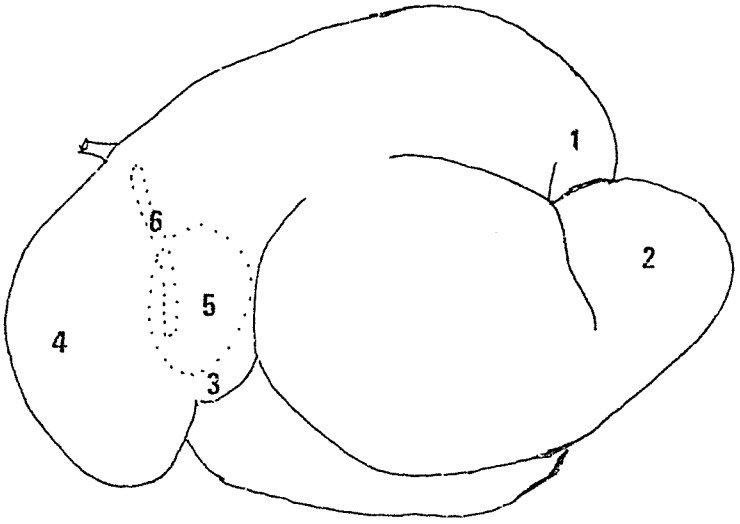
Figure 2.1 Tissue Sampling Sites

1981 lambing season

1 - left wall of caudodorsal blind sac
2 - left wall of caudoventral blind sac
3 - atrium ruminis, cranial sac
4 - reticulum (cranial wall)
5 - omasum (lamina)
6 - lips and floor of the reticular groove
(in lambs up to 34 days only and one adult)

1982 lambing season

2 - caudoventral blind sac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Lillie (1965). Dehydration was achieved through increasing concentrations of ethanol using a Shandon-Elliot automatic tissue processor (Watson Victor Ltd.) and cleared in chloroform and xylene before infiltration and embedding in three changes of fresh paraffin wax at 56°C (Appendix II). Six-micron-thick sections were cut on a microtome (C. Reichert, Germany), floated on warm water, affixed onto glass slides and stained with Haematoxylin and Eosin (H & E), Masson's Green Trichrome (MGT) and Toluidine Blue (TB). A step-by-step description of the staining methods are found in Appendix II.

The sections were then examined and photographed under a Leitz (Wetzlar) Ortholux microscope. Measurements were made on the epithelium by counting the individual cell layers at three locations (in the tips and sides of papillae and interpapillary areas) and subjected to analysis of variance to determine the significance of differences between locations.

2.5 Electron Microscopic Techniques

2.5.1 Conventional electron microscopy

The schedule for processing of tissues for conventional transmission electron microscopy recommended by Weakley (1972) was followed except for some modification as specified below. With this method however, conical papillae taken from the omasum and the reticular groove were not adequately processed and could not be observed in the electron microscope.

Fixation. Tissue samples collected from anesthetised animals were immediately immersed in a pool of ice cold 4 % glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.4, on a wax board. Papillae were isolated from the stomach wall and cut with razor blades into blocks not more than 1 mm^3 in size while still immersed in the fixative. These blocks of papillary tissue were further fixed in the same fixative for 2 to 4 hours at 4°C in capped amber-coloured bottles; recycled 5 ml bottle containers for enzymes used in clinical enzymology were

ideal for this use and were employed throughout the tissue processing schedule. After fixation, the tissues were either rinsed for a total time of 30 minutes in three changes of the same buffer solution used in the fixative to which 7 % sucrose had been added, or washed overnight in this buffer-sucrose mixture.

Post-fixation. The tissues were post-fixed in 1 % osmium tetroxide in either 0.2 M phosphate or cacodylate buffer solution, pH 7.4, in the dark, in a fume cupboard for one hour and then washed in either two changes of the same buffer solution or in distilled water.

Dehydration. The tissues were dehydrated in increasing concentrations of ethanol, absolute alcohol and two changes of propylene oxide. In most cases, as the post-fixation procedure usually finished late in the evening, the tissues were stored overnight in 70 % alcohol at 4°C. The next morning, the tissues were transferred to 95 % alcohol and then to absolute alcohol at 20 minutes each and then to two 15 minute changes of 100 % (anhydrous) alcohol previously stored in contact with silica gel. Dehydration was done at room temperature unless otherwise stated. It was important to ensure that caps were to be on the bottles to avoid evaporation of alcohol or propylene oxide and prevent drying of the tissues.

Infiltration. Infiltration was achieved by placing the tissue in a mixture of equal parts of embedding resin and propylene oxide at room temperature in the fume cupboard and deliberately leaving the caps off the bottles. The procedure was usually initiated by using the last change of propylene oxide from the dehydration procedure and adding to it an equal volume of the embedding resin mixture and leaving the uncapped bottles in the fume cupboard overnight.

Embedding. The embedding medium used throughout was Epon 812 resin (Fluka, Switzerland) mixed by following the procedure of Hayat (1970) to yield blocks which he

classified as being of medium hardness (Appendix III). The tissues were moved to fresh resin in embedding boats and placed in the oven for polymerisation at 60°C. Using this resin mixture, polymerisation was usually completed in 24 hours.

Sectioning and staining. a) One-micron-thick sections were cut on an ultra-microtome (LKB Ultrotome III) using glass knives, fixed onto glass slides on a hot plate (C. Reichert HK 120) at 80°C, stained with either 1 % toluidine blue or methylene blue followed by basic fuchsin and mounted with DPX mountant (BDH Chemicals Ltd.). These sections were used for histological orientation and evaluation of areas to be cut for electron microscopy and for measuring the mitotic indices of the epithelium (see Section 2.6). In addition, some sections were stained by the P.A.S. technique using the method of Weakley (1972). The staining procedures employed in resin sections are found in Appendix III.

b) Ultra-thin sections, pale gold, silver or silver grey (between 1200 - 500 Å) were cut using glass knives on the ultramicrotome and mounted on Formvar-coated or uncoated copper grids. Sections were then double stained using a saturated solution of uranyl acetate in 50 % alcoholo for three minutes followed by lead citrate for 1 - 2 minutes (Venable and Coggeshall, 1965).

Electron microscopy. The sections were viewed and photographed in a Phillips 200 transmission electron microscope operating at 60 kV.

2.5.2 Scanning electron microscopy

Scanning electron micrographs were taken of the reticulum of a one-day-old lamb killed in 1982 in which an abnormal structure in the mucosa was observed (see Section 3.1). The tissue was fixed in a modified Karnovsky's fixative in 0.1 M phosphate buffer. After dehydration through a graded series of acetone, the tissue was dried in liquid CO_2 by the critical point drying method. It was then coated with gold and

observed in a Cwikscan/100 field emission scanning electron microscope. The S.E.M. procedure was kindly undertaken by Mr. D. Hopcroft of the Department of Scientific and Industrial Research electron microscope laboratory in Palmerston North.

2.6 Mitotic Index

The mitotic index, a marker for the proliferative activity of renewing epithelial systems such as stratified keratinizing epithelia (Leblond, 1981) was measured in two lambs in each age group. H & E-stained paraffin and toluidine blue-stained resin sections were used and mitotic figures and nuclei in basal cells immediately adjacent to the basement membrane were counted consecutively. The mitotic index (M. I.) was then expressed as:

$$\text{M. I. (\%)} = \frac{\text{number of basal cells showing mitotic figures}}{\text{total number of basal nuclei observed}} \times 100$$ (Sakata and Tamate, 1976 a).

The mitotic index was examined on the basis of an average count of 2976 cells. Differences within lambs, between lambs and between age groups were examined for significance by applying Chi-square tests.

2.7 Na^+-K^+ATPase Cytochemistry

The strontium capture technique developed by Ernst (1972 a and b) to localise the Na^+-K^+-ATPase sites in secretory and reabsorptive epithelia was employed to visualise in the electron microscope the enzymatic sites in the ruminal epithelium. This technique uses strontium (Sr^{++}) as the capture ion to precipitate phosphate hydrolysed from p-nitrophenyl phosphate (p-NPP) by the ouabain-sensitive, K^+-dependent phosphatase component (known in the literature as K^+-NPPase) of the Na^+-K^+-ATPase enzyme complex. This technique gained popularity because it lends itself to light and electron microscopic analysis of enzyme distribution in cells and tissues, is easily correlated with biochemical assays of enzymatic activity and is unencumbered by "background" ATPase activity due to Mg^{++}-ATPase which does not bear any relationship with Na^+-K^+-ATPase (Ernst et al.,

1980; Firth, 1980).

Papillary samples (50 - 80 mg wet mass) taken from the caudoventral blind sac of the rumen from lambs killed in 1982 were fixed by immersion in 1 % paraformaldehyde-0.25 % glutaraldehyde in 0.1 M cacodylate buffer, ph 7.5, at room temperature. They were then incubated for phosphatases in a medium containing 20 mM disodium p-nitrophenyl phosphate (Sigma Chemical Co.), 20 mM $MgCl_2$ (BDH Chemicals Ltd.), 20 mM $SrCl_2$ (BDH Chemicals Ltd.), 30 mM KCl (BDH Chemicals Ltd.), and 250 mM Tris-HCl buffer (Sigma Chemical Co.). The pH was adjusted to 9.0.

The following sets of control experiments were used:

(a) Since the rumen epithelium contains alkaline phosphatase (Habel, 1963), an enzyme which also hydrolyses p-NPP at pH 9.0, papillary samples were incubated in the complete medium containing 10 mM L-cysteine (Sigma Chemical Co.) to differentiate phosphate deposits due to this enzyme from that derived from K+-NPPase. Cysteine is a potent inhibitor of alkaline phosphatase (Gordon, 1952; Ernst, 1975).

(b) To demonstrate the sensitivity of the K+-NPPase component to glycosides, papillary samples were incubated in the complete medium to which 10 mM ouabain (Sigma Chemical Co.) had been added.

(c) To test the K+-dependence of K+-NPPase, papillary samples were incubated in a medium from which KCl was omitted.

(d) To test the Mg++-dependence of the reaction, some tissues were incubated in $MgCl_2$-free medium.

(e) To compare sites of enzymatically produced precipitates with those produced non-enzymatically, papillary samples were incubated in either a medium without p-nitrophenyl phosphate or in a medium in which p-NPP was substituted with 10 mM inorganic phosphate.

The tissues were incubated either in the complete medium or in the controls listed above for 30 minutes.

The tissues were then rinsed in 100 mM Tris buffer, pH 9.0 and treated with 2 % $Pb(NO_3)_2$ to convert precipitated strontium phosphate to the more electron-dense lead phosphate for visualisation in the electron microscope. The sections were then washed in 0.1 M cacodylate buffer, pH 7.5 and post-fixed in 1 % osmium tetroxide in 0.1 M cacodylate buffer, pH 7.4 for 15 minutes. After osmication, some sections were treated with 1 % $(NH_4)_2S$ to convert lead phosphate to lead sulphide for examination in the light microscope.

The tissues were then dehydrated in a graded series of ethanol and embedded in Epon 812 resin. One-micron-thick sections for light microscopic examination were cut with glass knives and stained with crystal violet. Ultra-thin sections for electron microscopy were cut with glass knives, mounted on uncoated grids and examined with or without staining with 1 % lead citrate in a Phillips transmission electron microscope (E.M. 200) operating at a voltage of 60 kV.

For comparative purposes, the cytochemical localisation of Mg^{++}-ATPase in the epithelium was also undertaken using a modified Wachstein-Meisel procedure of Gray and Habel (1979). This procedure employed mild fixation and disodium adenosine triphosphate (disodium-ATP, BDH Chemicals Ltd.) as the substrate and lead (Pb^{++}) in low concentrations as the capture ion. Details of this procedure are found in Appendix IV.

2.8 Illustrations

The text figures contained in this thesis were from colour light micrographs and black and white electron micrographs and from line and free-hand drawings based on photographs and photomicrographs.

2.9 Statistical Methods

The statistical analyses were made with the help of PRIME computer using Minitab programme release 81.1 (Pennsylvania State University, 1980).

CHAPTER THREE

RESULTS

3.1 Gross Dissection

Dissection of the stomach of the adult sheep and of the 56 day-old lamb confirmed that the rumen is the largest compartment of the mature stomach, followed, in decreasing order, by the abomasum, reticulum and omasum (Figure 3.1). The reticulo-rumen was marked externally by grooves that corresponded to the internal muscular invaginations (pillars) which partially divided it into its cavities. The most conspicuous of these grooves was the reticulo-ruminal groove situated between the reticulum and the rumen. Internally, this corresponded to the reticulo-ruminal fold which separated the reticulum from the cranial sac of the rumen.

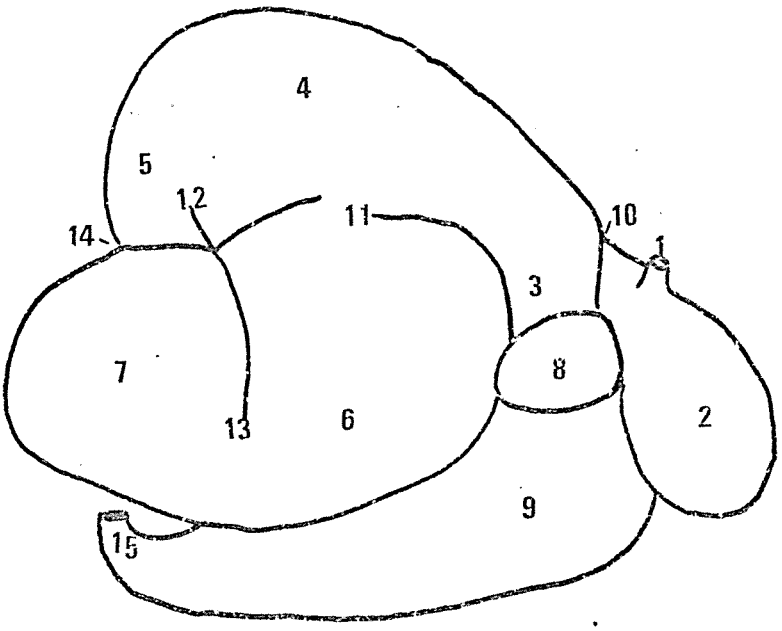
The transversely directed cranial groove in the cranial extremity of the rumen corresponded internally to the cranial pillar over which the cranial sac communicated with the dorsal sac. The cranial pillar was a large oblique and dorsocaudally directed muscular flap which was continued on each side by the right (visceral) and left (parietal) longitudinal pillars; the longitudinal grooves on the outer surface of the rumen were in corresponding positions to the right and left longitudinal pillars.

The dorsal sac was continued caudally by the caudo-dorsal blind sac from which it was demarcated by the short dorsal coronary grooves on the outer surface and their corresponding coronary pillars internally. The coronary pillars branched off from the cranially projected caudal pillar arising from the caudal wall of the rumen. Outside, the position of the caudal pillar was marked by the transversely directed caudal groove.

The cranial, caudal, left and right longitudinal grooves marked off the ventral sac from the dorsal sac. Internally, their corresponding pillars formed the rim of a large opening through which the dorsal sac

Figure 3.1 Diagrammatic representation of the stomach of the adult sheep; right side

1 - esophagus
2 - reticulum
3 - cranial sac or the rumen
4 - dorsal sac of the rumen
5 - caudodorsal blind sac
6 - ventral sac of the rumen
7 - caudoventral blind sac
9 - abomasum
10 - reticulo-ruminal groove
11 - right longitudinal groove
12 - right dorsal coronary groove
13 - right ventral coronary groove
14 - caudal groove
15 - pylorus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communicated with the ventral sac. Caudally, the ventral sac was continued by the caudoventral blind sac from which it was separated by the ring of muscular pillar formed by the ventral coronary pillars. Like the dorsal coronary pillars, the ventral coronary pillars also branched off from the caudal pillar. Externally, ventral coronary grooves were in corresponding positions.

Another striking feature of the interior of the forestomach was the structural arrangement of the mucosal lining (Figure 3.2). In the rumen, the internal surface was studded with numerous papillae which varied in shape and size and covered the entire surface. The papillae ranged from the short and compressed forms located at the longitudinal and coronary pillars which gave the pillars their distinctly ridged appearance, to the branched types which may be found in the transition between the rumen and the reticulum. The most typical of these papillae were the tongue or finger-shaped papillae which were distributed in the different sacs of the rumen. They varied from 1 - 5 mm in length, the shortest being generally found in the dorsal wall and the longest in the ventral lying parts of the rumen - e.g., the ventral parts of the atrium ruminis, the floors of the ventral sac and of the caudodrsal and caudoventral blind sacs. However, even in these areas, the papillae still differed in length and papillae less than 1 mm high could also be observed.

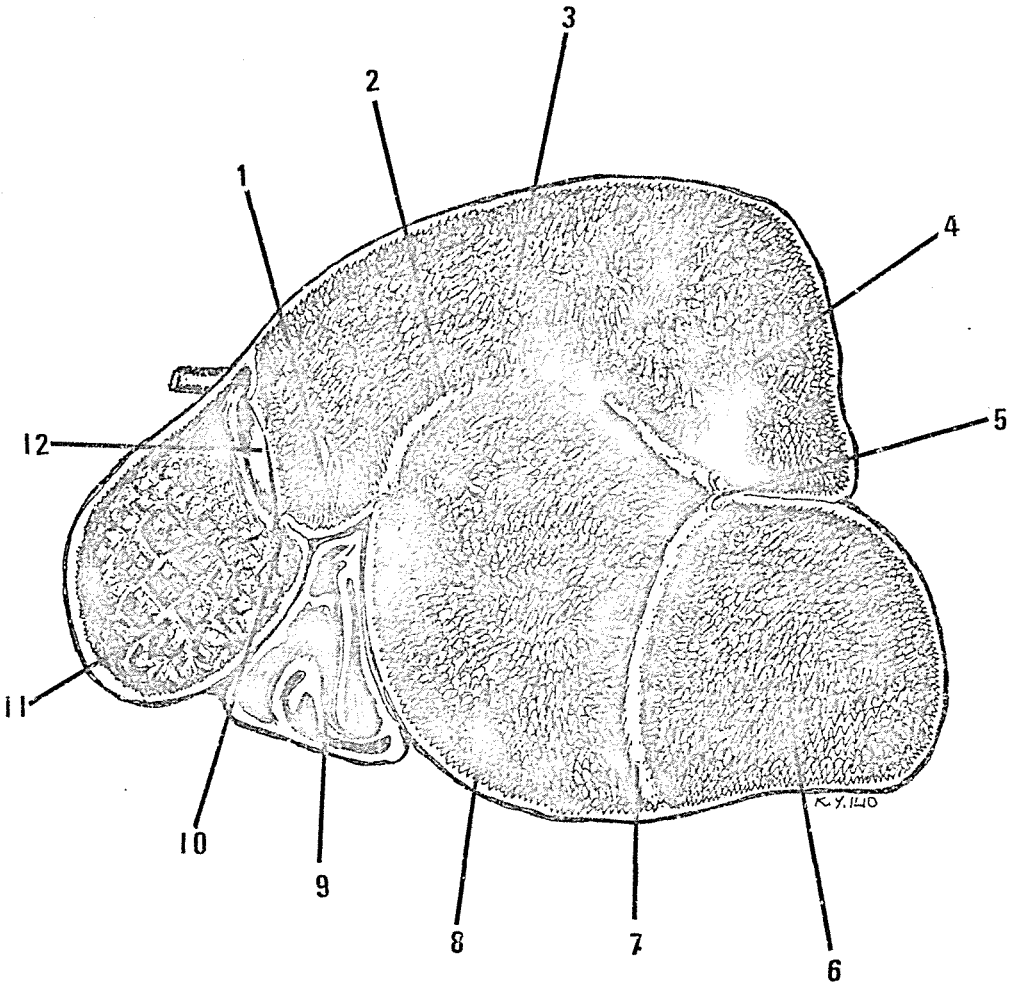
The internal surface of the reticulum was arranged into compartments bordered by intersecting mucosal folds or ridges, thus giving it its honey-comb appearance. The ridges and the floors of the compartments were also studded with papillae which were conical in shape.

In the omasum, four orders of length of the laminae were found. The surfaces of the laminae were also studded with papillae, the most common of which were conical.

The reticular groove was marked with longitudinal folds which were also papillated as they reached the reticulo-omasal orifice. These papillae were also conical but with distinctly curved or hooked tips, especially those near the orifice.

Figure 3.2 Reticulo-rumen of sheep to show position of internal structures

1 - atrium ruminis, cranial sac
2 - right longitudinal pillar
3 - dorsal sac of the rumen
4 - caudodorsal blind sac
5 - caudal pillar
6 - caudoventral blind sac
7 - right ventral coronary pillar
8 - ventral sac of the rumen
9 - abomasum
10 - reticulo-ruminal fold
11 - reticulum
12 - reticular groove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In contrast to the adult stomach, the largest compartment in one-day-old lambs was the abomasum, followed by the rumen, reticulum and omasum (Figure 3.3). The forestomach at this age had all the characteristic internal features of the adult stomach, except that the papillae were smaller and, in some cases, indistinct. In the rumen, the papillae were less than 1 mm in length and gave the mucosa a velvety appearance. In the reticulum, the honeycomb appearance of the mucosa and the papillae were already well-established. In the omasum, 4 orders of laminae were found.

In one of the one-day-old lambs dissected in 1982, a peculiar structure was found in the mucosa of the cranial wall of the reticulum. This structure was about 4 cm in length and was formed by a tubular elevation of the mucosa which followed a serpentine-like pattern on the reticular wall. The surrounding mucosa was apparently normal. Scanning electron micrographs of this structure (Figure 3.4) revealed it to have a highly keratinized and relatively smooth surface. It was surrounded by conical papillae at its sides which arose from what appeared to be deformed reticular crests. The structure was not investigated further.

3.2 Histology

3.2.1 General observations (Figures 3.5 and 3.6)

Sections of whole papillae from the rumen, reticulum and omasum of the adult sheep stained with H & E, Masson's trichrome and toluidine blue (Figure 3.5 A, B and C) showed that the papillae were composed of richly vascularised connective tissue cores surrounded by stratified keratinizing epithelium. The connective tissue core appeared to be predeominantly composed of densely packed collagen fibres which extended outwardly into the epithelium as the papillary process on which the basal cells of the epithelium were arranged (Figure 3.5 A). In the conical papillae of the omasum however, the connective tissue core may also contain acid mucopolysaccharides, as it stained reddish purple in toluidine blue-stained sections (Figure 3.5 C).

The deeper layers of the epithelium were folded into

Figure 3.3 Diagrammatic representation of the stomach of a one-day-old lamb (actual size). Right side.

1 - dorsal sac of the rumen
2 - ventral sac of the rumen
3 - cranial sac of the rumen
4 - reticulum
5 - omasum
6 - abomasum

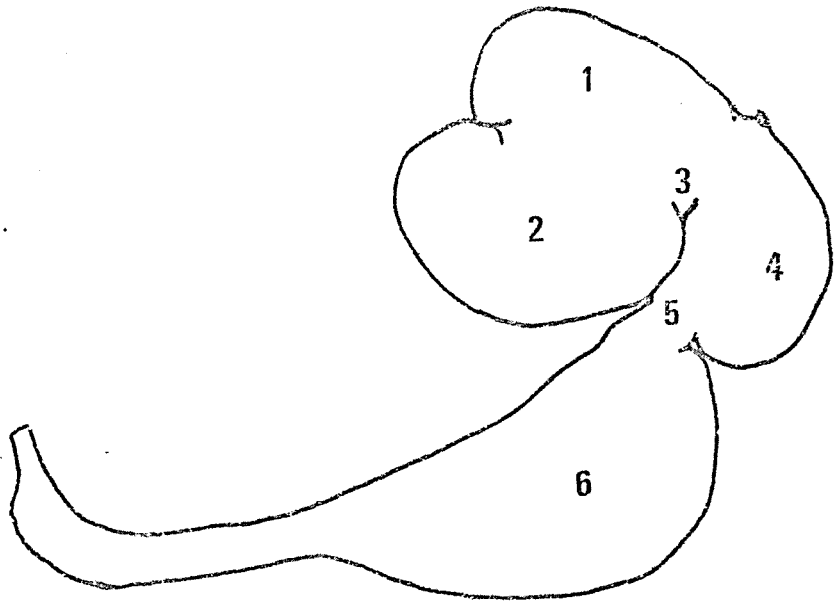
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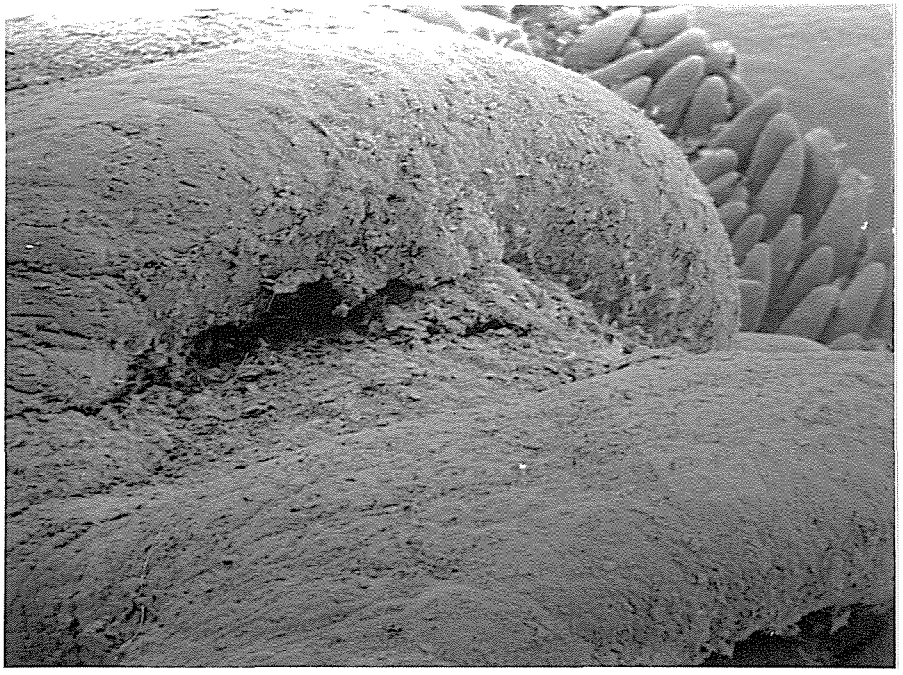
Figure 3.4 Scanning electron micrograph of the abnormal structure found in the reticulum of a one-day-old lamb killed in 1982. Note its highly keratinized and smooth surface. x 40.



epithelial pegs or bulbs which interlocked with the papillary processes from the connective tissue core to form the so-called epithelium-connective tissue interface. The papillary processes which were prominent at papillary tips especially in the rumen were invested with large blood vessels (Figure 3.5 A). No blood vessels were found in the epithelium.

Histological sections of mucosae taken from the rumen, reticulum, omasum and reticular groove of the adult sheep showed that they were lined by stratified keratinizing epithelia. As shown in Figure 3.6 A, B and C, the epithelial from these different locations in the forestomach are composed of five general cell layers which appear to merge into one another without distinct boundaries: stratum basale or the basal layer; stratum spinosum or the spinous layer; stratum granulosum or the granular layer; stratum transitionale or the transitional layer; and stratum corneum or the cornified or keratinized layer.

There did not appear to be any major differences between the epithelium taken from the different sampling sites in the forestomach, consequently, the epithelium will be described here as one and, unless otherwise specified, will be referred to as the forestomach epithelium. However, since the number of cell layers above the stratum basale and the amount of flattening and vesiculation in the cells of the keratinized layer are not uniform, the general descriptions in the sections following may not be equally applicable to all parts of the epithelium.

The first layer of cells situated at the bottom of the stratified epithelium was identified as the stratum basale (Figure 3.6 A). This consisted of a single layer of columnar cells with large, either ovoid or irregularly-shaped nuclei with 1 - 3 nucleoli. A few basal cells displayed mitotic figures.

Irregularly interspersed between the cells of the stratum basale or between the stratum basale and the layer of cells above it were distinct light-staining cells with either round or elongated nuclei (Figure 3.6 A and C).

Figure 3.5 Light micrographs of papillae taken from different locations in the forestomach of the adult.

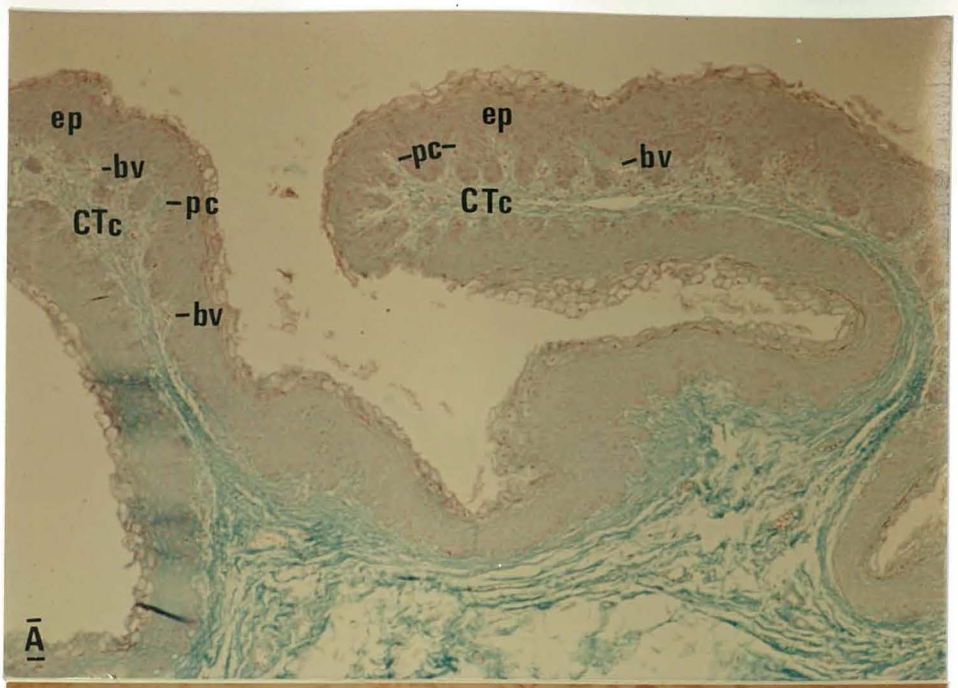
A. Tongue-shaped papillae from the rumen (cranial sac). MGT-stained paraffin section. x 100

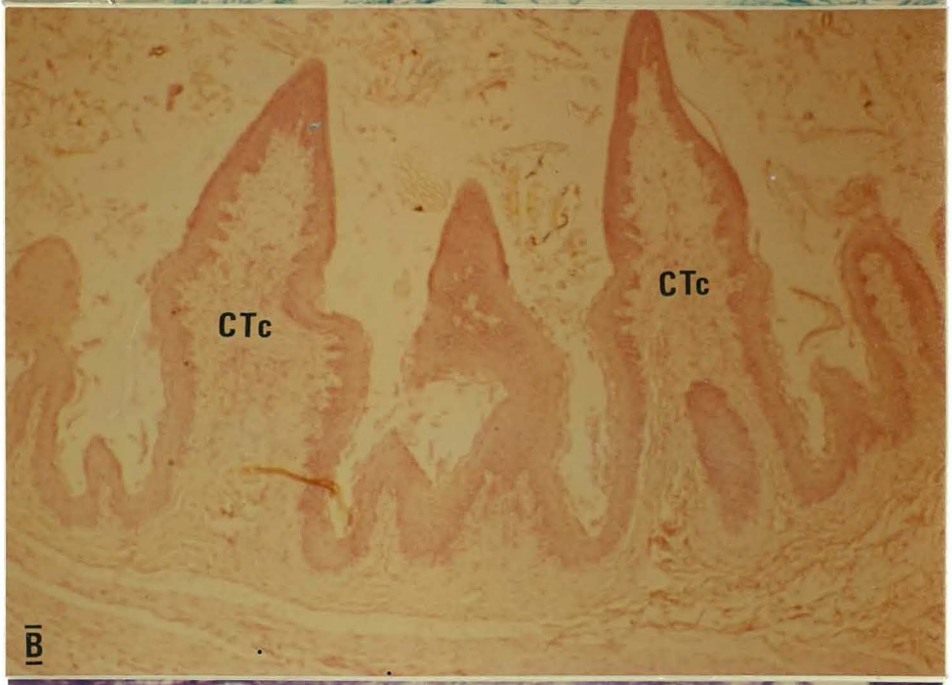
B. Conical papillae from the reticulum. H & E-stained paraffin section. x 90.

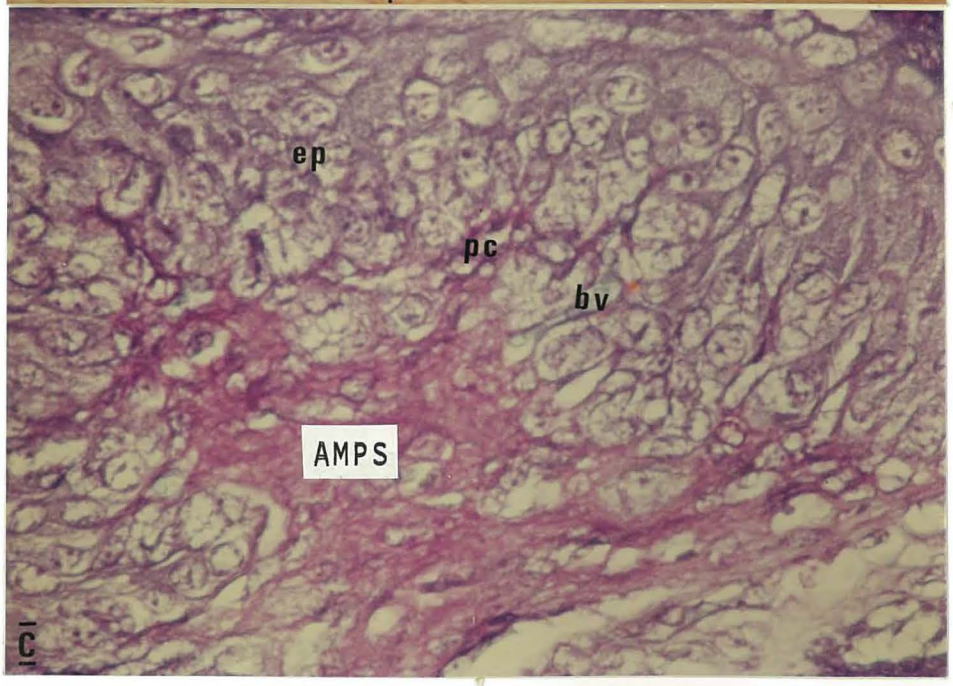
C. Basal portion of the conical papillae from the omasum. Toluidine blue-stained paraffin section. x 650.

Legend for Figure 3.5:

ep - epithelium
CTc - connective tissue core
pc - papillary process
bv - blood vessel
AMPS - acid mucopolysaccharide


ep
-bv
CTc
-pc
-bv
ep
-pc-
CTc
-bv
A


CTc
CTc
B


ep
pc
bv
AMPS
C

Some of these light-staining cells were occasionally encountered in the upper levels of the epithelium (Figure 3.6 C; see also Figure 3.8 C). A few of them contained metachromatic granules in the cytoplasm as seen in toluidine blue-stained sections.

The next 1 - 4 layers of cells above the stratum basale were identified as the stratum spinosum (Figure 3.6 A). This layer consisted of polyhedral cells which were larger than the basal cells and showed prominent intercellular bridges which were more conspicuous in resin sections. No cells in mitosis were observed in this layer.

Above the stratum spinosum was the stratum granulosum which was composed of 1 - 3 individual cell layers (Figure 3.6 A). The upper cellular levels of this layer consisted of distinctly flattened cells with basophilic keratohyalin granules in the cytoplasm as seen in H & E-stained sections. The lower cellular levels of this layer were indistinguishable from the upper cells of the stratum spinosum.

The most superficial cells of the epithelium which consisted of 1 - 6 layers composed the stratum corneum (Figure 3. 6 A). The cells comprising this layer were of two forms: (1) flattened heavily keratinized cells which may contain pyknotic nuclei as found in the tips of the ruminal papillae and in the conical papillae of the reticulum (Figure 3.6 B) and omasum, and (2) swollen or vesicular cells with thickened cytoplasmic membranes and pale-staining cytoplasm which may also contain pyknotic nuclei as found in the sides of the papillae of the rumen and in interpapillary areas (Figure 3.6 A and C). Both types of cells were found together in some areas of the stratum corneum with the flattened keratinized cells lying deeper than the swollen keratinized cells. Metachromatic material was found in between the cells of the stratum corneum as seen in toluidine blue-stained sections (Figure 3.6 A).

In between the stratum granulosum and the stratum corneum were found, swollen or vesicular cells with pale-staining cytoplasm quite distinct from the swollen cells of the stratum corneum in that their cell membranes

Figure 3.6 Light micrographs of epithelia taken from the forestomach of the adult

A. Epithelium, rumen (ventral sac); Toluidine blue-stained resin section. x 650.

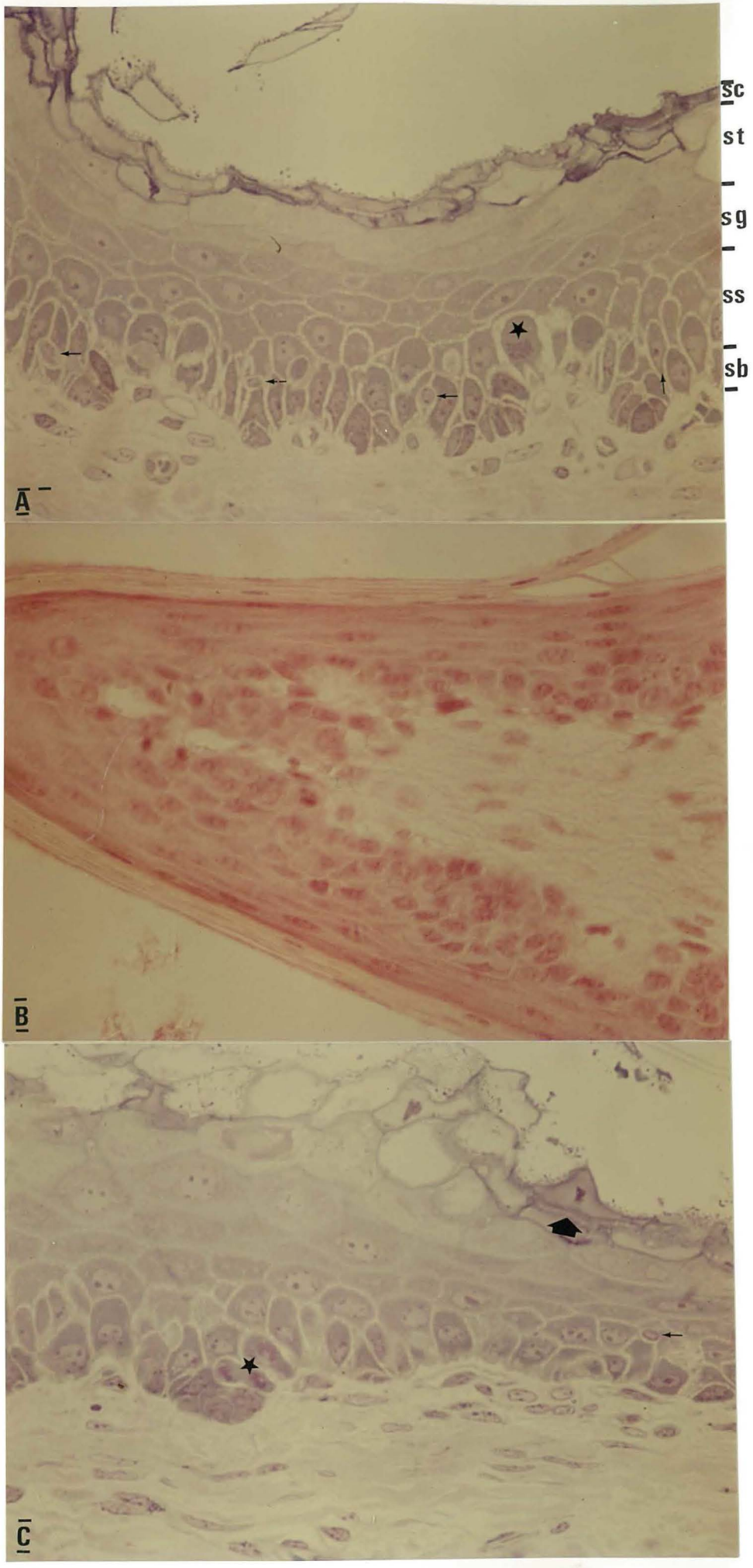
B. Epithelium, reticulum; H & E stained paraffin section. x 650.

C. Epithelium, omasum; Toluidine blue-stained resin section. x 650.

Legend for Figure 3.6:

sb - stratum basale
ss - stratum spinosum
sg - stratum granulosum
st - stratum transitionale
sc - stratum corneum

horizontal arrows - light-staining cells
vertical arrows - basal cell with the appearance of migrating towards the next layer
star - cells showing mitosis
large arrow - metachromatic material in the intercellular spaces in the stratum corneum


sc
st
sg
ss
sb
A
B
C

were not thickened (Figure 3.6 A). These were identified as being the cells comprising the stratum transitionale. Generally, this stratum formed a complete layer of cells 1 - 2 cell deep and separated from the swollen keratinized cells of the stratum corneum by 1 - 2 layers of flattened keratinized cells. However, in some areas of the epithelium, the stratum transitionale may be incomplete or only a few vesicular cells may be found. Only a few transitional cells were observed at the light microscopic level in the reticulum or in the conical papillae of the omasum, where the keratinized layers were thick and composed of flattened and heavily keratinized cells.

In some stretches of the epithelium, especially in the parts which are situated on the tips of the papillary processes, the epithelium may be only a few cell deep. In these cases, any of the three middle layers (stratum spinosum, stratum granulosum and stratum transitionale) may be discontinuous. However, some kind of keratinized layer was always present.

3.2.2 Histological appearance at different ages

Unless otherwise indicated, the following descriptions are based on sections taken from the rumen.

3.2.2.1 Birth to 24 hours of age (Figure 3.7)

At birth, the papillae of the rumen were joined for about two-thirds of their lengths (Figure 3.7 A). The epithelium (Figure 3.7 B) was 9 - 25 cell deep and was loosely differentiated into:

(1) a layer of cells comprising the stratum basale;

(2) an intermediate zone of cells with pale-staining cytoplasm, about 5 - 25 cell deep; and when present,

(3) a superficial keratinized zone, the stratum corneum, composed of 1 - 2 layers of cells. A discontinuous layer of transitional cells was also found just below the keratinized layer.

The stratum basale (Figure 3.7 B) was composed of cells with dark-staining cytoplasm and ovoid nuclei with 2 - 3 nucleoli. Light-staining cells as well as mitotic figures (Figure 3.7 B) were occasionally found in this layer. The line formed by the bases of adjacent basal

Figure 3.7 Light micrographs of the forestomach mucosa taken from one-day-old lambs

A. Partially joined papillae, rumen (cranial sac); keratinized cells appear reddish. MGT-stained paraffin section. x 100

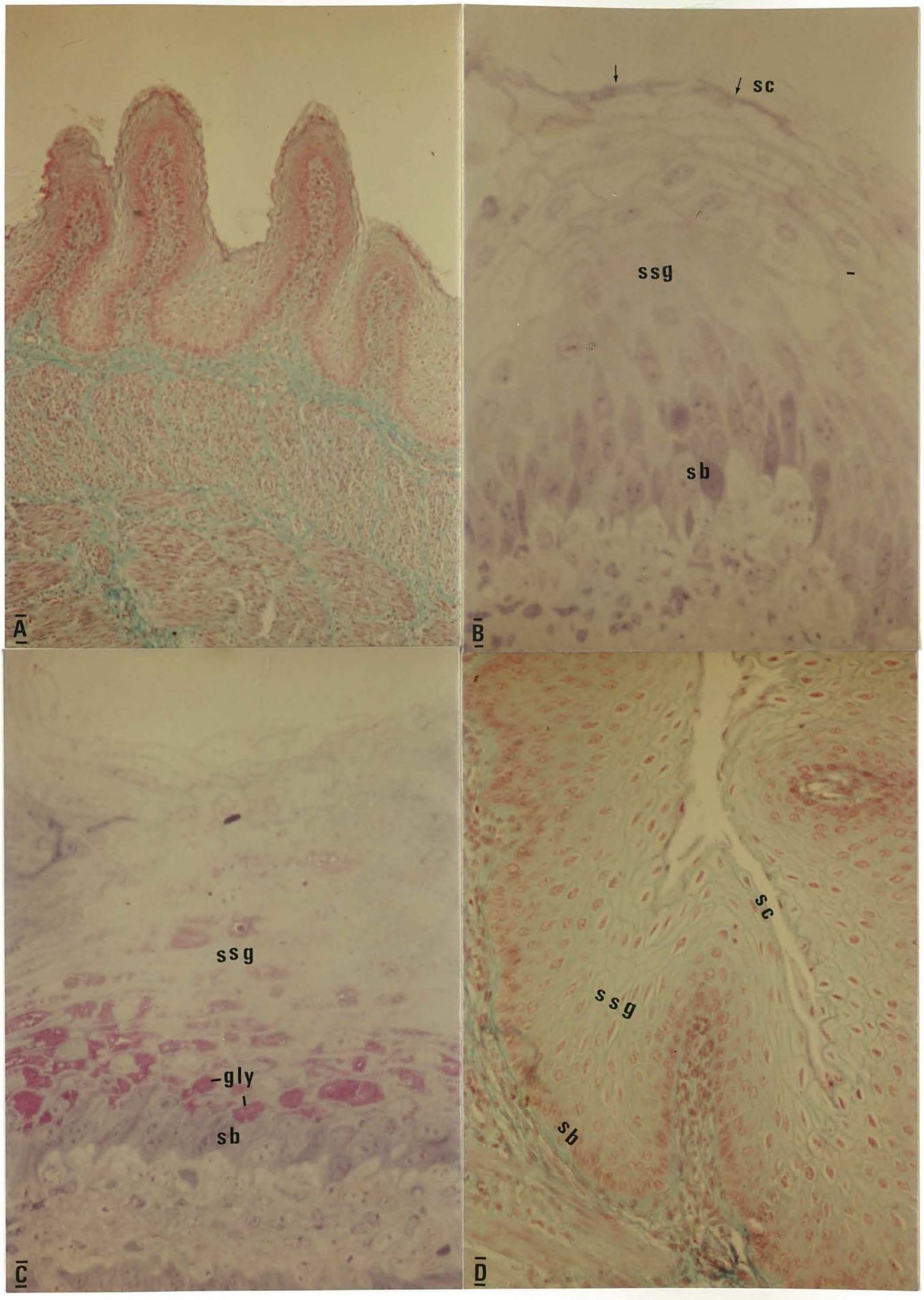
B. Epithelium, rumen (ventral sac); a few keratinized cells (arrows) appear in the superficial layers. Toluidine blue-stained resin section. x 650.

C. Epithelium, rumen (cranial sac); glycogen granules are abundant in the cytoplasm of intermediate cells; the basal cells appear non-reactive. P.A.S.-stained resin section. x 650.

D. Epithelium, omasal laminae; a thin layer of partially keratinized cells appear at the most superficial level. MGT-stained paraffin section. x 260.

Legend for Figure 3.7:

sb - stratum basale
ssg - stratum spinosum and stratum granulosum
sc - stratum corneum
gly - glycogen
arrows - keratinized cells


A
B
sc
ssg
sb
C
ssg
gly
sb
D
sc
ssg
sb

cells was relatively smooth and had few undulations.

The intermediate zone is equivalent to the stratum spinosum and the stratum granulosum combined. In sections stained with H & E and toluidine blue (Figure 3.7 B), this zone was seen to be composed of large polyhedral cells with pale-staining cytoplasm and ovoid nuclei. In P.A.S.-stained sections, the cytoplasm of these cells was occupied by P.A.S.-positive, diastase-sensitive glycogen granules (Figure 3.7 C). In contrast, the basal cells and the cells in the most superficial level did not show P.A.S. reactivity.

In areas from which transitional cells or keratinized cells were apparently absent, the cells of the intermediate layer tended to become flattened as they reached the surface (Figure 3.7 B and D).

The stratum corneum, when present, was composed of flattened cells with dense cell membranes which appeared reddish or orange in trichrome-stained sections and/or swollen cells with thick plasma membranes (blue-green in trichrome-stained sections) with or without nuclei (Figure 3.7 D).

3.2.2.2 12 days (Figure 3.8)

The ruminal papillae at this stage formed distinct entities (Figure 3.8 A). The epithelium was between 6 - 15 cell deep and could be differentiated into four or five cellular strata as found in the adult (Figure 3.8 B). In toluidine blue-stained sections, the stratum basale can be seen to be composed of columnar cells and occasional light-staining cells with purple oval or elongated nuclei (Table III). Although the direction of movement cannot be ascertained in the micrograph, some of these light-staining cells appeared to have crossed over from the underlying connective tissue (Figure 3.8 C). The line formed by the bases of the basal cells was relatively smooth with few undulations.

The stratum spinosum was formed by 2 - 4 layers of large polyhedral cells and the stratum granulosum was composed of 1 - 2 layers of keratohyalin granule-bearing cells. The stratum transitionale formed an incomplete layer of cells beneath the stratum corneum. Both vesicular

Figure 3.8 Light micrographs of the ruminal mucosa taken from 12-day-old lambs.

A. Papillae, cranial sac. Papillae has formed distinct entities. MGT-stained paraffin section. x 100 (N.B. extreme blue-green coloration is due to the printing of the photograph.)

B. Epithelium, cranial sac; note the development of a thin stratum corneum. Toluidine blue-stained resin section. x 650.

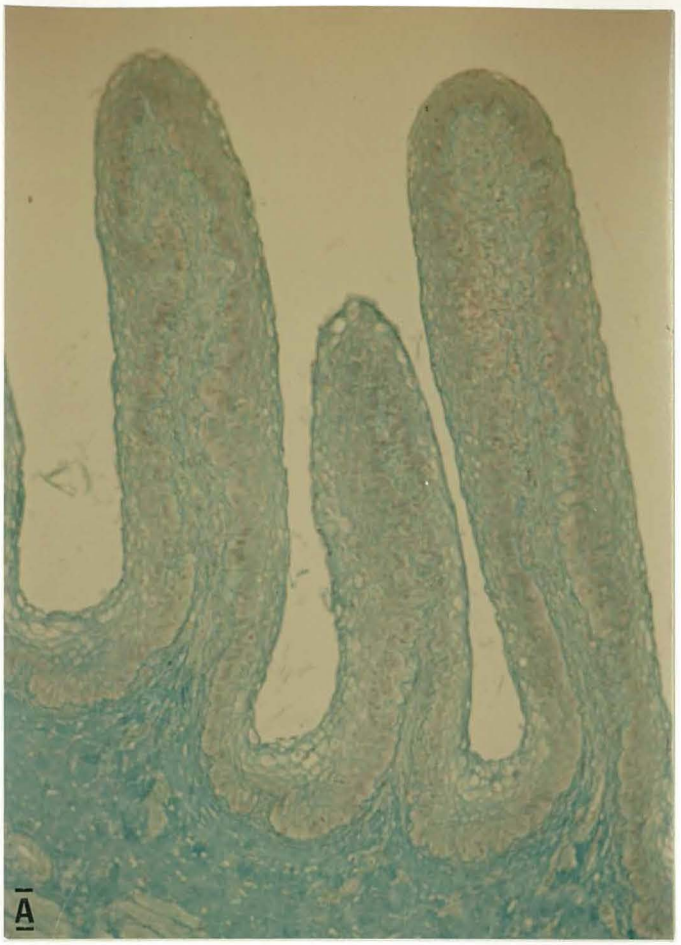
Legend for Figure 3.8 B

Large arrow - light staining cell in the stratum spinosum

Double arrows - lymphocyte-like cell closely apposed with two light-staining cells

Single arrow - light-staining cell

Star - cell showing mitotic figures


A

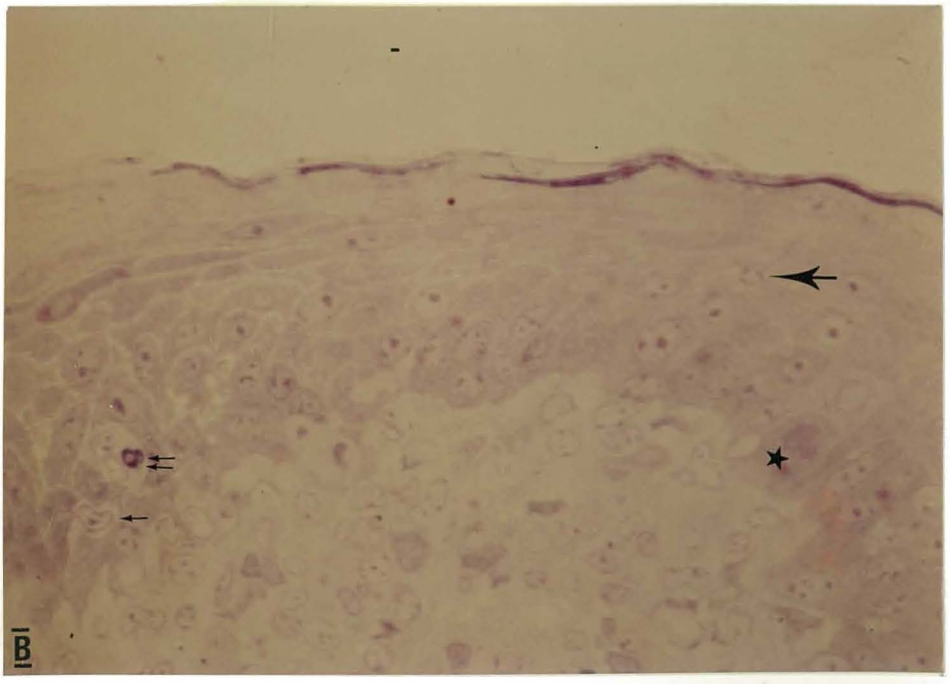
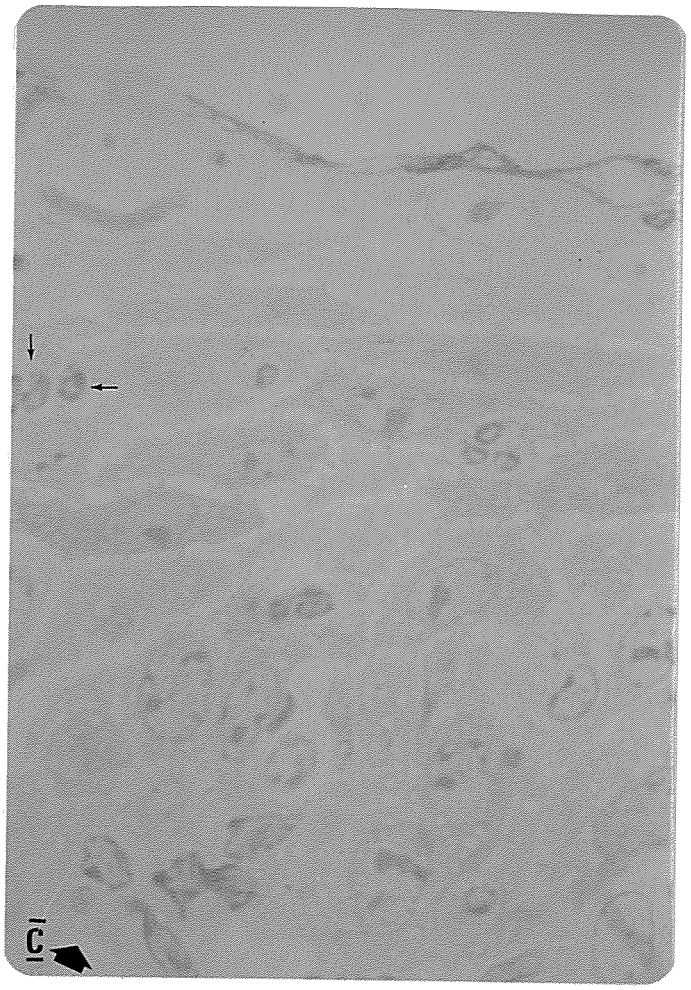
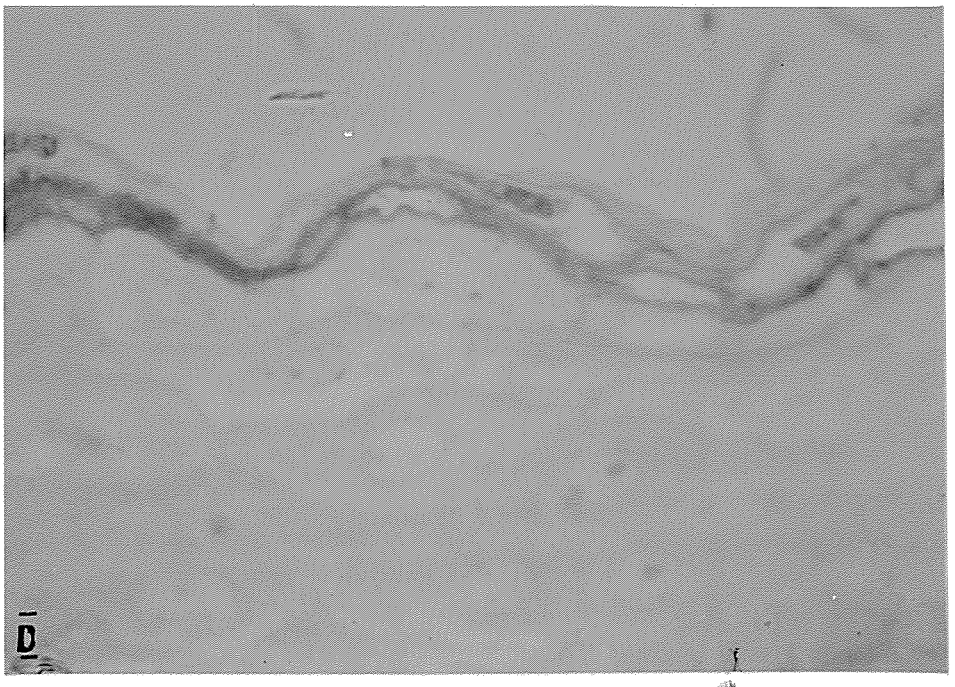

B

Figure 3.8 (Continued) Light micrographs of the ruminal epithelium taken from 12-day-old lambs.

C. Epithelium, cranial sac. Light-staining cells (large arrows) appear to cross over from the underlying connective tissue. Other light-staining cells are numerous in the upper cellular levels. Small horizontal arrow points to a lymphocyte-like cell which is closely apposed to another light-staining cell (vertical arrow). Toluidine blue-stained resin section. x 1650.

D. Epithelium, cranial sac. Note slight P.A.S.-positivity in the intercellular spaces in the stratum corneum. P.A.S.-stained resin section. x 1650.


C


D

and flattened cells were found in the stratum corneum in the tips and sides of the papillae while only vesicular cells were found in the stratum corneum in interpapillary areas. The stratum corneum in these areas were 1 - 3 cell deep. P.A.S. staining revealed slight P.A.S. (diastase-resistant) positivity in between cornified cells (Figure 3.8 D) which was also observed as metachromatic with toluidine blue (Figure 3.8 C).

3.2.2.3 23 days (Figure 3.9)

The epithelium (Figure 3.9 A) at this age was composed of 4 - 13 individual cell layers and on the average, consisted of 1 layer of basal cells; 1 - 2 layers of spinous cells; 1 layer of granular cells; an almost complete layer of transitional cells; and 1 - 2 layers of keratinized cells which were vesicular at the sides of the papillae and at interpapillary areas (Figure 3.9 A).

As in the previous age, in toluidine blue-stained sections, distinct light-staining cells with purple nuclei were seen interspersed between the columnar cells. Mitotic figures were also observed in some of the basal cells.

Undulations of the deeper layers of the epithelium which can be recognised as epithelial pegs or bulbs were first seen at this age. They were first seen to occur at papillary tips where the deeper layers of the epithelium became folded apparently to accomodate the growing papillary processes from the connective tissue core. The formation of epithelial pegs or bulbs was especially observed in trichrome-stained preparations (Figure 3.9 B).

Toluidine blue and P.A.S.-staining also showed metachromasia (Figure 3.9 A) and slight positivity respectively in the stratum corneum.

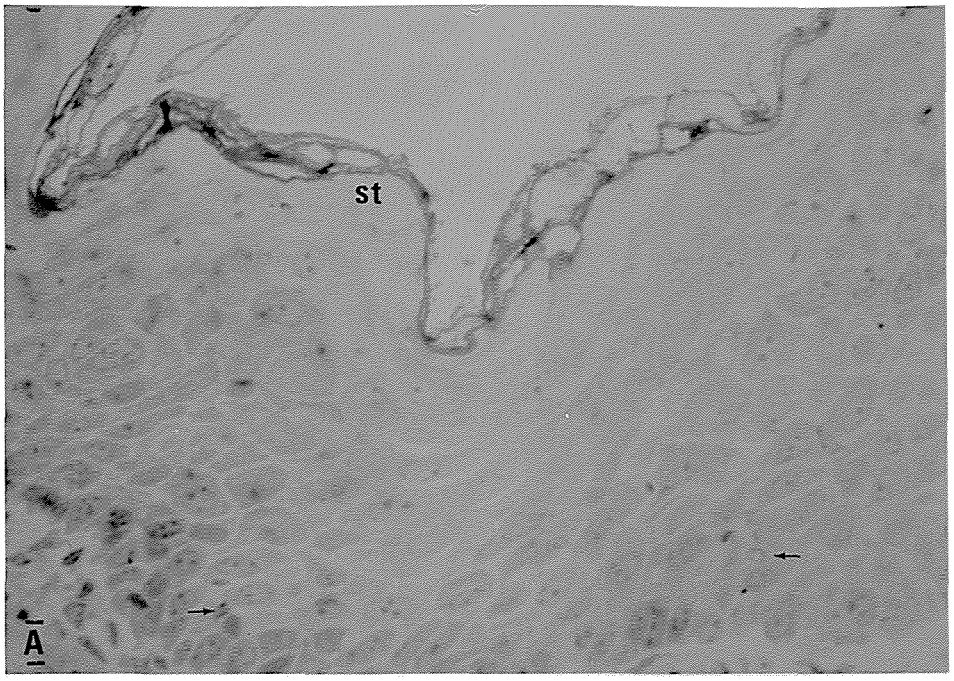
2.2.2.4 34 days (Figure 3.10)

The epithelium at this age was composed of 4 - 16 individual cell layers (Figure 3. 10 A). The appearance of the cellular strata was similar to the previous age except that the number of swollen cells in the stratum corneum had increased especially at the sides of the papillae. The papillary tips (Figure 3.10 B) and

Figure 3.9 Light micrographs of the ruminal mucosa taken from 23-day-old lambs.

A. Epithelium, dorsal sac; transitional cells (st) form an almost complete layer beneath the stratum corneum; note presence of both flattened and swollen cells in the keratinized layer. Arrows point to nuclei of light-staining cells. Toluidine blue-stained resin section. x 650.

B. Tip of papillae. Papillary processes from the connective tissue core and epithelial bulbs are extensive; st = transitional cell. MGT-stained parafin section. x 260.


st
A

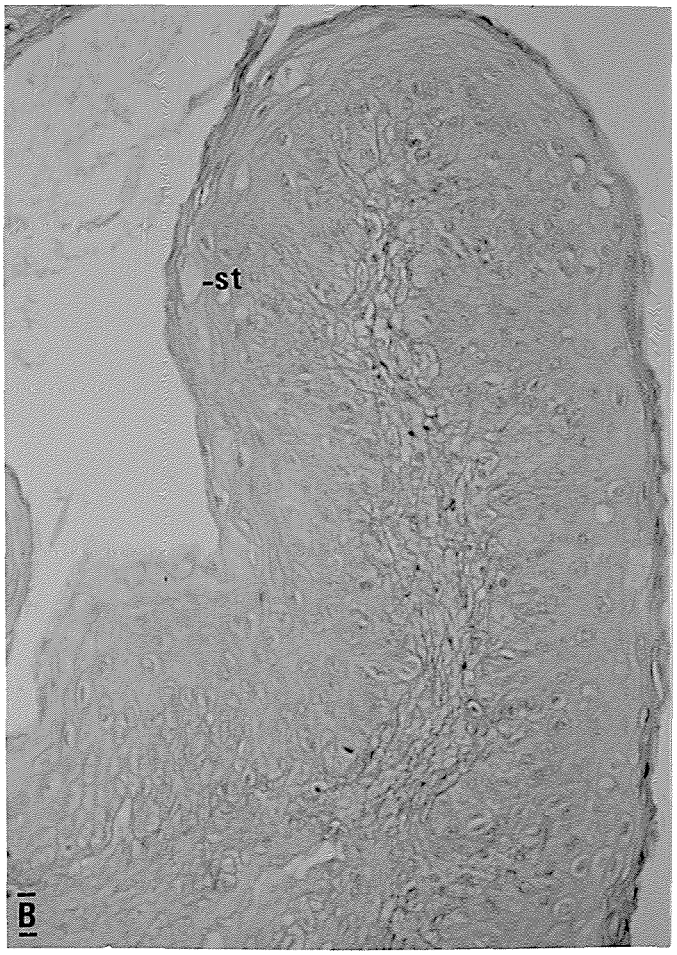
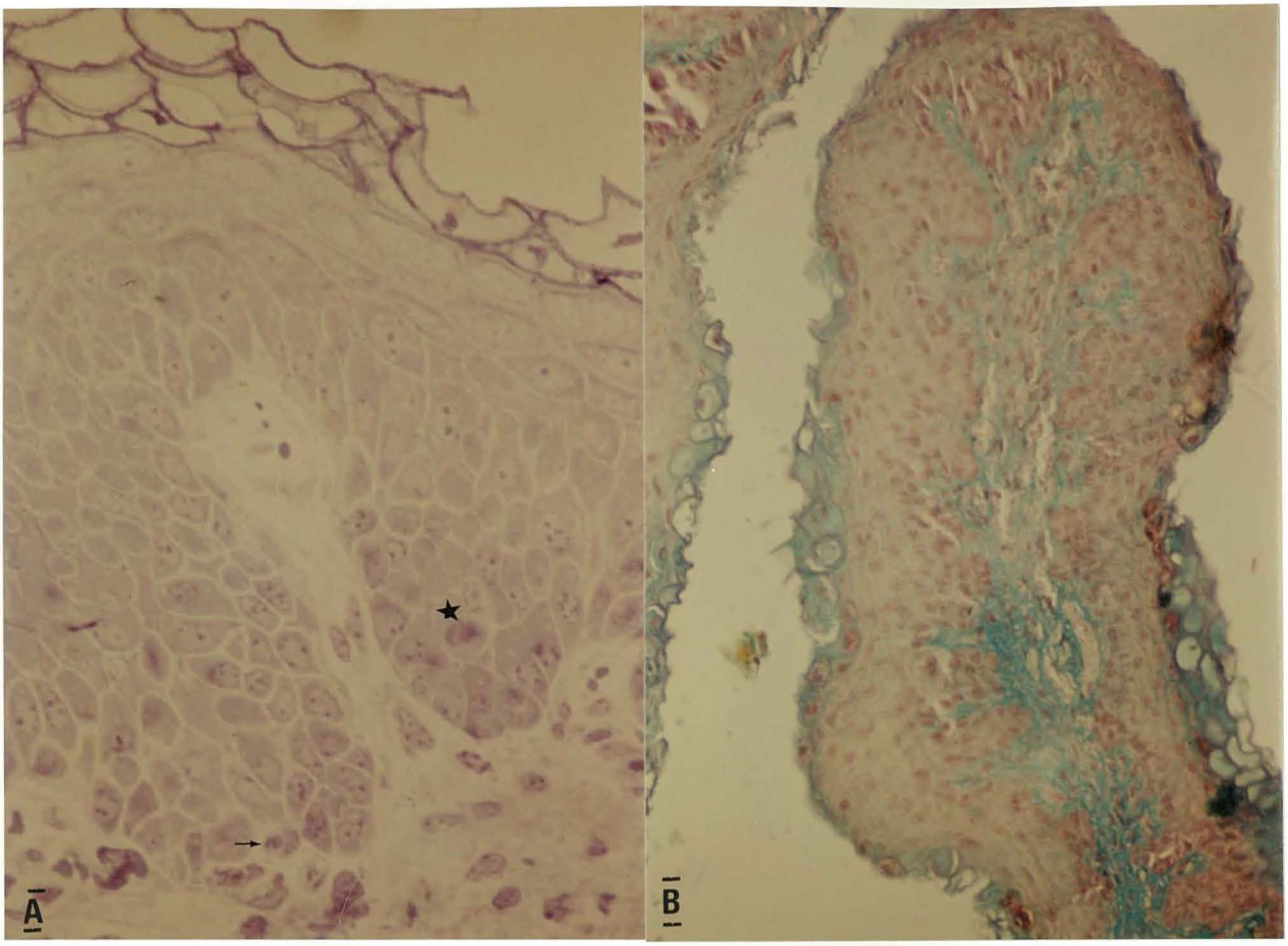

-st
B

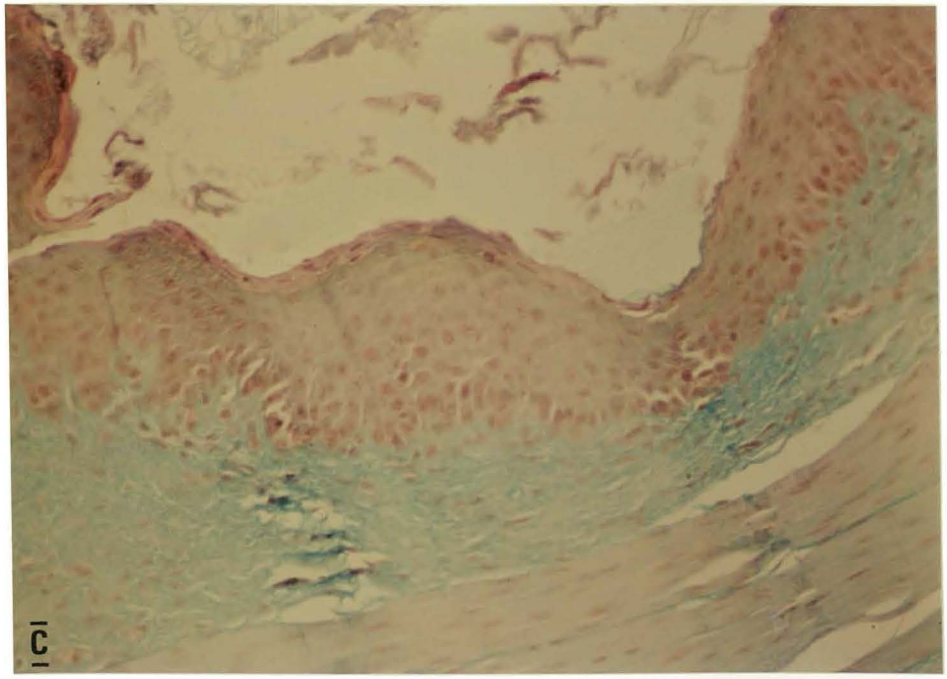
Figure 3.10 Light micrographs of the ruminal mucosa taken from 34-day-old lambs.

A. Epithelium, ventral sac. Note dilated blood vessel investing the papillary process; arrow points to a light-staining cell displacing a basal cell nuclei; star indicates cell displaying mitotic figures. Toluidine blue-stained resin section. x 650 .

B. Papilla, cranial sac. The tip of the papilla is covered with flattened keratinized cells while the swollen keratinized cells are found at the sides of the papilla. MGT-stained paraffin section. x 260 .

C. Interpapillary area, cranial sac. Note the presence of flattened keratinized cells in the keratin layer. MGT-stained paraffin section. x 260 .


A
B


C

interpapillary areas (Figure 3.10 C) were covered by a thin layer of flattened keratinized cells.

Epithelial bulb-papillary process interactions were prominent at this age and were found in all areas of the papillae. Blood vessels investing the papillary processes were large and dilated. P.A.S. and toluidine blue-staining reactions were similar to the previous age and cells with light-staining cytoplasm in the stratum basale were more numerous than in the previous ages (Table III).

3.2.2.5 45 days (Figure 3.11)

The general appearance of the epithelium was similar to the epithelium in the adult (Figure 3.11 A). The epithelium was composed of 4 - 12 individual cell layers composed typically of: a layer of basal cells, 1 layer of spinous cells, 1 layer of granular cells, a complete layer of transitional cells and 1 - 5 layers of keratinized cells with the more superficial layers composed of swollen cells. Like in the adult, in some areas of the epithelium, any of the intermediate layers may be discontinuous - i.e., only four cellular layers may be found although the stratum corneum was always present.

Slight P.A.S.-positivity and metachromasaia with toluidine blue were also found in the stratum corneum (Figure 3.11 A and C). Only a few mitotic figures were observed in the stratum basale while light-staining cells were more numerous. Cells with pyknotic nuclei were also seen in the stratum gasale (Figure 3.11 B).

Epithelial bulb-papillary process interactions were more prominent at this age with some epithelial bulbs reaching deep into the connective tissue core (Figure 3.11 B).

3.2.2.6 56 days (Figure 3.12)

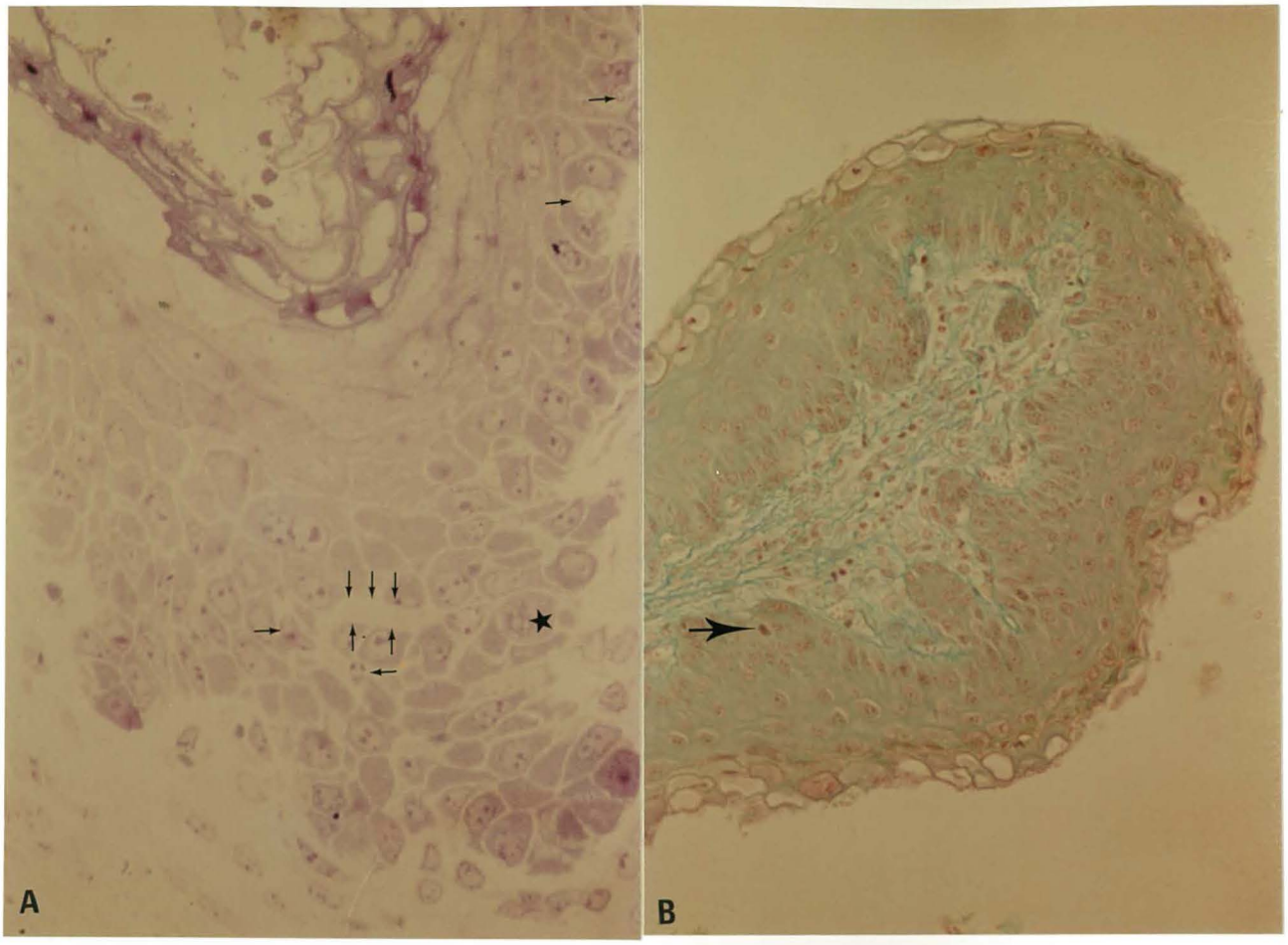
The general appearance of the epithelium at this age resembled that in the adult (Figure 3.12 A). The epithelium was composed of 5 - 9 layers of cells comprising of: 1 layer of basal cells, 1 layer of spinous cells, 1 layer of granular cells, 1 layer of transitional cells, 1 layer of flattened keratinized cells and 1 - 2 layers

Figure 3.11 Light micrographs of the ruminal mucosal taken from 45-day-old lambs.

A. Epithelium, cranial sac. Horizontal arrows point to light-staining cells; vertical arrows point to possible cell process of a light-staining cell; the star indicate a cell showing mitotic figures. Toluidine blue-stained resin section. x 650.

B. Tip of the papillae, ventral sac. Note extensive papillary process-epithelial bulb interactions; blood vessels investing the papillary process are highly dilated; arrow indicates an apoptotic body. MGT-stained paraffin section. x 260.

C. Epithelium, dorsal sac. Note P.A.S.-positive reaction in the stratum corneum. Arrow points to a retained nucleus in the stratum corneum. P.A.S.-stained resin section. x 650.


A
B

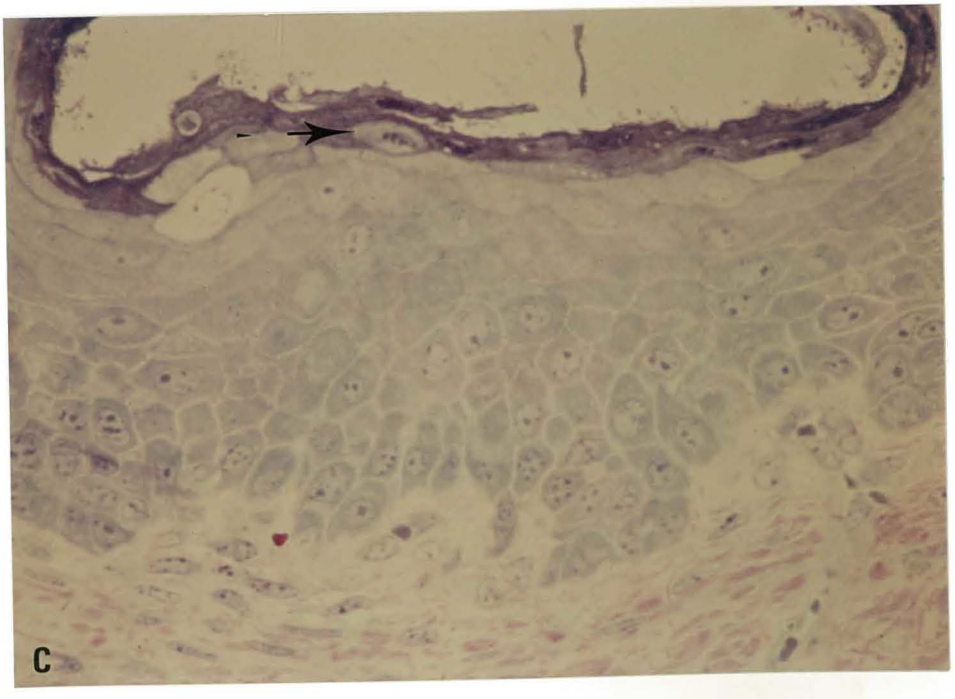
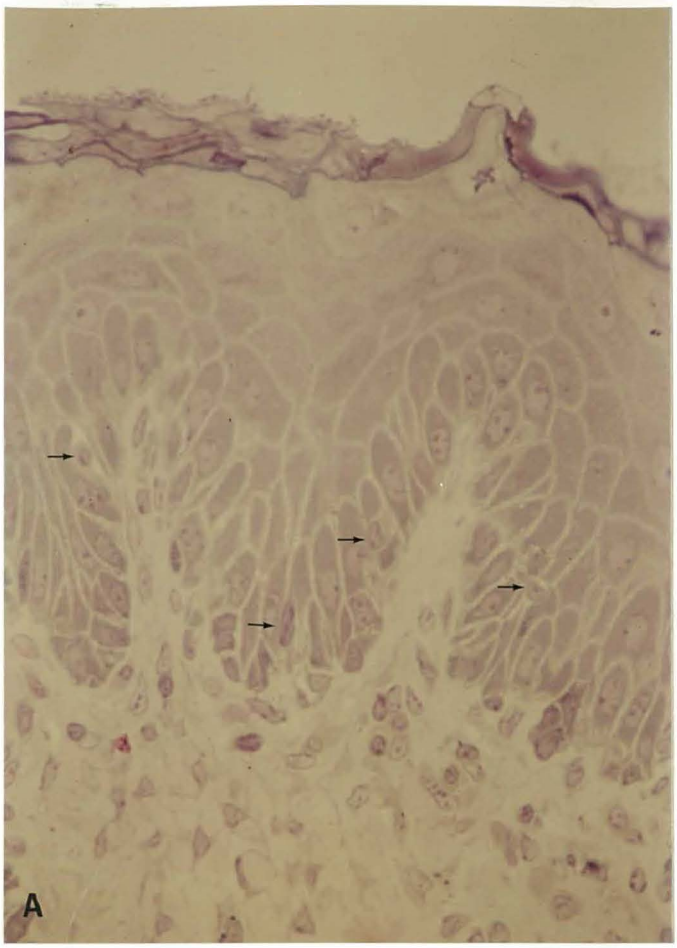
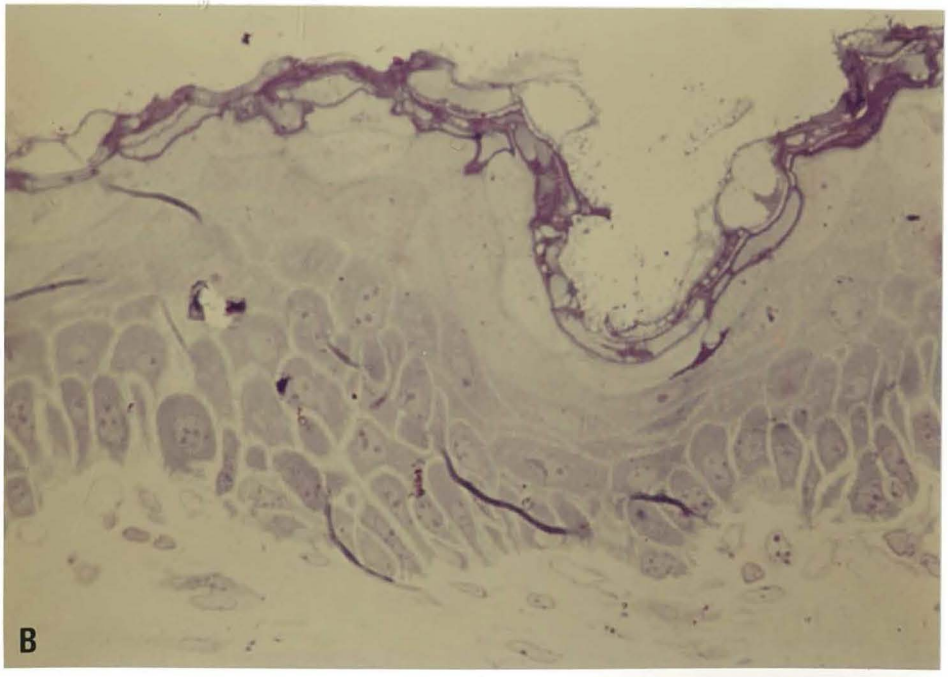

C

Figure 3.12 Light micrographs of the ruminal epithelium taken from 56-day-old lambs.

A. Epithelium, ventral sac. Arrows indicate numerous light-staining cells in the basal layer of the epithelium. Toluidine blue-stained resin section. x 650.

B. Epithelium, ventral sac. Note P.A.S.-positive reaction in the stratum corneum. The stratum transitionale forms a complete layer of cells beneath the stratum corneum. P.A.S.-stained resin section. x 650.


A


B

of swollen keratinized cells. As in the previous age, and in the adult, stratification of the epithelium may also be variable with any of the intermediate layers in some sections appearing to be discontinuous.

P.A.S.-positivity (Figure 3.12 B) and toluidine blue metachromasia (Figure 3.12 A) in the stratum corneum were also observed. The epithelial bulb-papillary process interactions in all areas were quite prominent and large blood vessels were seen in the papillary processes of the connective tissue core. Almost no mitotic figures were observed in the basal layers of the epithelium and the light-staining cells were more numerous than in the previous ages (Table III).

3.2.3 Changes in the number of layers of the epithelium in different ages

The number of individual cell layers of the forestomach epithelium in different ages were counted in sections taken from three locations in the rumen: the tips and sides of papillae and the interpapillary areas and are shown in Table IV.

The epithelium was thickest in 24 hour-lambs ($p < .05$). It then showed a decrease in 12 day-old lambs ($p < .01$) and again in 23 day-old lambs ($p < .01$). At the next two ages (34 and 45 days), the number of cell layers remained fairly constant before decreasing again in 56 day-old lambs ($p < .01$) at which stage the number of cell layers did not differ significantly from that in the adult.

3.3 Mitotic Index

The mitotic index of the forestomach epithelium was measured in the dorsal, cranial and ventral sacs of the rumen in two lambs per age group, except that only one 45 day-old lamb and one adult sheep were used. The results of the mitotic counts are given in Table V. They show a wide fluctuation in the mitotic indices. However, the values in all the 45 readings were less than 1 % (range: 0.065 - 0.984 %). There were no significant differences in the mitotic indices between the three sampling sites in any given lamb and the data from the three sites in

Table III Number of light-staining cells in the forestomach epithelium in selected ages expressed as a percentage against the number of basal cells

Age	Light-staining cells counted	Total number of basal cells conted	%
24 hours	37	5982	0.62
12 days	26	820	3.17
34 days	195	3840	5.08
56 days	489	6843	7.15

Table IV Number of Individual Cell Layers in the Ruminal Epithelium in Different Ages (Sample Means $\bar{X}$, Standard Deviation S.D.)*

Age	$\bar{X}$	S.D
24 hours	14.17**	6.75
12 days	9.83	2.23
23 days	8.00	2.65
34 days	8.06	2.35
45 days	7.97	2.39
56 days	7.03	1.22
adult	7.51	2.42

*n = 35 measurements for each age

**24 h > 12 d > 23, 34, 45 d > 56 d, adult

TABLE V. Mitotic Indices (%) of the Ruminal Epithelium Taken from Pairs of Lambs at Different Ages[1]

Age	MITOTIC INDEX							
	Dorsal Sac (DS)		Cranial Sac (CS)		Ventral Sac (VS)		DS + CS + VS	
	Lamb		Lamb		Lamb		Lamb	
	A	B	A	B	A	B	A	B
24 hours	0.387[a] (11[b]/2851[c])	0.868 (44/5070)	0.780 (39/5000)	0.766 (22/2871)	0.678 (32/4723)	0.433 (15/3461)	0.652 (82/12574)	0.710 (81/11482)
12 days	0.752 (34/4522)	0.349 (10/2866)	0.871 (28/3251)	0.457 (17/3720)	0.748 (26/3475)	0.515 (15/2914)	0.785 (88/11212)	0.442 (42/9500)
23 days	0.309 (9/2911)	0.438 (2/457)	0.520 (15/2884)	0.324 (13/4011)	0.609 (18/2951)	0.667 (10/1500)	0.480 (42/8746)	0.419 (25/5968
34 days	0.840 (25/2975)	0.376 (5/1331)	0.803 (24/2990)	0.667 (4/600)	0.984 (56/5691)	0.791 (12/1518)	0.901 (105/11656)	0.609 (21/3449)
45 days	0.369 (10/2709)	-----	0.407 (6/1475)	-----	0.633 (20/3159)	-----	0.490 (36/7343)	-----
56 days	0.386 (11/2851)	0.500 (20/4000)	0.065 (1/1538)	0.351 (18/2850)	0.191 (6/3136)	0.145 (2/1383)	0.239 (18/7525)	0.389 (32/8233)
adult	-----	-----	-----	-----	-----	-----	0.215 (7/3266)	

[a] Mitotic Index (%) = $\frac{\text{[b] number of basal cells showing mitotic figures}}{\text{[c] total number of basal cells counted}} \times 100$

[1] Lambs 'A' and 'B' differed with each age group

each animal were therefore pooled for further analysis, as in Table V.

Animals within the same age group showed similar indices, except that in 12 day-old lambs, the values differed significantly ($p < .005$). Comparisons between successive age groups revealed (Figure 3.13) that the indices had decreased significantly ($p < .01$) between the first and 12th days and then decreased even further ($p < .001$) in the 23 day-old lambs. From this age, the index then increased in 34 day-old lambs ($p < .005$) before decreasing again in 45 day-old lambs ($p < .005$) and yet further in 56 day-old lambs ($p < .01$). The adult value did not differ significantly from that obtained at 56 days.

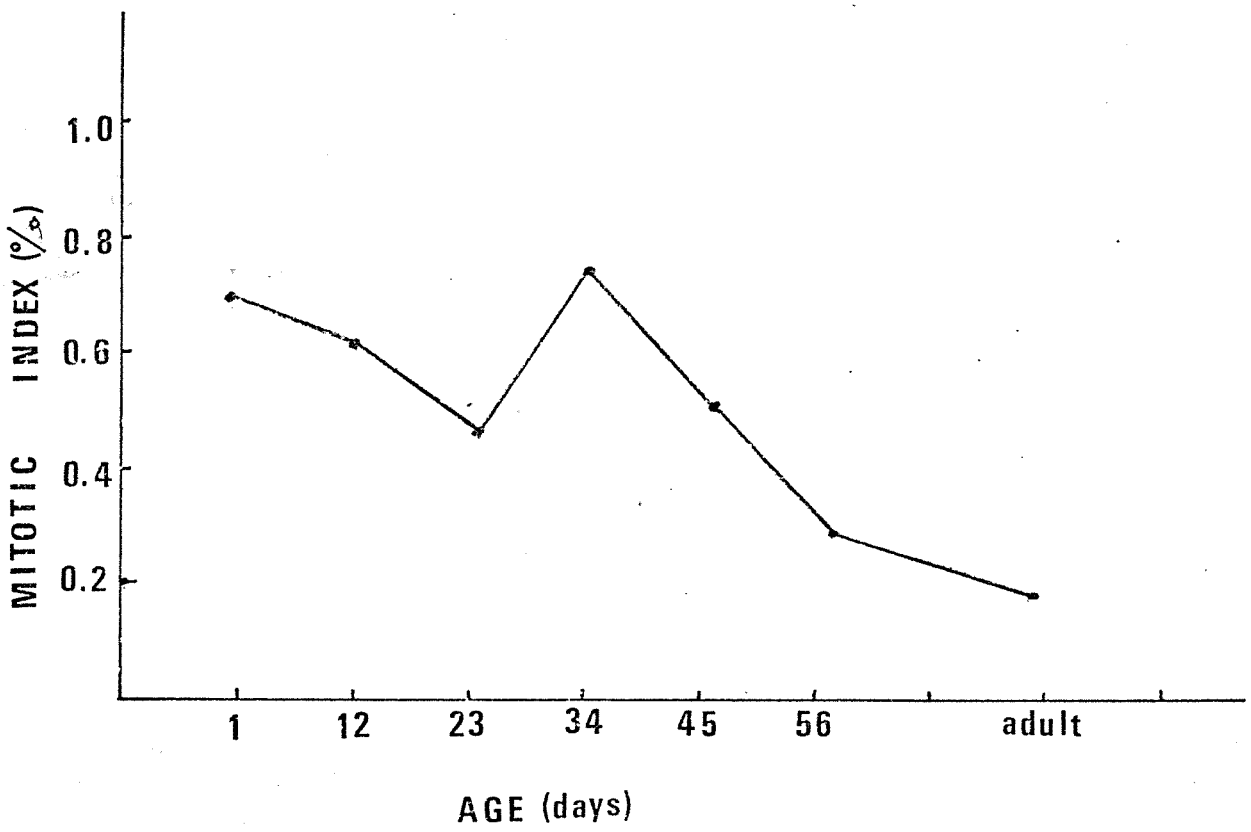
3.4 Electron Microscopy

3.4.1 General observations

The same general pattern of organisation of the forestomach epithelium as seen in light microscopic preparations was recognised in electron micrographs of the epithelium in the adult. In low power micrographs (e.g. Figure 3.14), the epithelium was observed to be a stratified keratinizing epithelium differentiated into a series of five general cellular strata: stratum basale, stratum spinosum, stratum granulosum, stratum transitionale and stratum corneum. When the cells comprising these strata were examined at successively higher levels, it was possible to follow in sequence, events in their differentiation.

In general, from the basal layer where the cells were columnar in shape with their long axes perpendicular to the basal lamina, the cells migrated to the stratum spinosum where they became large and increased the number of their desmosomal connections. From this layer upwards, the cells became flattened with their long axes parallel to the luminal surface. In the stratum granulosum, conspicuous granules (keratohyalin and membrane-coating granules) developed in the cytoplasm. At the stratum transitionale, keratinization or cornification started with the degeneration of cellular organelles and the dispersion of keratohyalin granules. In the stratum corneum, cell organelles disappeared

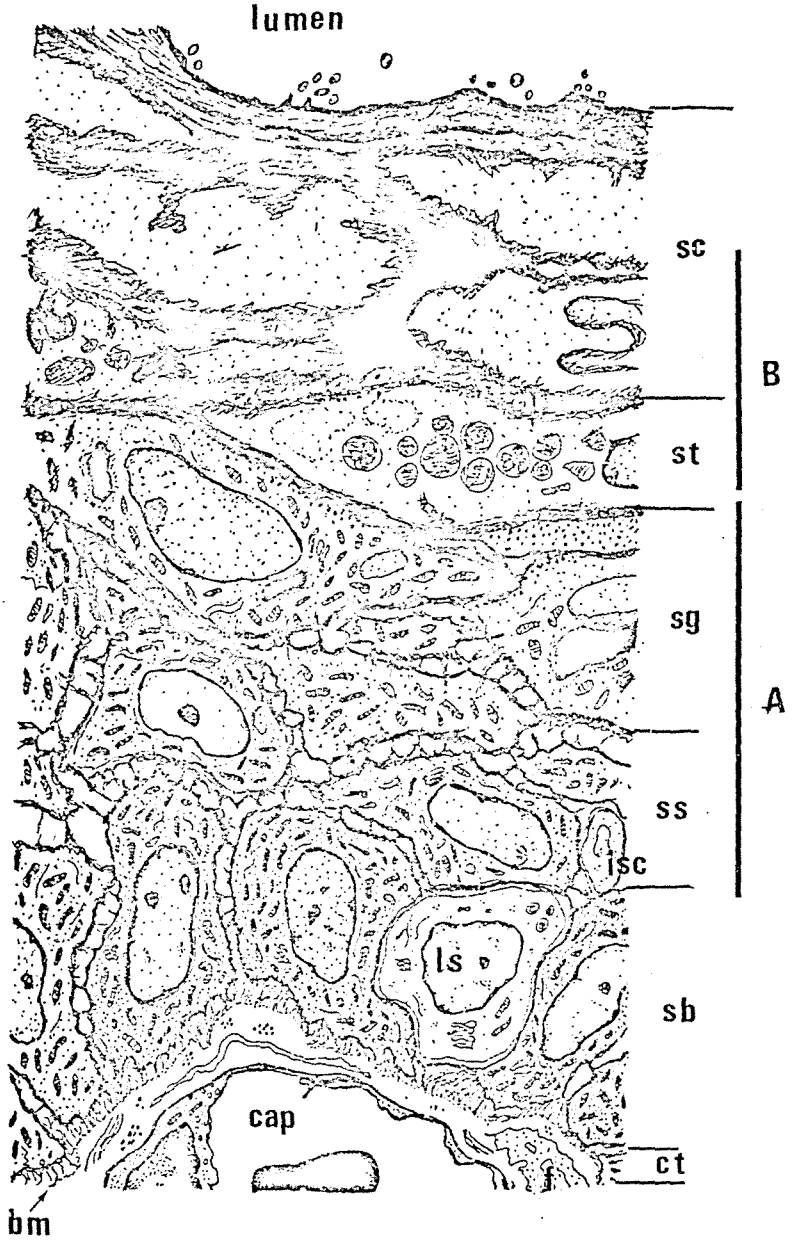
Figure 3.13 Mean mitotic indices in the ruminal epithelium in different ages*



* 45 days and adult - based on one animal

Figure 3.14 Semi-diagrammatic representation of the ruminant forestomach epithelium. Details of cell junctions are omitted.

Legend:
sb - stratum basale
ss - stratum spinosum
sg - stratum granulosum
st - stratum transitionale
st - stratum corneum
ls - light-staining cell
lsc - process of light staining cell
ct - connective tissue; f - fibroblast
bm - basement membrane
cap - capillary
A - levels where differentiation products - keratohyalin granules, Endoplasmic reticulum - protein and membrane-coating granules are formed.
B - levels where keratinocytes are transformed into keratinized cells; MCGs are discharged into the intercellular spaces


lumen
sc
B
st
sg
A
ss
lsc
ls
sb
cap
ct
bm

(although degenerated nuclei were seen to persist) and replaced by a mass of keratin. Ultimately, desquamation of the keratinized cells occurred. Bacteria were seen to adhere to desquamating cells.

At the ultrastructural level, the boundaries delineating the stratum basale, the stratum transitionale and the stratum corneum were well defined; only the boundary between the stratum spinosum and stratum granulosum was not clear-cut. The epithelium was separated from the underlying connective tissue by an electron dense basement membrane about 0.04 μm in thickness. The basement membrane followed a slightly wavy course defined by the line formed by the tips of the microvillus-like processes of the basal cells. Numerous fibroblasts which appeared to be actively synthesising collagen fibres were found in the subepithelial layer. Collagen fibres were also abundant in the subepithelial space. Blood vessels with tenuous and sometimes fenestrated endothelium lay close to the basement membrane.

3.4.2 Observations on the cytology of epithelial cells

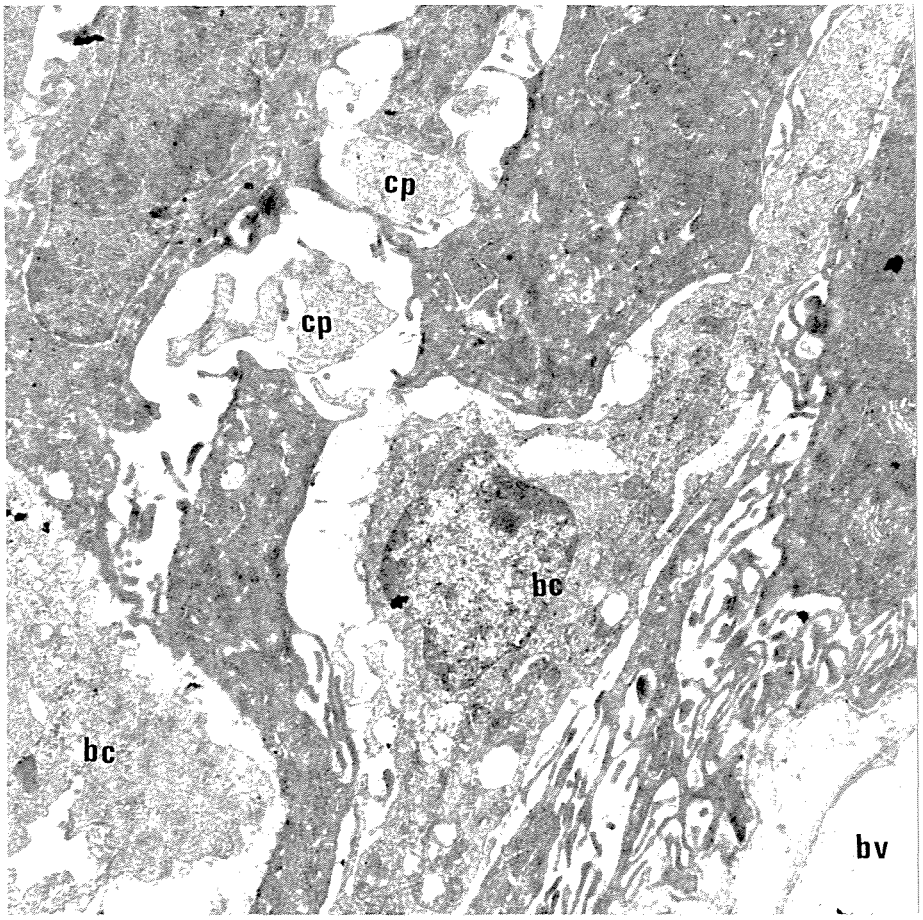
Unless otherwide stated, the descriptions in this section generally apply to the epithelium taken from the different locations in the forestomach of the adult and unless otherwise stated, may also apply to the epithelium in younger animals.

3.4.2.1 Cells of the stratum basale (Figures 3.15 - 3.17)

Two types of cells were found in the basal layer of the epithelium. These were the columnar cells and the light-staining branching cells interspersed between the columnar cells (Figure 3.15).

A. Columnar cells (Figure 3.16). The most striking feature of the columnar cells of the basal layer was the large number of mitochondria present in the cytoplasm (Figure 3.16 A). These mitochondria were situated both above and below the ovoid nuclei and many contained electron-dense bodies. They appeared to be of varying shapes and sizes and were not orientated in any order. Their cristae

Figure 3.15 Electron micrograph of the epithelium taken from the forestomach of the adult sheep. Stratum basale, x 8100. Note light-staining branching cells (bc) and their cytoplasmic processes (cp) between the columnar basal cells. The blood vessel (bv) has an attentuated endothelium.


cp
cp
bc
bc
bv

were prominent and had varying conformations; it was not uncommon to find a mitochondrial crista completely partitioning a mitochondrion (Figure 3.16 B).

Scattered within the cytoplasm of basal cells were numerous ribosomes, most of which were free, in clusters or associated with membranes of the endoplasmic reticulum (Figure 3.16 C). Centrioles were also observed although they were rather infrequent. The Golgi apparatus was prominent and lay close to the nucleus. Lysosomal bodies and vesicles of varying sizes containing materials of differing densities were also distributed throughout the cytoplasm (Figure 3.16 B). Some of these vesicles were membrane bound others were located at the periphery of the cell. Tonofilaments occurring either free or in bundles were scattered throughout the cell. These tonofilaments which were also seen associated with ribosomes were especially numerous in the basal cells of the omasal and reticular groove epithelia.

The basal cells were surrounded by prominent intercellular spaces which were interrupted rather infrequently by desmosomes with short tonofilaments attached to them (Figure 3.16 C). Pentalaminar junctions (gap junctions) occasionally joined adjacent basal cells (Figure 3.16 D).

Finger-like cytoplasmic processes extended from the cell surface into the lateral and apical intercellular space (Figure 3.16 E). In the basal surface, these cytoplasmic processes formed an array of microvillus-like processes extending over the wide distance (the so-called basal sinus) between the main cell body and the basement membrane. The tips of these processes were observed to come together to close off the basal sinus and were anchored to the basement membrane by hemidesmosomes. No mitochondria were generally found in the cell processes and a few basal cells without microvillus-like processes were also observed.

B. Light-staining Branching Cells (Figure3.17). The light-staining cells which had been seen in the light microscope were also easily distinguished in electron micrographs by their relatively clear cytoplasm and sinuous

Figure 3.16 Electron micrographs of the forestomach epithelium taken from the adult. Stratum Basale.

A. Columnar basal cells and underlying connective tissue. x 9000. Note abundant mitochondria in the basal cell cytoplasm. The basolateral membrane surfaces are thrown into numerous cytoplasmic processes. The intercellular spaces between basal cells are wide. Connective tissue fibroblasts are seen.

B. Detail of the cytoplasm of columnar basal cells. x 17,550. Note C-shaped mitochondria and mitochondrial cristae completely separating the mitochondria. ly - lysosomal body; t - tonofilaments in bundles.

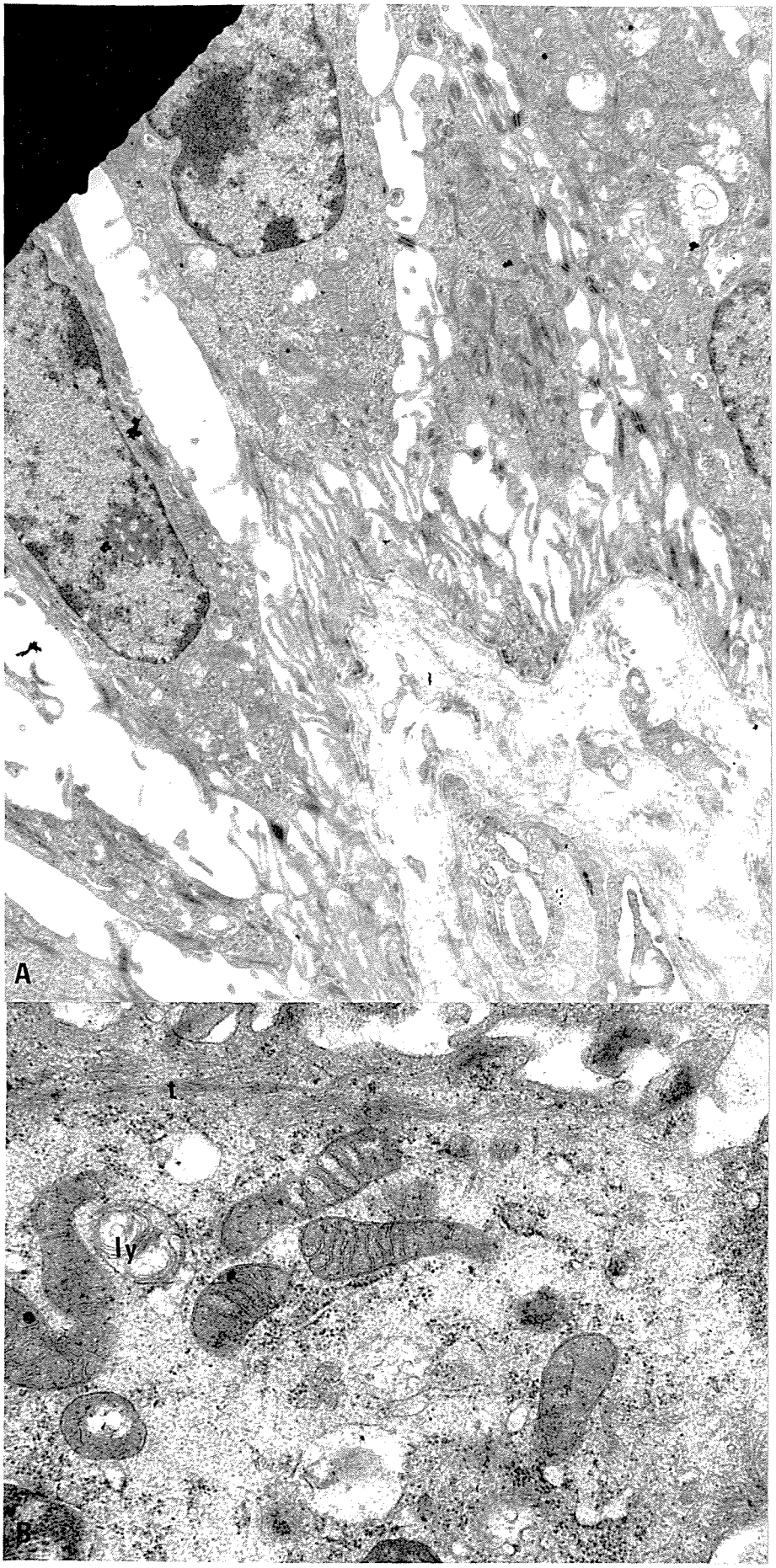
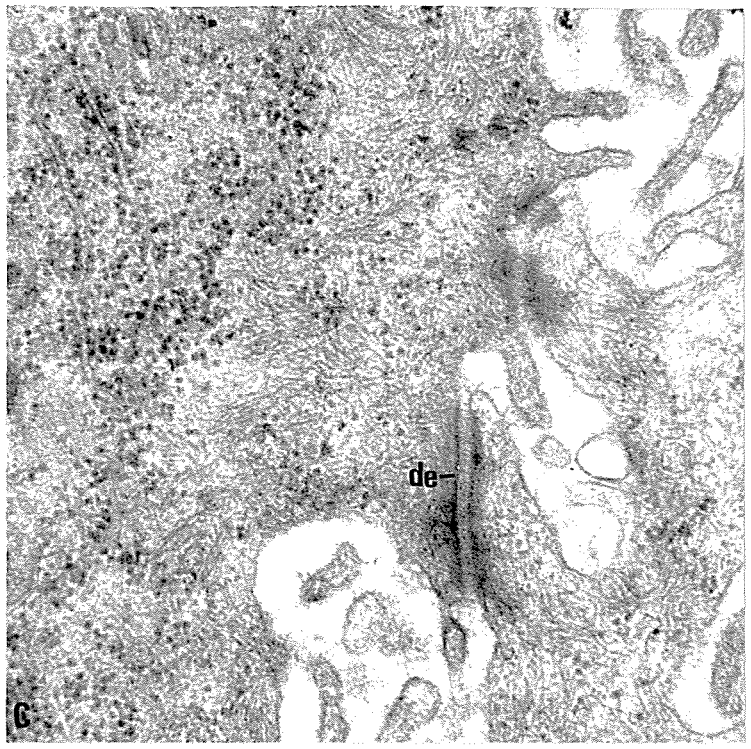

A
t
ly
B

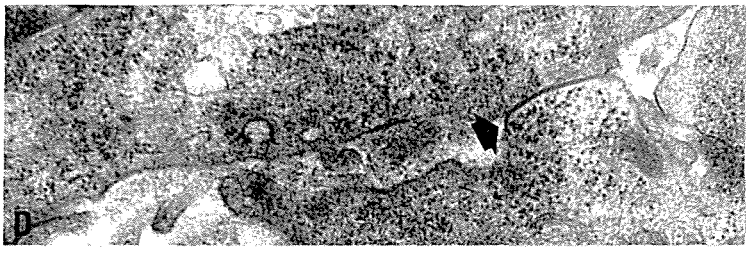
Figure 3.16 (Continued) Electron micrographs of the epithelium taken from the forestomach of the adult sheep. Stratum Basale.

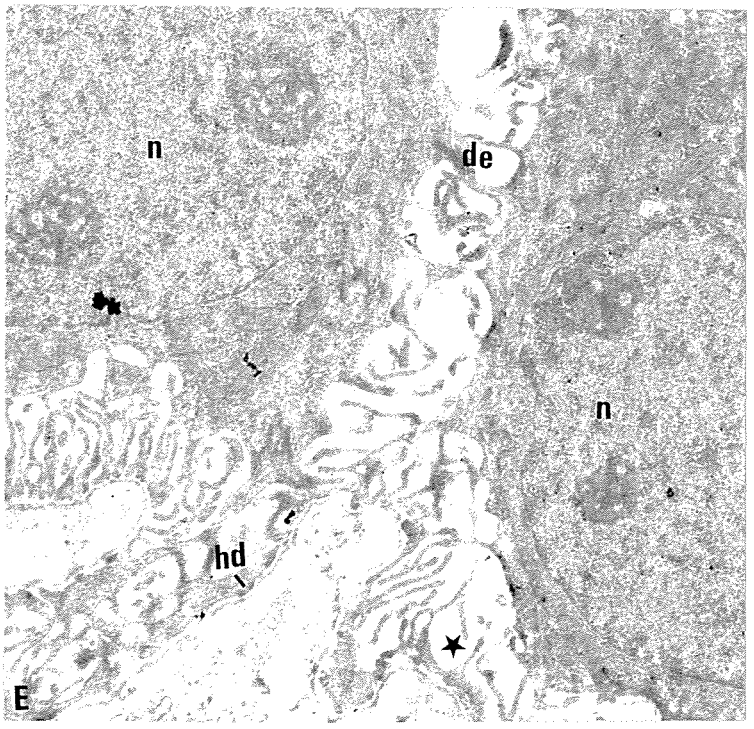
C. Detail of basal cell cytoplasm and desmosomal connection (de) between two adjacent basal cell. x 26,200 Note rough endoplasmic reticulum, tonofilaments and free ribosomes in the cytoplasm. The lateral membrane surfaces are thrown into cytoplasmic process.

D. Gap junctions between two neighbouring basal cells (arrows). x 17,550.

E. Detail of microvillus-like array of processes in the basal surface of basal cells. Hemidesmosomes (hd) anchor basal cell processes to the basement membrane. Star indicates the basal sinus. x 7865.


de
C


D


n
de
n
hd
E

irregular outline. These cells were broadly classified into two categories:

1. Light-staining cells with branching cytoplasmic processes and which did not form any interconnections with neighbouring cells (Figure 3.15 and 3.17 A). These cells were interspersed between the basal cells in the epithelium taken from all sites in all ages. They were light-staining in electron micrographs because of the relative paucity of cellular organelles in the cytoplasm. These cells had either deeply indented or smoothly outlined nuclei. A few mitochondria, Golgi apparatus, endoplasmic reticulum either associated or not associated with ribosomes, free ribosomes, lysosomes, multi-vesicular bodies, dense bodies containing concentric lamellae and other inclusions were found in the cytoplasm as well as in some of the processes. The processes, which were often long and tortuous, were seen in between the basal cells or between the cells in the upper layer of the epithelium, and they sometimes displaced the nucleus.

Within this category, the Langerhans cell was positively identified (Figure 3.17 B). This cell had the characteristic cytoplasmic granules (Birbeck granules) which were few but were found anywhere in the cytoplasm including in the cytoplasmic processes. However, most of the light-staining cells, although generally similar to Langerhans cells, were difficult to categorise because they lacked recognisable Birbeck granules or other identifying inclusions characteristic of other branching cells found in most mammalian keratinizing epithelia (e.g., melanosomes and Merkel cells), and appeared to be equivalent to the so-called "indeterminate cells" (Breathnach, 1980).

Other cells were round in outline with electron dense nuclei similar to those found in lymphocytes (Figure 3.17 C). Still others had, in their cytoplasm, granules of differing densities, most of which showed a periphery of coarsely granular patches while other showed lamellated or crystalline internal arrangements (Figure 3.17 D). In some cells with these granules, a few strands of rough endoplasmic reticulum and free ribosomes appeared in the cytoplasm, while in others, rough endoplasmic reticulum was prominent and,

Figure 3.17 Electron micrographs of the epithelium taken from the forestomach of the adult sheep. Stratum Basale.

A. Light-staining cell with various cytoplasmic inclusions. Note the absence of cell junctions which attach it to neighbouring basal cells. x 13600.

B. Cell process of a light-staining cell between basal cells. Arrow indicates the Birbek granule which identify this particular cell to be a Langerhans cell. x 22,500.

C. A lymphocyte-like cell in the epithelium. x6435.

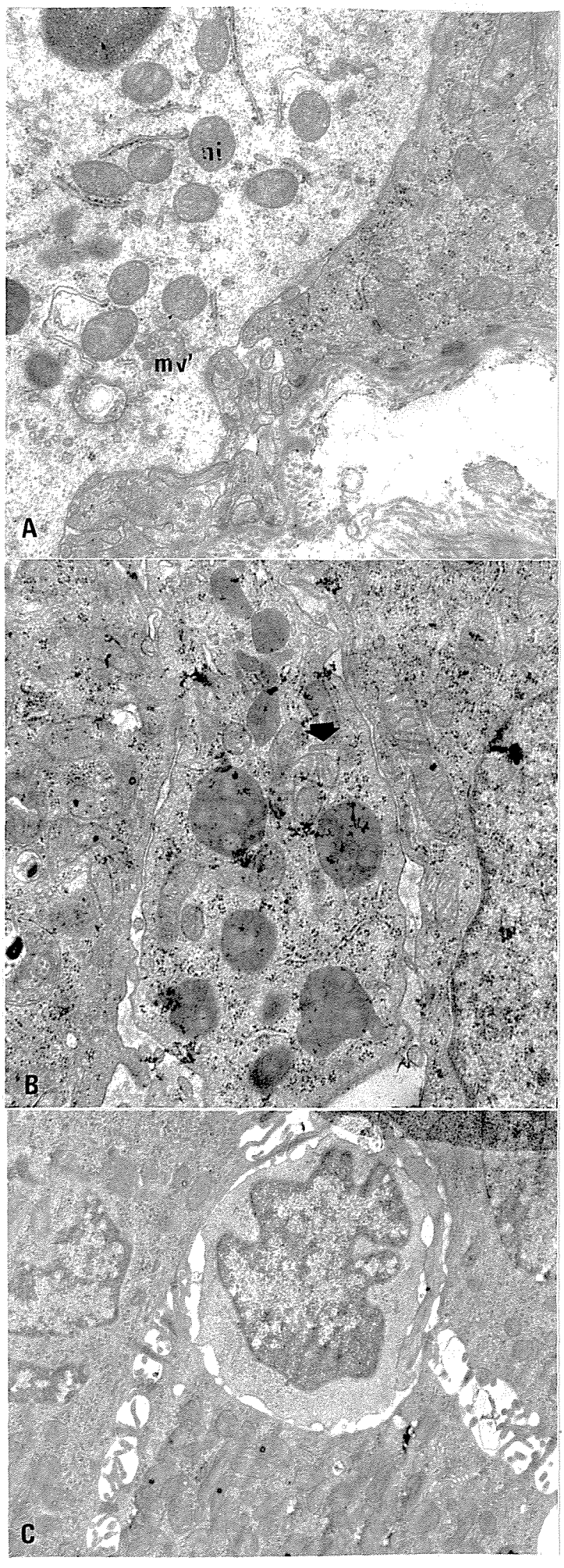
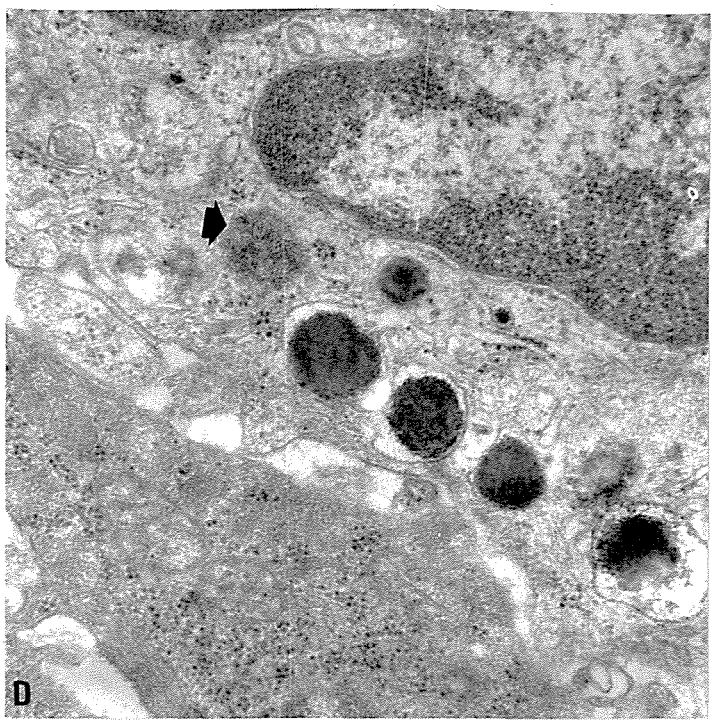

ni
mv
A
B
C

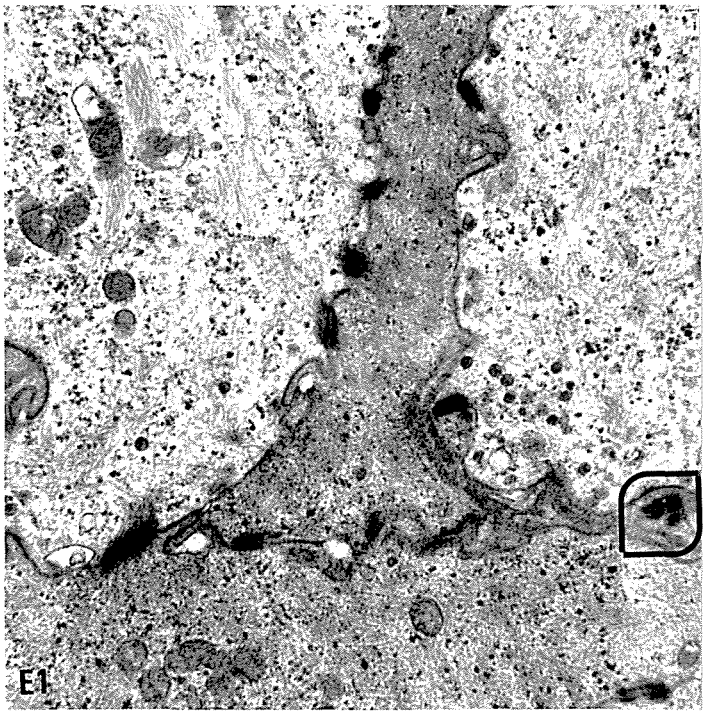
Figure 3.17 (Continued) Electron micrographs of the epithelium taken from the forestomach of the adult sheep. Stratum Basale.

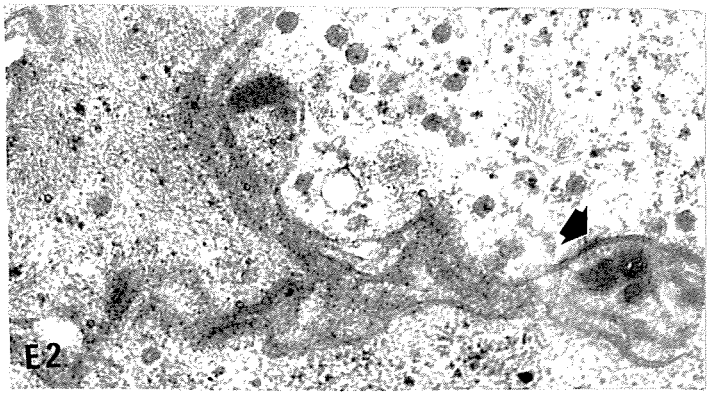
D. Detail of light-staining cell containing granules of differing densities; most of the granules are with coarse granular patches in periphery while one shows lamellation or para-crystalline arrangement (arrow). x 35,700.

E 1. Light-staining cell which form desmosomal attachment with neighbouring basal cells. Note dense-cored granules situated to one side of the cytoplasm. Demaracted area corresponds to the synapse-like structure indicated by the arrow in Figure 3.17 E2. x 13,600.

E 2. Detail of dense-cored granules and synapse-like structure (arrow) associated with a few dense-cored granules. x 23,400.


D


E1


E2

together with mitochondria and Golgi apparatus occupied the spaces between the granules. Blunt cytoplasmic processes were present on the cell surfaces of these cells.

2. Light-staining cells which formed interconnections with neighbouring cells (Figure 3.17 E). These cells were characterised by their generally ovoid nuclei and the relative paucity in the number of formed organelles in the cytoplasm. Unlike the cells in the first category, these cells were connected to neighbouring cells by desmosomes. A few mitochondria, ribosomes, endoplasmic reticulum and tonofilaments were observed in the cytoplasm. The Golgi apparatus was well-developed and was situated near the nucleus. Small dense-cored granules situated to one side of the cell near the cell surface were also observed. Cells in this category were usually seen in the reticular epithelium.

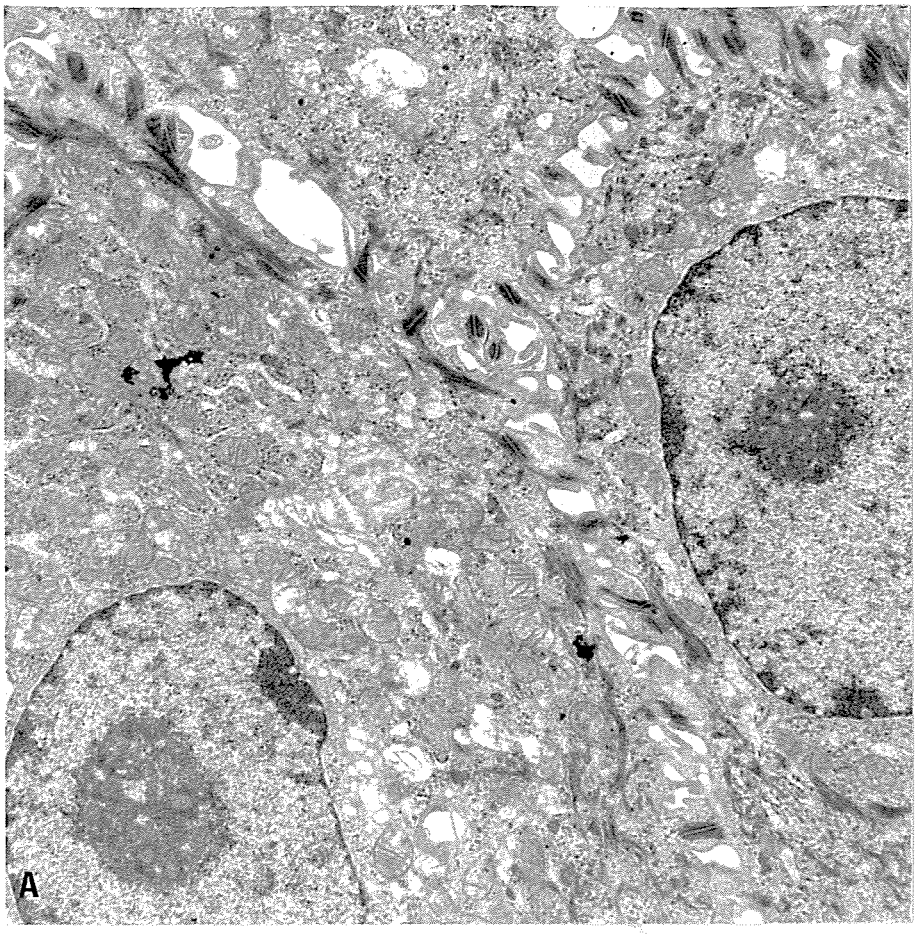
3.4.2.2 Cells of the stratum spinosum (Figure 3.18)

The most prominent features of the cells of the stratum spinosum were their large size and the increased size of the bundles of tonofilaments in the cytoplasm and in the desmosomal connections with adjacent cells (Figure 3.18 A). The bundles of tonofilaments were usually observed parallel to the cell surface and joined with desmosomes. At the boundary between the stratum basale and the stratum spinosum, the desmosomes were situated at the ends of tortuous cytoplasmic processes which appeared unconnected to any cell (Figure 3.18 B). The tonofilaments which joined the desmosomes in this level were short. In the upper levels of the stratum spinosum, the cells were flattened, their cytoplasmic processes became straight with the desmosomes still retaining cell-to-cell contact. The desmosomes were well-developed and characterized by parallelism of the cell membranes, an intercellular space occupied by a plug of dense material bisected by a denser central layer, dense cytoplasmic plaques backing the inner membrane leaflets and long bundles of cytoplasmic tonofilaments converging on the inner aspect of each plaque (Figure 3.18 C). Gap junctions were also found joining adjacent spinous cells (Figure

Figure 3.18 Electron micrographs of the epithelium taken from the forestomach of the adult sheep. Stratum Spinosum.

A. Cells of the stratum spinosum. Mitochondria, bundles of tonofilaments, rough endoplasmic reticulum (some filled with granular Er-protein) and ribosome clusters are abundant in the cytoplasm. Golgi bodies are dilated. Desmosomes are quite prominent. x 21,450.

B. Tortuous cytoplasmic processes located between the stratum basale and stratum spinosum. Desmosomes are found joining the ends of the processes. Gap junctions (arrows) are also found. x 27,500.


A

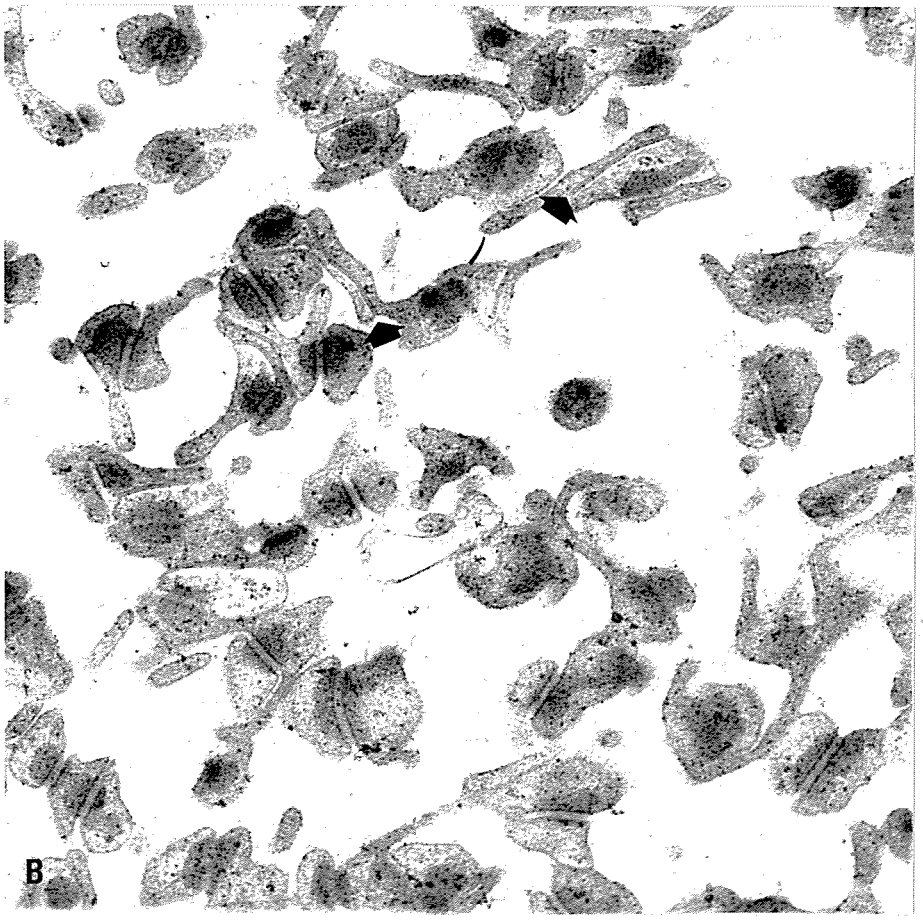
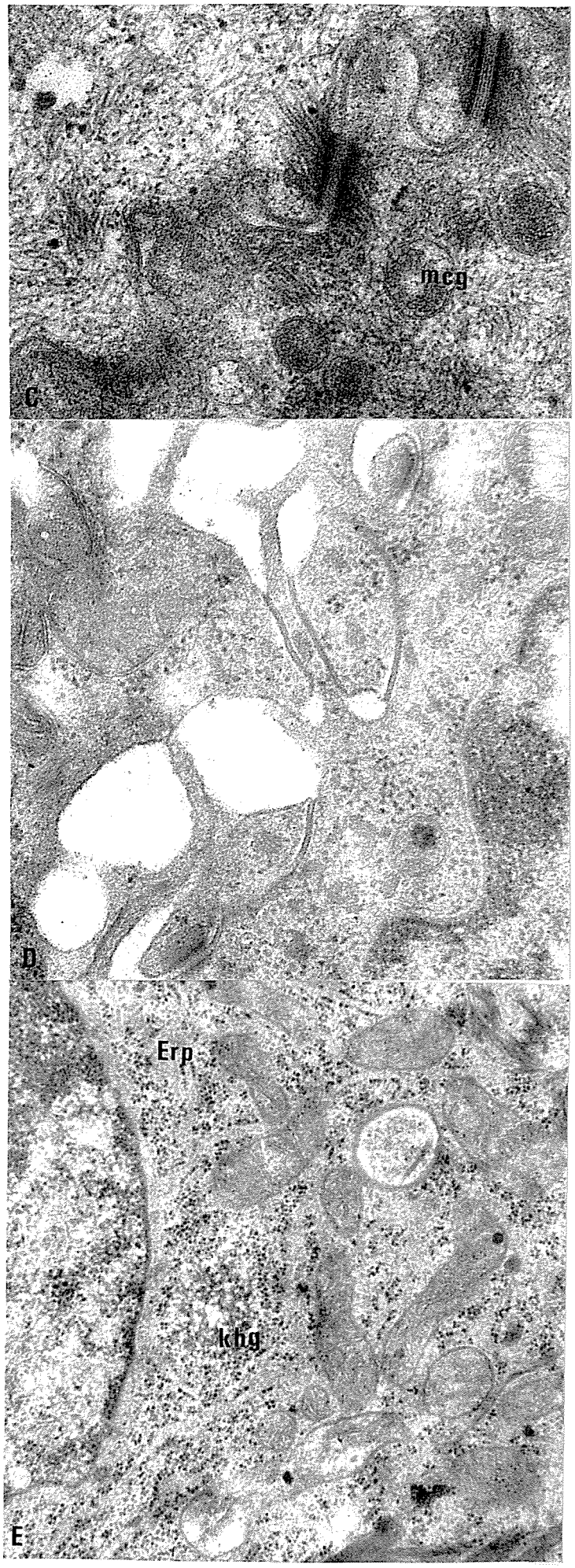

B

Figure 3.18 (Continued) Electron micrographs of the epithelium taken from the forestomach of the adult sheep. Stratum Spinosum.

C. Detail of desmosomes in the intermediate layers of the epithelium. Note MCGs with tubular granules. x 33,750.

D. Detail of intercellular junctions between spinous cells. Gap junctions and desmosomes. x 45954.

E. Detail of cytoplasmic structures in the upper spinous cells. Er-protein (Erp) is found free in the cytoplasm. Cisternae of rough endoplasmic reticulum are dilated with Er-protein. A small keratohyalin granule is surrounded by ribosomes. x26,200.


mcg
C
D
Erp
khg
E

3.18 D).

Large numbers of mitochondria were also a feature of the cells of the stratum spinosum (Figure 3.18 A). As in basal cells, they were of varying shapes and sizes and did not appear to be orientated in any order. Free ribosomes were numerous throughout the cytoplasm. The cisternae of rough endoplasmic reticulum especially in the upper layers were dilated with filamentous material, the so-called Er-protein (Figure 3.18 E). Er-protein was also seen free in the cytoplasm (Figure 3.18 E). Some membrane-coating granules and keratohyalin granules were found in the upper cell layers. The keratohyalin granules were small electron-dense oval bodies predominantly located in the perinuclear region of the cell. Most were vacoulated and surrounded by ribosomes (Figure 3.18 E). Others were freely in contact with the surrounding cytoplasm incorporating tonofilaments.

3.4.2.3 Cells of the stratum granulosum (Figure 3.19)

The cytoplasm of granular cells contained numerous keratohyalin granules as well as membrane-coating granules (Figure 3.19 A and B). The keratohyalin granules were large and surrounded by ribosomes; some were situated near the Golgi apparatus. The membrane-coating granules were about 0.1 - 0.3μm in diameter and more numerous towards the periphery of the cells especially on the side near the lumen. Membrane-coating granules were especially observed in sections passing through the cell periphery (Figure 3.19 B).

At high magnifications, there appeared two forms of membrane-coating granules based on their internal organizations. The more common was the membrane-coating granule limited by trilaminar membrane structure containing usually aggregated centrally placed ring-like or tubular granules (Figure 3.19 C). In the second type, lamellations consisting of alternating electron-dense and electron-lucent bands were found (Figure 3.19 D). Membrane-coating granules with intermediate internal structure - i.e., a central core of lamellations and surrounded by granules in the periphery were also found.

Figure 3.19. Electron micrographs of the epithelium taken from the forestomach of the adult sheep. Stratum Granulosum.

A. Montage of keratinocytes in the upper level of the epithelium. Reduced from 13,600 magnification.

In the granular cells (sg), the keratohyalin granules are in large masses; the cisternae of endoplasmic reticulum are dilated with Er-protein. A lysosomal body (ly) is seen indicating that the granular cell is already in the second phase in the keratinization process. Note that the intercellular spaces between the granular cells are narrow or virtually obliterated.

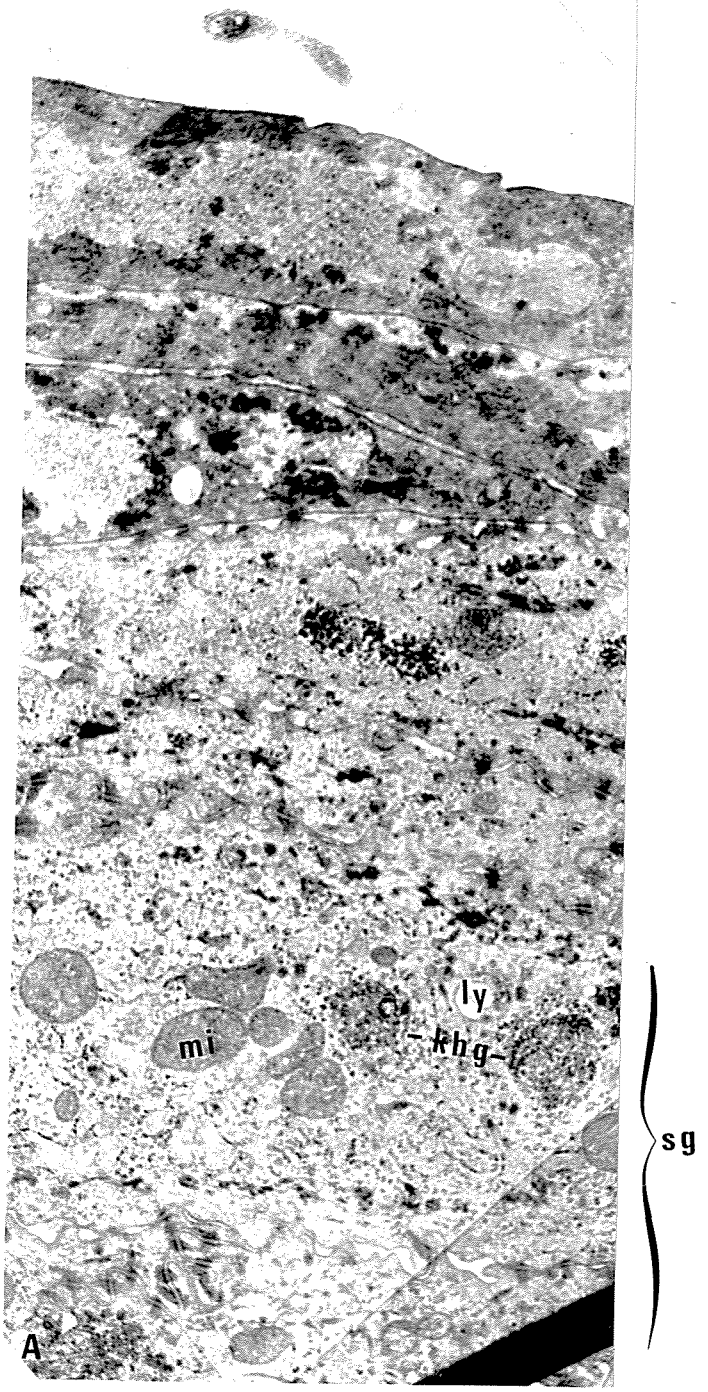
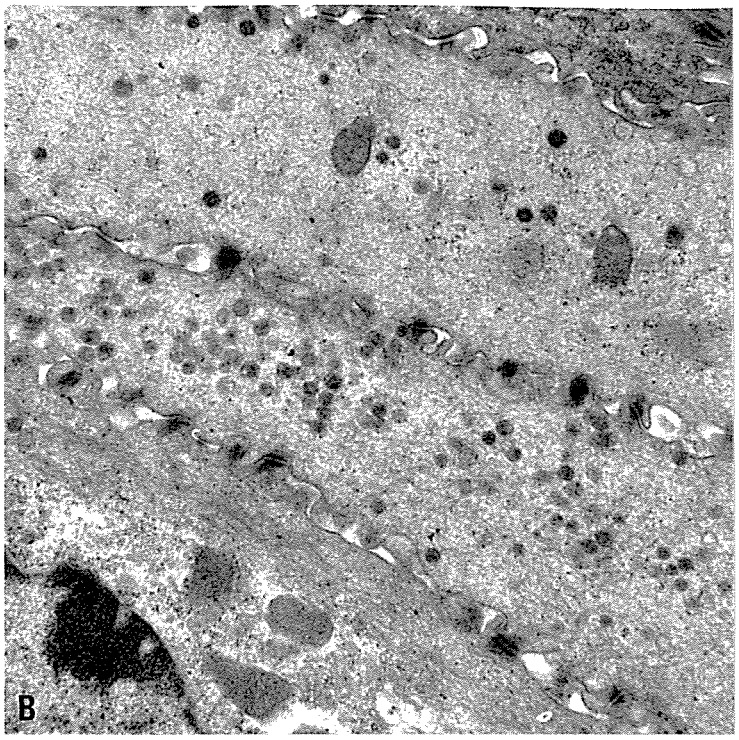

ly
mi
sg
A

Figure 3.19 (Continued) Electron micrographs of the epithelium taken from the forestomach of the adult sheep. Stratum Granulosum.

B. Membrane-coating granules (MCGs) in granular cells. x 13,600.

C. Detail of the internal structure in MCGs. Arrows indicate MCGs with tubular internal granules. Star indicates an MCG with a lamellated internal appearance. x 134,310.


B

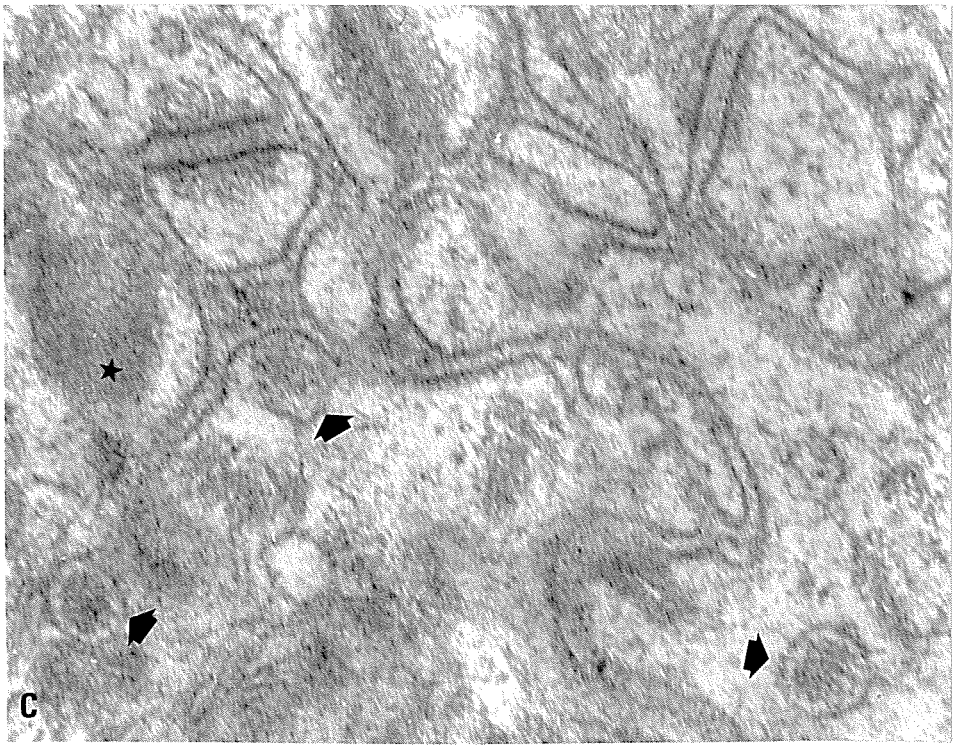
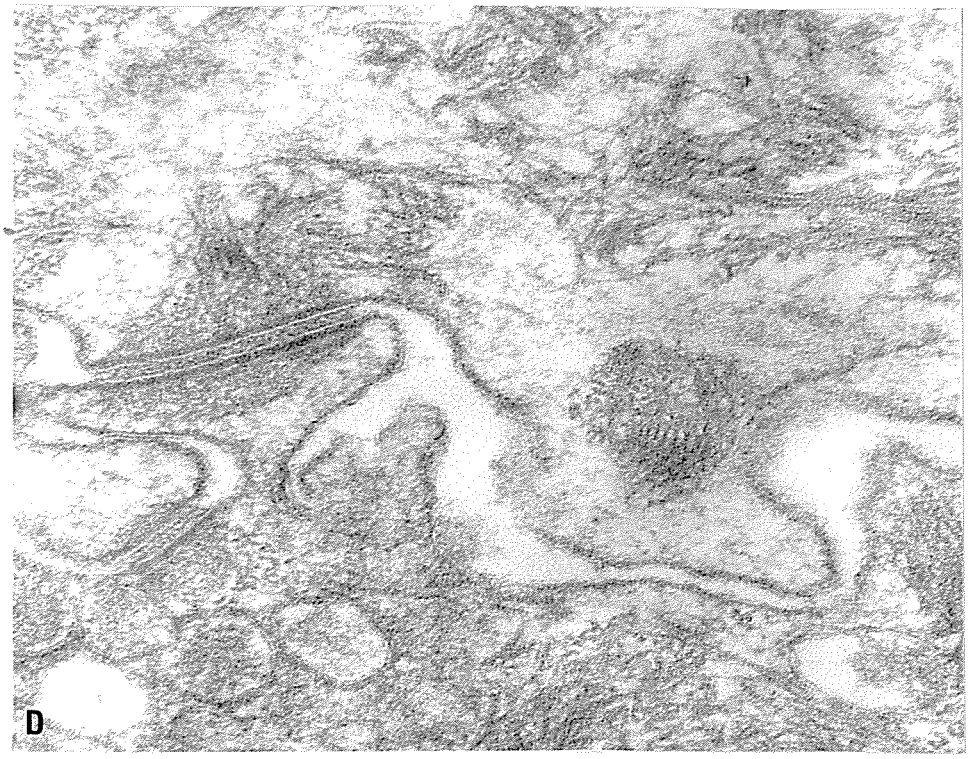
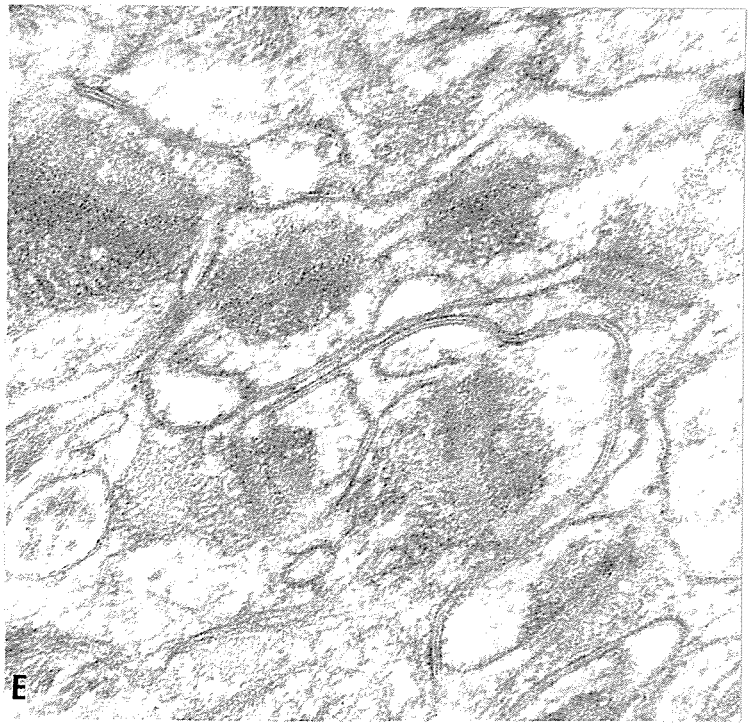

C

Figure 3.19 (Continued) Electron micrographs of the epithelium taken from the forestomach of the adult sheep. Stratum Granulosum.

D. Detail of MCG with lamellated internal structure. x 134,310.

E. Long strands of gap junctions between cells of the stratum granulosum. x 85,500.


D


E

Mitochondria were also numerous in the granular cell cytoplasm, some showing cristal swelling. Free ribosomes, dilated Golgi apparatus, bundles of tonofilaments and few lipid droplets were found throughout the cytoplasm. The cisternae of the rough endoplasmic reticulum were highly dilated with Er-protein which was also found free in the cytoplasm (Figure 3.19 A).

The intercellular spaces surrounding the flattened cells of the stratum granulosum were decidedly narrow and in most places, were obliterated by desmosomes, close apposition of adjacent cell membranes and by gap junctions. This was especially observed in the outer limit of this layer where the intercellular spaces were virtually obliterated by long strands of gap junctions between neighbouring cell membranes (Figure 3.19 E). Zonula occludentes (tight junctions) were also seen here especially associated with desmosomes or with gap junctions. The so-called internalised gap junctions (or annular junctions) were also observed (see Figure 3.18 E).

3.4.2.4 Cells of the stratum transitionale (Figures 3.19 A and 3.20)

Varying degrees of degradation of the formed cell organelles and cell cornification were observed in the cells of the stratum transitionale. The most conspicuous features of the cells in this layer were the presence of numerous lysosomal bodies in the cytoplasm, the aggregation of keratohyalin granules into large masses which were later observed to be dispersed in the cytoplasm and the marked dilation of the cisternae of endoplasmic reticulum with Er-protein which were also seen dispersed in the cytoplasm together with the components of keratohyalin granules (Figures 3.19 A; 3.20 A). The lysosomal bodies appeared to be autolysosomes and varying stages of their development were observed in a transitional cell (Figure 3.20 A). Dispersion of Er-protein and keratohyalin granules displaced the degraded cellular constituents including the nucleus.

Tonofilament bundles were now in greater density in the peripheral cytoplasm and appeared to have coalesced.

Figure 3.20 Electron micrographs of the epithelium taken from the forestomach of the adult sheep. Stratum Transitionale.

A. Montage showing a prominent transitional cell. Reduced from 13,600 magnifications. Autolysosomes are abundant; note MCGs are present in the cells immediately below the transitional cell but markedly reduced in the transitional cell itself.

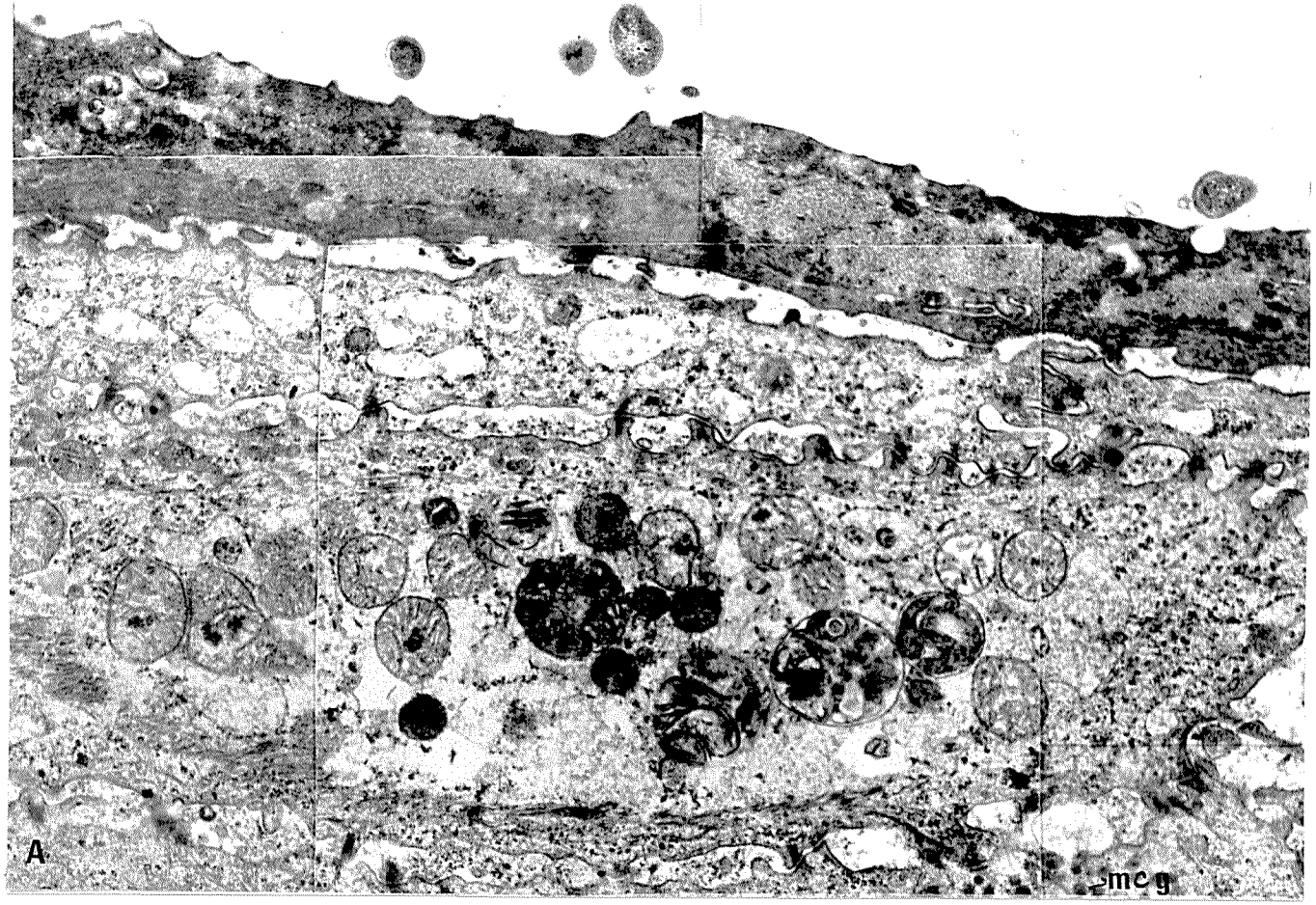
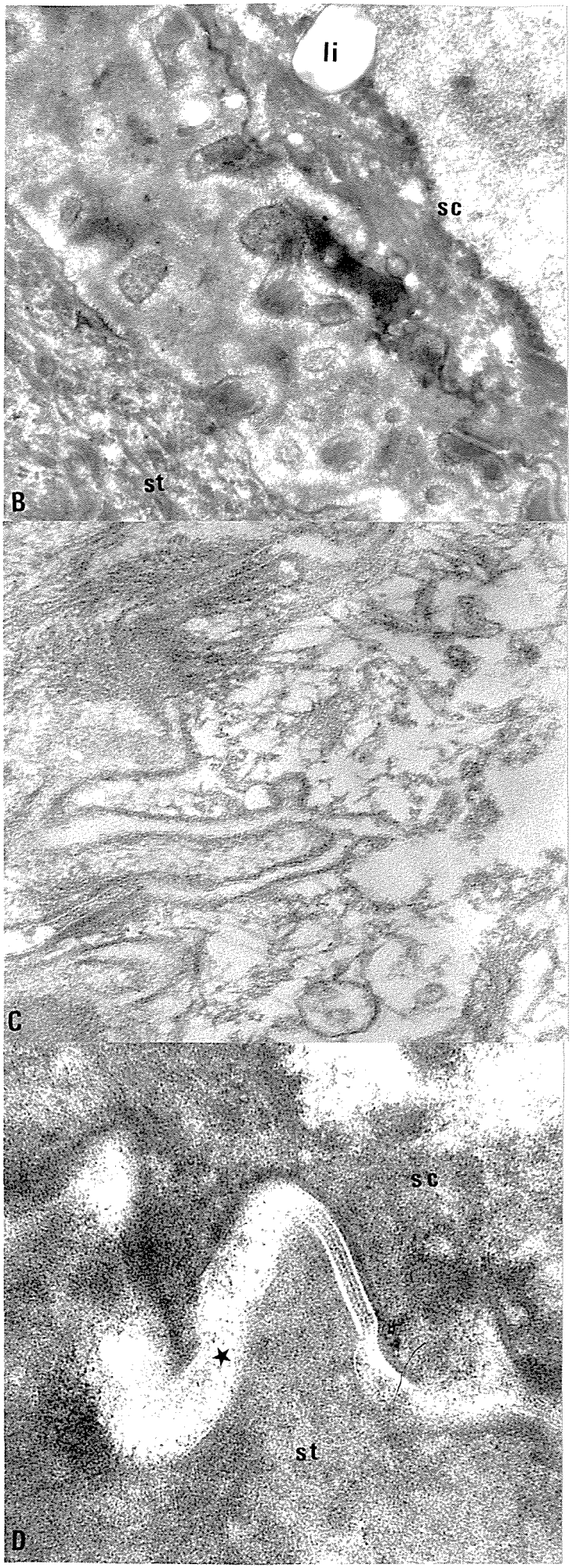

A
mcg

Figure 3.20 (Continued) Electron micrographs of the epithelium taken from the forestomach of the adult sheep. Stratum Transitionale.

B. Electron-dense material in the intercellular space between the stratum transitionale (sc) and the stratum corneum (sc); some intact MCGs are found in the intercellular space. Note the thickened membrane of the cornified cell. A lipid droplet (li) is found in the cornified cell. x 13,600.

C. MCG with the appearance of being extruded into the intercellular space. x 85,500.

D. Modified desmosomes between the transitional cell (st) and corneal cell (sc). Note thickened cytoplasmic membrane of the corneal cell. Star indicates lamellated material in the intercellular space. x 47,400.


li
sc
st
B
C
sc
st
D

Membrane-coating granules have markedly decreased in the transitional cell cytoplasm and appeared to have been extruded together with the degraded cell organelles into the intercellular spaces (Figure 3.20 B and C). Electron-dense material occupied the now widened intercellular spaces (Figure 3.20 B). Fuzzy material was seen adhering to the outer surfaces of the cell membranes (Figure 3.20 B). Lamellations were also seen in the intercellular spaces (Figure 3.20 D) as well as intact membrane-coating granules (Figure 3.20 B).

Desmosomes joined the transitional cells to neighbouring lateral and apical cells. They were prominent on the ends of the long cytoplasmic processes and appeared to be modified between the stratum transitionale and stratum corneum. Modified desmosomes were characterised by apposition of two parallel trilaminar cytoplasmic membranes, one of which, on the horny cell side had a peripheral dense layer fused to its inner leaflet which was continuous with the desmosomal attachment plaques (Figure 3.20 D).

In the reticulum, although no swollen transitional cells were found in histological sections, flattened cells with similar ultrastructure to swollen transitional cells in the rumen were observed (see Figure 3.21 B).

3.4.2.5 Cells of the stratum corneum (Figure 3.21)

The most prominent features of the cells of the keratinized layer were their thickened cytoplasmic membranes and their internal structure. The cornified cell was surrounded by a trilaminar membrane with thickened inner leaflet. Based on the ratio of their internal constituents, two kinds of keratinized cells were found. The first was the vesicular keratinized cell (Figure 3.21 A) in which two regions were distinguished: (1) an electron-dense periphery composed of coalesced tonofilaments embedded in a dense matrix of keratohyalin and Er-protein material; and (2) an electron lucent centre composed of fine fibrillary material from the Er-protein. The second type of keratinized cell was flattened and heavily keratinized (Figure 3.21 B).

Figure 3.21 Electron micrographs of the epithelium taken from the forestomach of the adult sheep. Stratum corneum.

A. Montage of the upper level of the epithelium showing a prominent vesicular cell (sc); st - transitional cell; sg - granular cell. x 13,600.

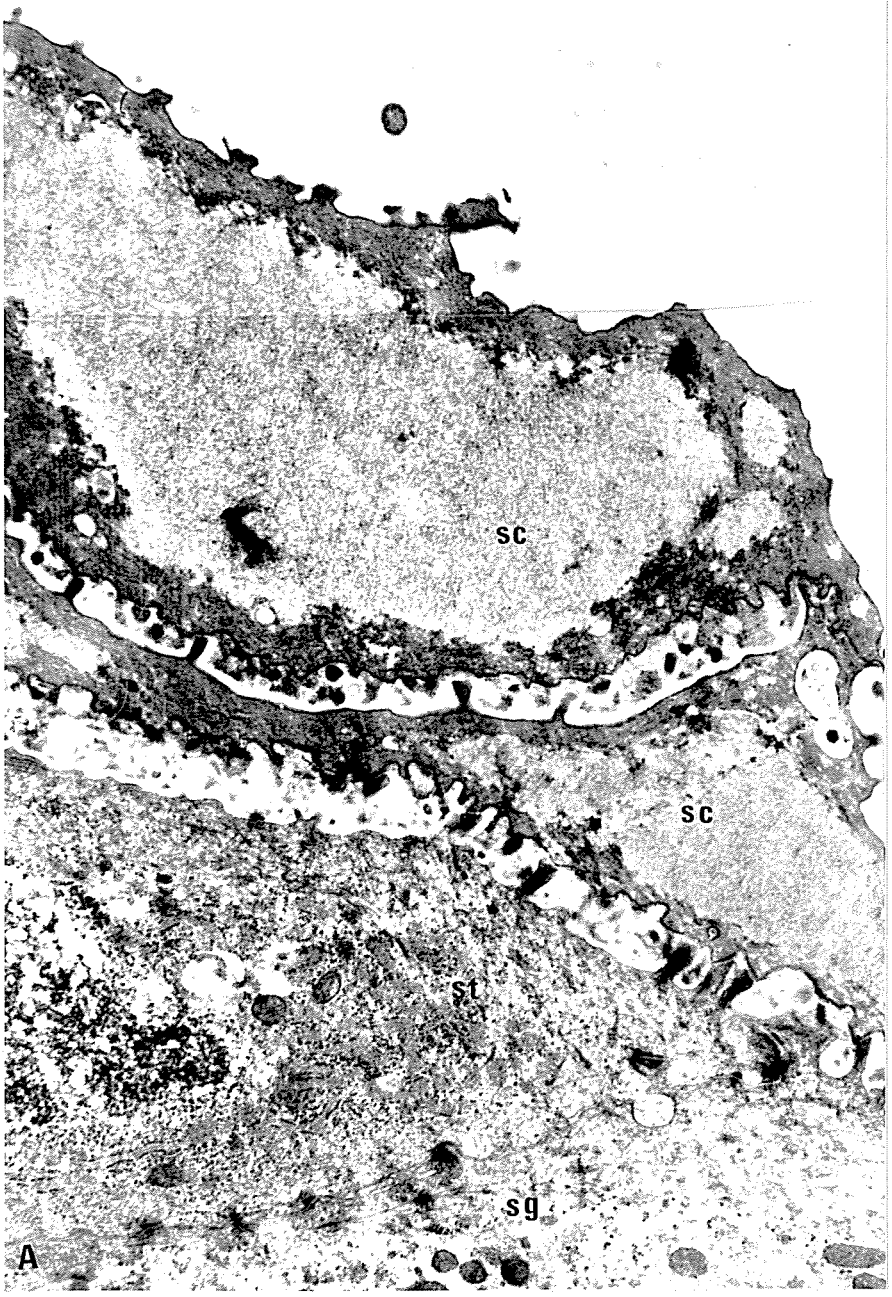

sc
sc
st
sg
A

Figure 3.21 (Continued) Electron micrographs of the epithelium taken from the forestomach of the adult sheep. Stratum Corneum.

B. Montage of the upper levels of the epithelium of the reticulum. Reduced from 13,600 magnifications.

The keratinized layer is composed of flattened keratinized cells. In the lower levels, only a few keratohyalin granules are found. li - lipid droplet; Erp - Er-protein.

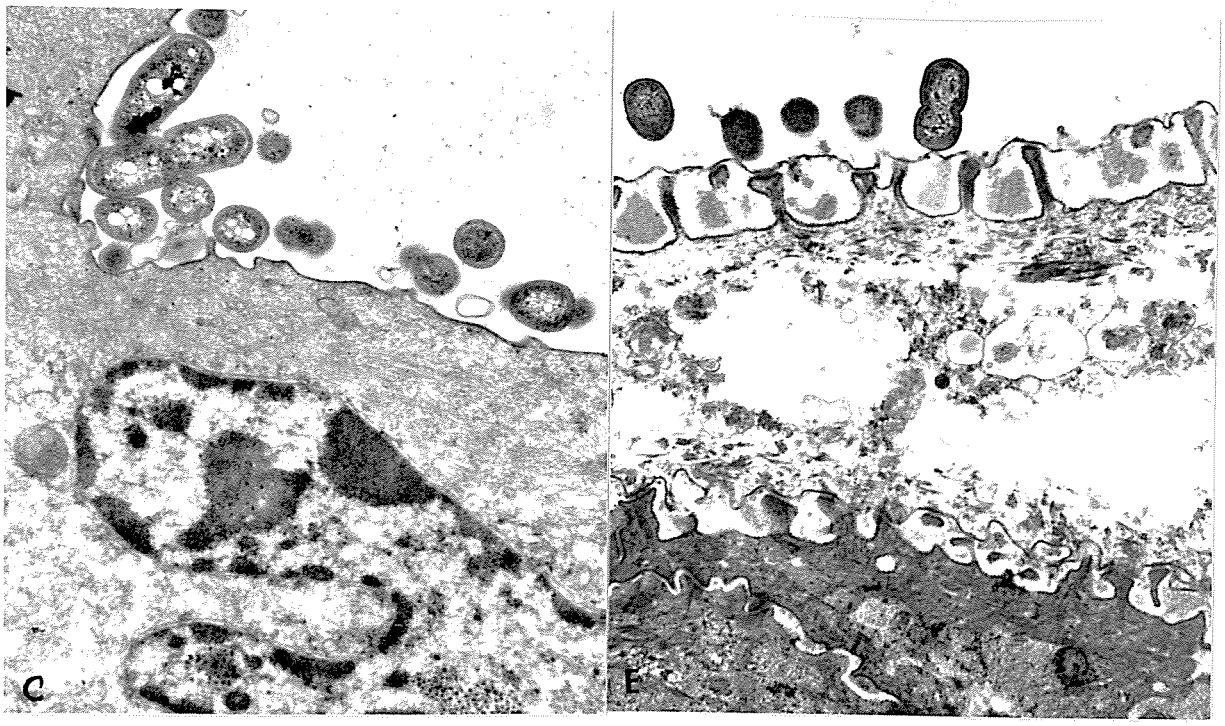

li
Erp
B

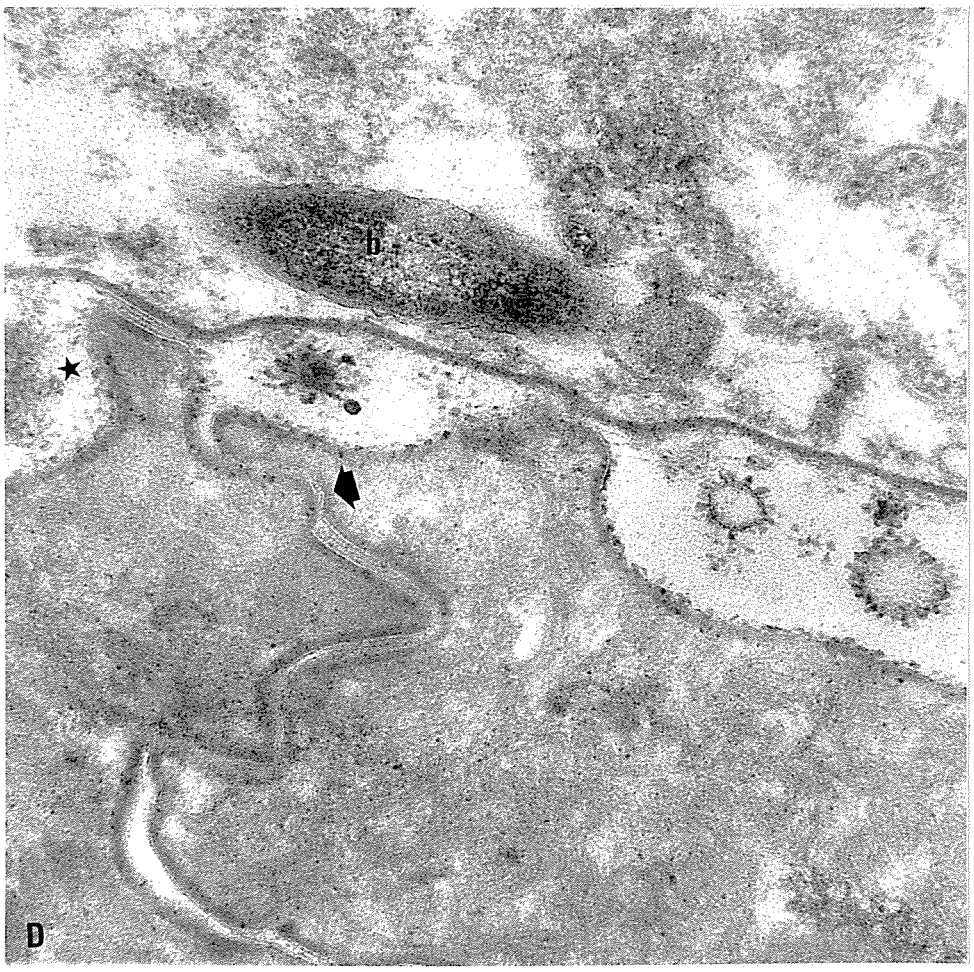
Figure 3.21 (Continued) Electron micrographs of the epithelium taken from the forestomach of the adult sheep. Stratum Corneum.

C. Retained nuclei in the keratinized layer displaying degradation. Note adherent bacteria. x 13,600.

D. Junctional complex between two cells of the stratum corneum - apical zonulae occludens (tight junction, arrow) and desmosomes. Note intact MCGs in the intercellular spaces. Star indicates lamellations in the intercellular spaces; b - bacterial cell. x 74,481

E. Bacteria adherent to degraded cornified cell. x 13,600.


C
E


b
D

Its internal structure was composed of tonofilaments suspended in a dense amorphous matrix composed of a mixture of dispersed of Er-protein and keratohyalin granules. Occasionally, nuclei were observed in both types of keratinized cells (Figure 3.21 C), but they appeared to be in advanced state of degradation - i.e., margination of chromatin material was observed. Lipid droplets were frequently seen especially in flattened keratinized cells. Glycocalices covering the luminal surface of the flattened keratinized cells were observed to have a fine fibrillary structure giving it a fuzzy appearance.

In sections where the keratinized cells were several cells deep, desmosomal remnants were found to join the cells to each other (Figure 3.21 B). Below desquamating cells, zonulae occludentes (tight junctions) were frequently observed in association with modified desmosomes to close off the intercellular spaces from the lumen (Figure 3.21 D).

Desquamation of cornified cells, especially of the vesicular type appeared to be partly mediated by bacteria. In many sections, adherent bacteria were observed on the superficial cells of the epithelium (Figure 3.21 E).

3.4.3 Observations on the ultrastructure of the epithelium in different ages

The following accounts summarise the distinguishing features of the ultrastructure of the epithelium during development of lambs from birth to 56 days of age as observed in the present study.

3.4.3.1 Birth-24 hours (Figure 3.22)

Although in low power micrographs the neontal epithelium appeared similar to that of the adult epithelium - i.e., stratification of epithelial cells and flattening of the most superficial cells, its ultrastructure presented a different picture.

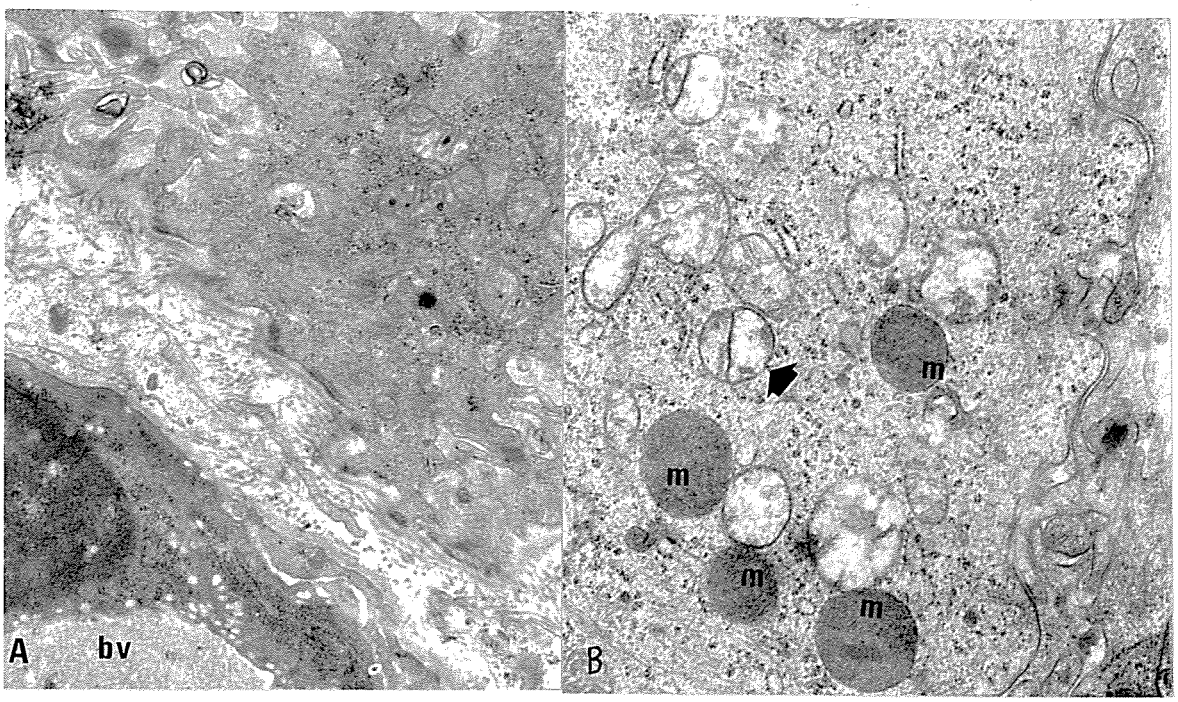
In the stratum basale, the columnar basal cells contained only a few mitochondria (Figure 3.22 A). However, ribosomes, endoplasmic reticulum and free tonofilaments were numerous. There was a high synthetic activity in the cells especially in the production of tonofilaments,

Figure 3.22 Electron micrographs of the epithelium taken from one-day-old lambs.

A. Stratum basale. A few mitochondria are present in the cytoplasm; the basal surface of the cell is smooth and the endothelium of the blood vessel (bv) is thick. Note numerous micropinocytic vesicles in the blood vessel endothelium. x 13,600.

B. Large mucous granules (m) in the basal cell of the epithelium Note mitochondria (arrow) completely partitioned by a crista. x 17,500.

C. Glycogen granules (dense-staining granules) in the cytoplasm of intermediate cells. x 6435.


A
bv
B
m
m
m
m

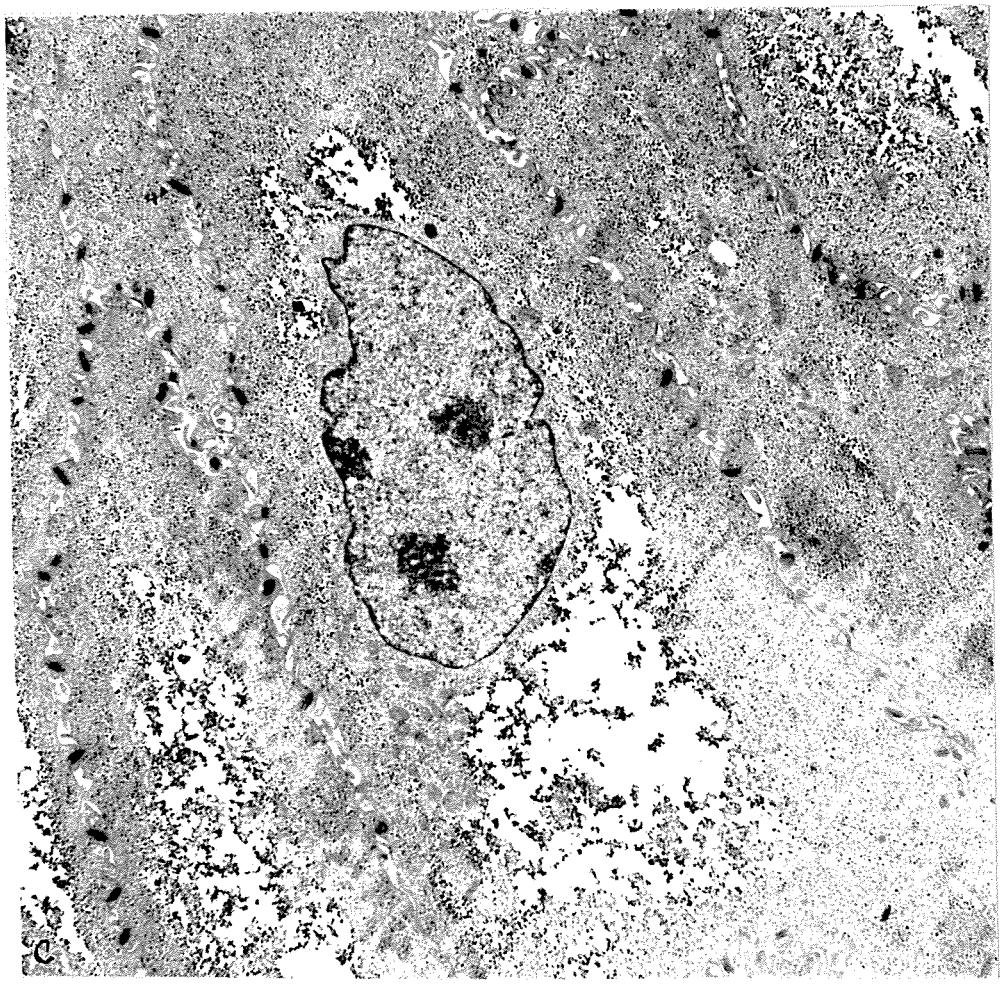

C

Figure 3.22 (Continued) Electron micrographs of the forestomach epithelium taken from one-day-old lambs.

D. Dilated cisternae of endoplasmic reticulum containing Er-protein (star) in the intermediate layers. Some membrane-coating granules (mcg) are seen in the cell periphery. x 26,200.

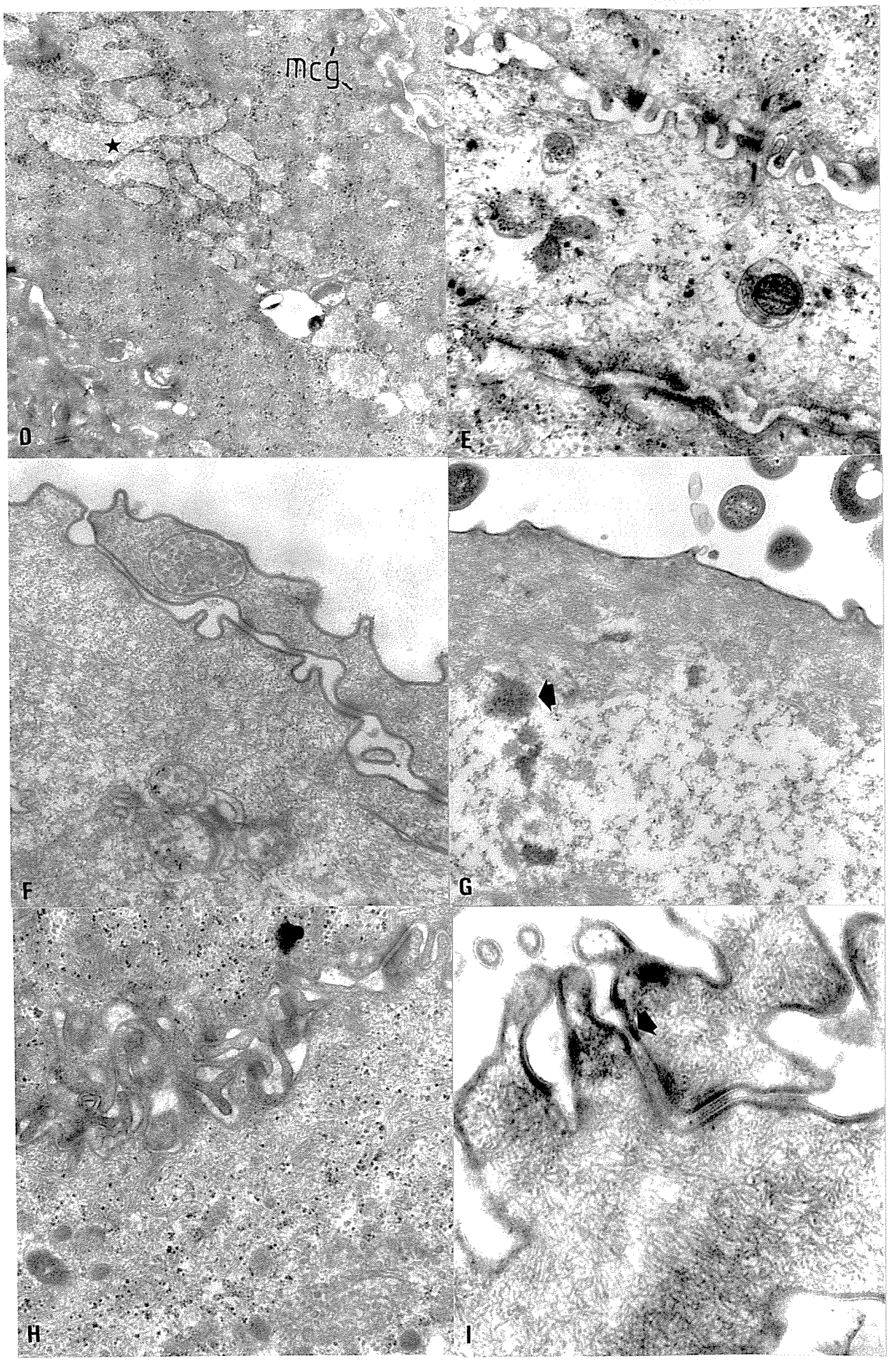
E. Small (rod-shaped) keratohyalin granules and lysosomal bodies in a cell in the intermediate layer. Some keratohyalin granules are seen incorporating tonofilaments. x 19,200.

F. Partially keratinized cells in the stratum corneum. A multi-vesicular body is present in the most superficial cell. x 17,500.

G. A partially keratinized cell in the stratum corneum with nuclear remnants (arrow) in the cytoplasm. Note the clear space in the middle of the cell. x 17,500.

H. Interlocking cell processes between cells in the intermediat layers. x 19,200.

I. A tight junction (zonula occludens) between two partially keratinized cells. x 64,200.


mcg
D
E
F
G
H
I

some of which were observed to be associated with ribosomes. The cisternae of the Golgi apparatus were moderately dilated and membrane bound vesicles similar to membrane-coating granules were seen in their vicinity. Large mucous granules were also found in the basal cells (Figure 3.22 B).

Cells in the process of division were frequently found in the basal layer, and, like the Langerhans cells which were already present at this age, were stained lightly. Blood vessels found close to the basement membrane had numerous micropinocytic vesicles in the endothelium (Figure 3.22 A). The endothelium surrounding the blood vessels was thicker compared to that found in the adult.

Epithelial cells in the deeper layers of the intermediate zone contained dark-staining masses of glycogen granules in their cytoplasm (Figure 3.22 C). In the upper levels of this zone, some cells were showing signs of keratinization (Figure 3.22 D and E). These cells displayed highly dilated rough endoplasmic reticulum filled with Er-protein (Figure 3.22 D) and small rod-shaped keratohyalin granules which appeared to incorporate tonofilaments (Figure 3.22 E). A few membrane-coating granules (Figure 3.22 D) and lysosomes (Figure 3.22 F) were also observed.

The resulting keratinized cells were flattened and had thickened cytoplasmic membranes (Figure 3.22 F). The tonofilaments were not arranged in bundles and although they were also observed to be concentrated near the periphery of the keratinized cell, they were not suspended in an electron-dense matrix. A clear space was seen in the middle of a keratinized cell (Figure 3.22 G) in which some fibrillary materials were scattered. Lysosomes (Figure 3.22 F) and nuclear remnants (Figure 3.22 G) were also observed in the keratinized cell. Adherent bacteria were also present on the surfaces of the most superficial cells (Figure 3.22 G).

The system of hemidesmosomes and desmosomes anchoring basal cells to the basement membrane, and epithelial cells

to each other, respectively, were well-established at this age (Figure 3.22 A). Desmosomes were especially well-developed in the intermediate zone (Figure 3.22 C). No gap junctions were observed in the intermediate zone except in some areas in the cranial sac where short strands were observed.

In the basal surface, the array of microvillus-like processes was absent and the basal sinus was narrow (Figure 3.22 A). The lateral surface of the basal cells were either simple or thrown into finger-like cytoplasmic processes. Interlocking cell processes in the intermediate zone narrowed the intercellular spaces towards the surface of the epithelium (Figure 3.22 H). At the most superficial layer, a type of junctional complex were observed between the neighbouring superficial cells which closed the intercellular spaces from the luminal surface: this complex was composed of a zonula occludens near the lumen, followed by two modified desmosomes and in some cases, another zonula occludens (Figure 3.22 I). The luminal surface of the most superficial cells may be thrown into cytoplasmic processes.

3.4.3.2 12 days (Figure 3.23)

The epithelium at this age resembles that in the adult in that fully keratinized cells had appeared in the most superficial layer of the epithelium and the cells in the lower levels contained large numbers of mitochondria (Figure 3.23 A). Some basal cells contained endoplasmic reticulum which were arranged concentrically (Figure 3.23 B); other showed degenerative changes such as the appearance of lysosomal bodies which were either lamellated or contained electron dense myelin figures (Figure 3.23 A). Numerous micropinocytic vesicles which appeared either being discharged and/or taken in by the basal cell were observed in the basal surface. Indentation of the cytoplasm resulting from pinocytosis or exocytosis appeared to initiate the formation of the microvillus-like array of processes in the basal surfaces of basal cells (Figure 3.23 C).

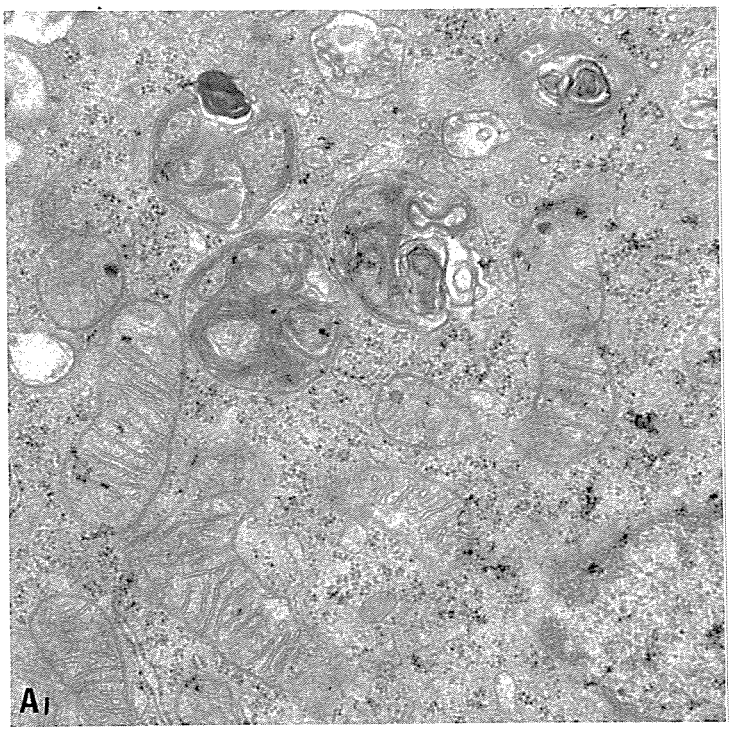
A few glycogen granules were observed in the cytoplasm of spinous cells in this age (Figure 3.23 D). Membrane-

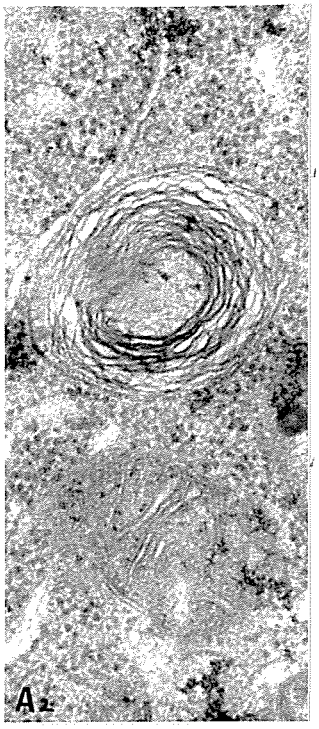
Figure 3.23 Electron micrographs of the epithelium taken from the forestomachs of 12-day-old lambs.

A1. Mitochondria and lysosomal bodies with myelin figures in a basal cell. x 60,939.
A2. A concentric-whorled lysosomal body. x 109,890.

B. Concentric lammelar bodies in the stratum basale. x 56,166.

C. Formation of the microvillus-like array of processes in the basal surface of basal cells. x 21,450.


A1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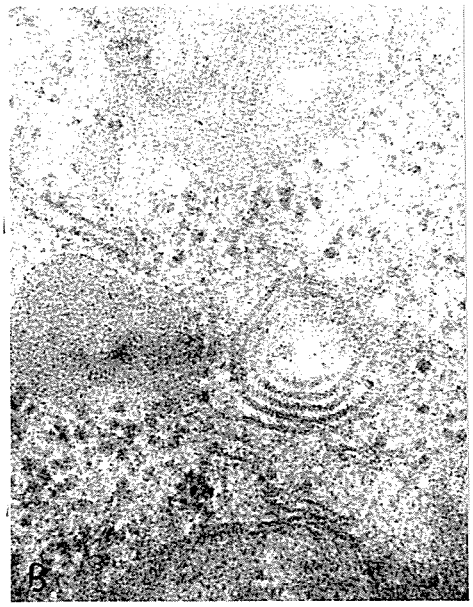

B

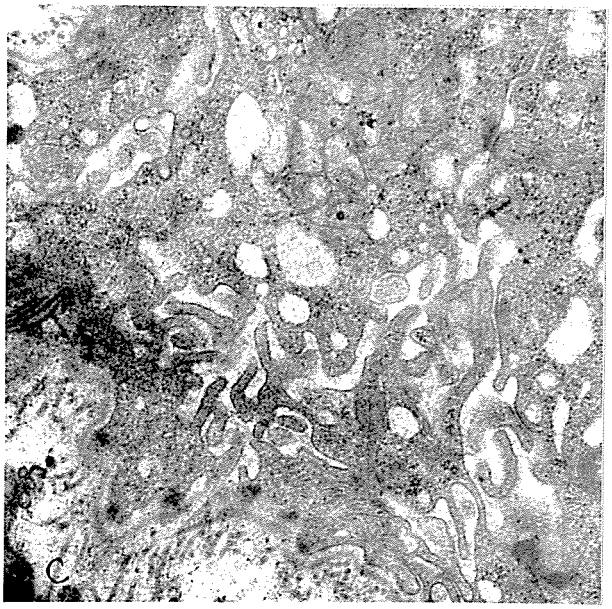
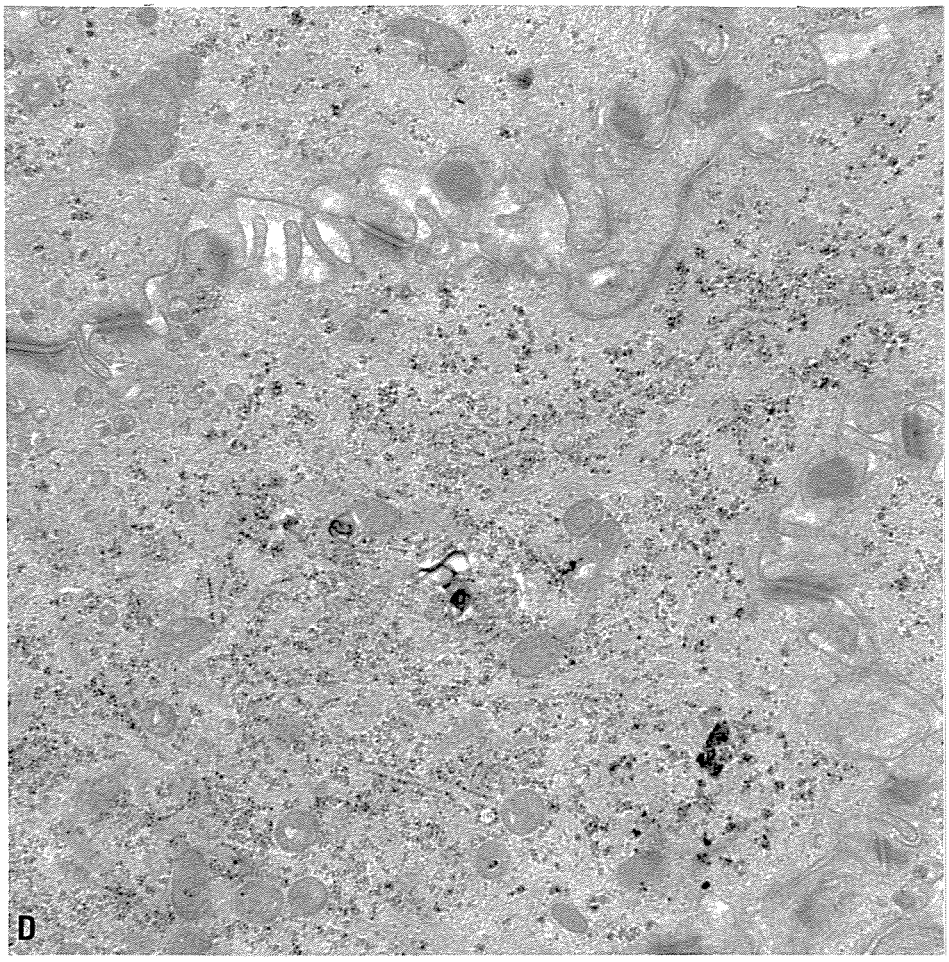

C

Figure 3.23 (Continued) Electron micrographs of the epithelium taken from the forestomachs of 12-day-old lambs.

D. A few glycogen granules (gly) and membrane-coating granules (mcg) in the stratum spinosum. x 17,550.

E. Membrane-coating granules (mcg) in the vicinity of the Golgi apparatus (go). Some of the membrane-coating granules appear to migrate toward the cell periphery. x 13,600.


D

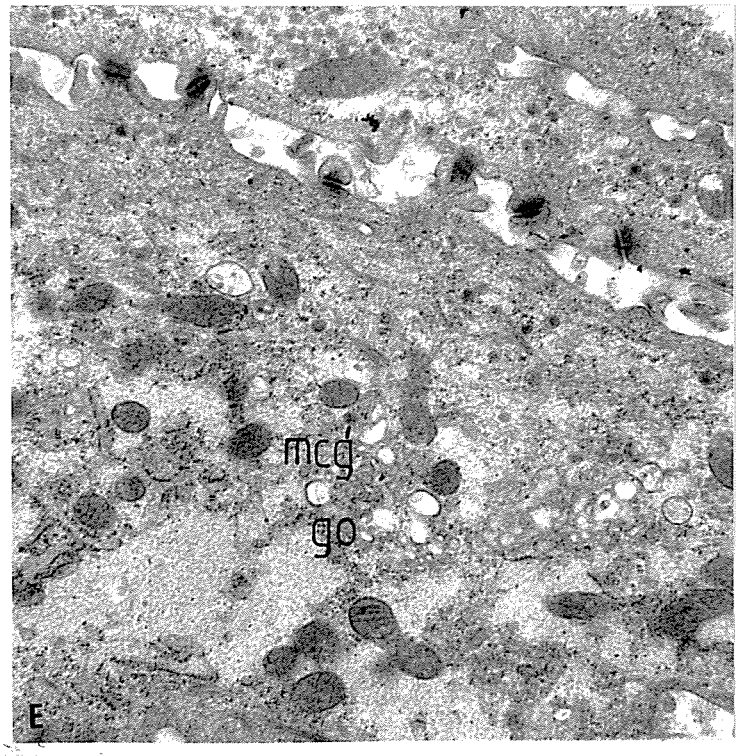
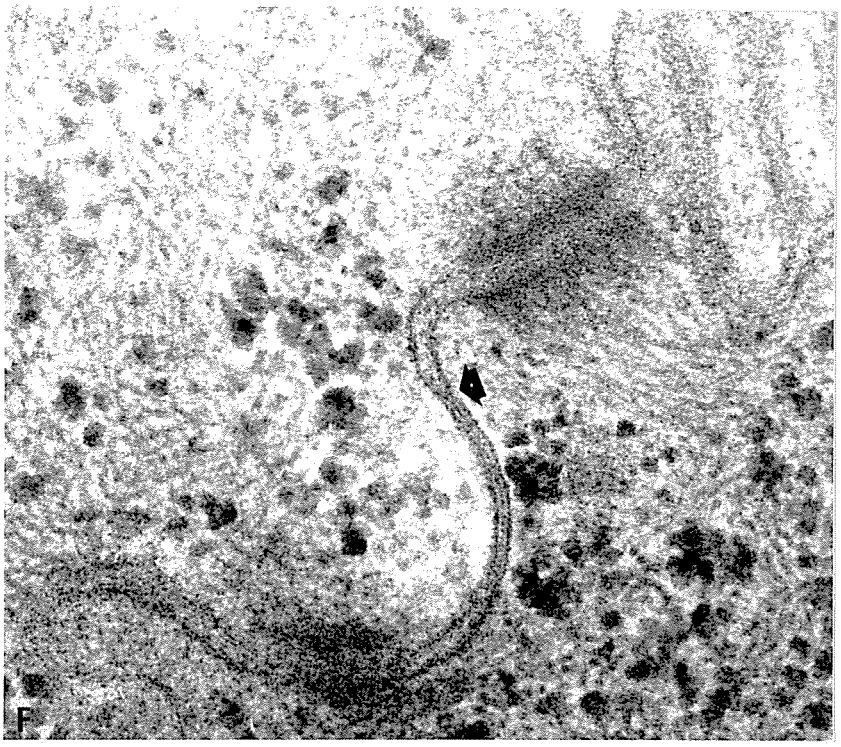
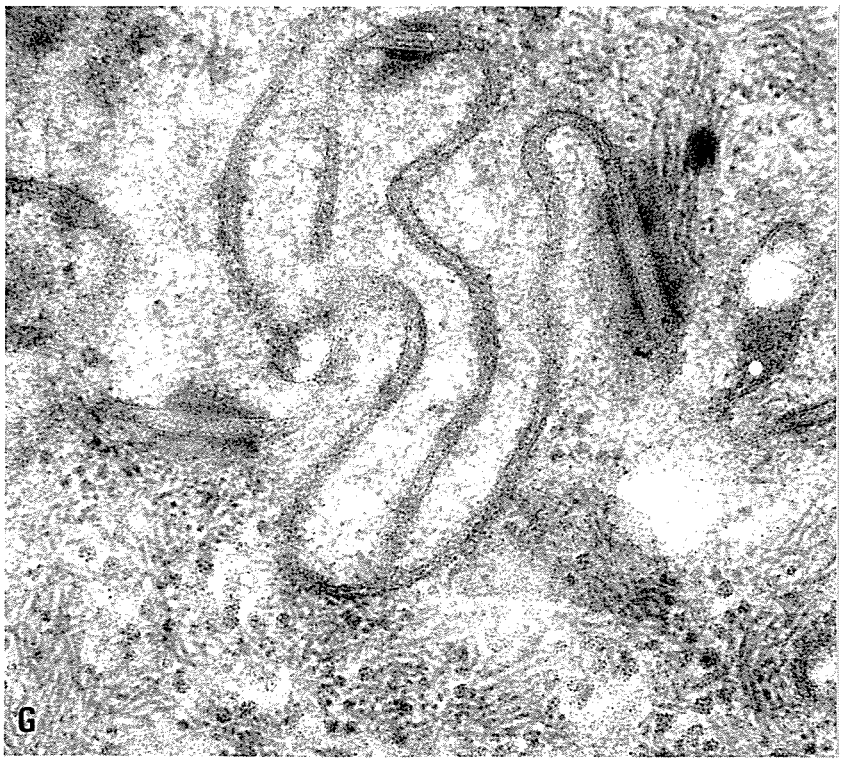

mcg
go
E

Figure 3.23 (Continued) Electron micrographs of the epithelium taken from the forestomachs of 12-day-old lambs.

F. Gap junction in the stratum granulosum. The arrow indicates a tight junction in the margin of the gap junction. x 56,166.

G. Non-junctional membrane appositions in the stratum granulosum. Note granular material between closely apposed cells. x 64,000.


F


G

coating granules were now numerous in the upper levels of the spinous layer and in the granular layer (Figure 3.23 D). Membrane-coating granules first appeared in the vicinity of highly dilated Golgi apparatus located near the nucleus (Figure 3.23 E). They were then seen in the cytoplasm appearing to migrate towards the periphery of the cell to accumulate near the cell surface to be discharged into the intercellular spaces. At the level of the transitional layer, membrane-coating granules were practically absent in the cell periphery.

The first appearance of a definite system of gap junctions in the epithelium was seen at this age. The junctions were occasionally seen as moderately long strands between basal cells and more often as short strands between spinous cells. At the granular cell layer, long strands of gap junctions were associated with desmosomes, tight junctions and close membrane appositions to seal off the intercellular spaces (Figure 3.23 F). Close membrane appositions were characterised by long and close parallelism of two adjacent cell membranes without forming a recognisable cell junction (Figure 3.23 G). The space between closely apposed membranes was reduced to less than 0.02μ and appeared to be occupied by granular material of moderate density.

3.4.3.3 23 and 34 days (Figure 3.24)

At these ages, signs of keratinization, such as dilation of rough endoplasmic reticulum with Er-protein, were already seen in basal cells (Figure 3.24 A). The bundles of tonofilaments became thicker and in the stratum spinosum, they were seen to practically traverse the entire diameter of the cell before joining desmosomes (Figure 3.24 B). Progressive amplification of the cytoplasmic processes in the surfaces of basal cells resulted in the basal surface in the widening of the basal sinus. As in neonatal animals, the endothelium of the blood vessels near the epithelium had numerous micropinocytic vesicles but their cytoplasm appeared thinner.

Gap junctions were observed in the basal cell processes

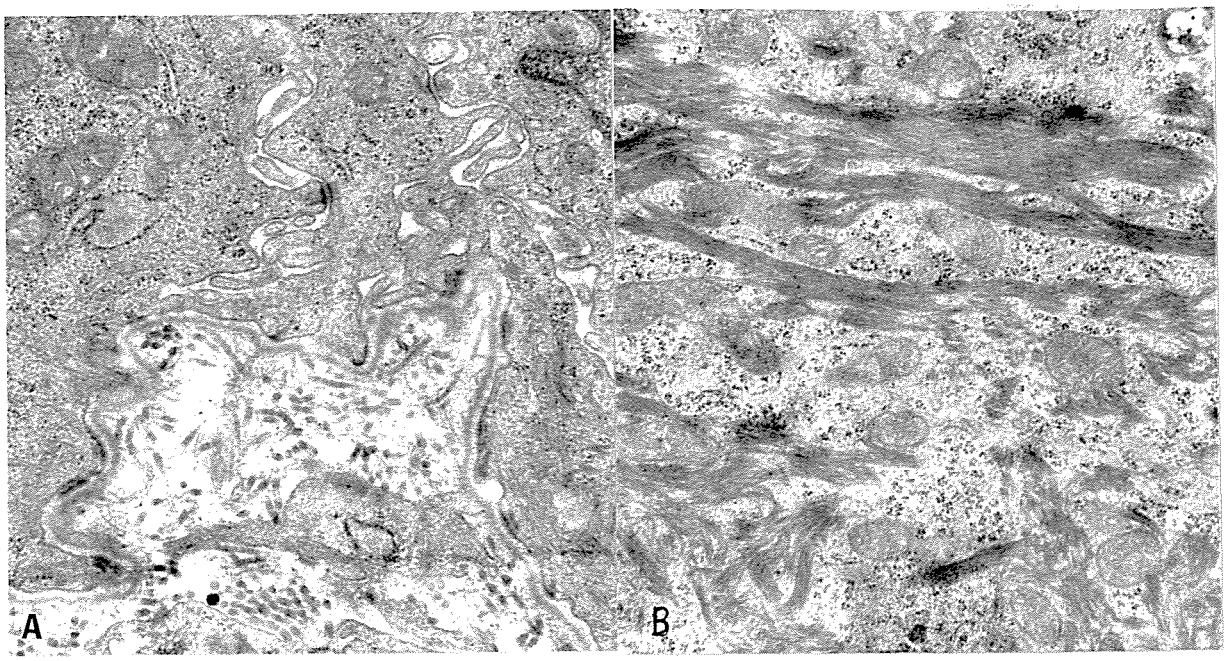
Figure 3.24. Electron micrographs of the epithelium taken from the forestomachs of 23- and 34-day old lambs.

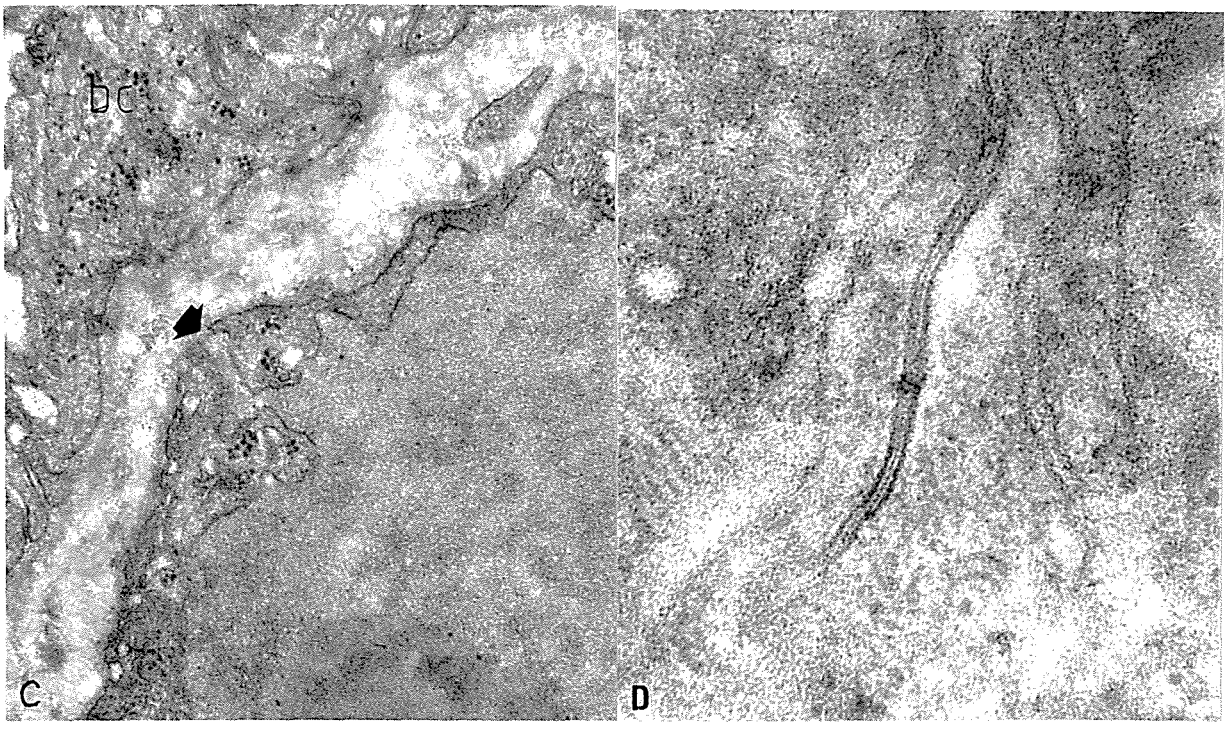
A. Basal cell. Note the dilated endoplasmic reticulum containing Er-protein. Collagen fibres are abundant in the subepithelial space. x 17,500.

B. Thick tonofilament bundles in the stratum spinosum. x 17,500.

C. Thin and fenestrated (arrow) endothelium in the blood vessel in the subepithelial space. The basal cell processes in the basal cells (bc) are quite developed. x 35,700.

D. A gap junction between basal cell processes. x 110,200.


A
B


bc
C
D

of the 34 day-old epithelium (Figure 3.24 D). This type of junction was found in all levels of the epithelium up to the outer limits of the stratum granulosum. Tight-junction-desmosomal complexes were readily recognised in the stratum corneum especially between the cells just beneath the cells being degraded by bacteria. Desmosomal elements were also seen to persist in the cytoplasmic processes of the most superficial cells.

3.3.3.4 45 and 56 days (Figure 3.25)

The ultrastructure of the epithelium at these ages resembled that in the adult. In the stratum basale, the microvillus-like array of processes were extensive and the Langerhans cells were more numerous. Lysosomal bodies were also observed in the basal cell cytoplasm. The endothelium of the highly dilated blood vessels close to the basement membrane was much thinner compared to the previous ages and appeared fenestrated (Figure 3.25 A) Gap junctions were found in great numbers and lengths in the epithelia at these ages. Although they were also found connecting basal cells, they were more numerous in the stratum spinosum and stratum granulosum, being especially long in the stratum granulosum where they were found in association with desmosomes and in some cases with tight junctions (Figure 3.25 B). Internalised gap junctions (or annular junctions) were also found in the cells of both the stratum spinosum and stratum granulosum (Figure 3.25 C) some of which appeared to be invaginating (Figure 3.25 D). No other special features were observed in the epithelia at these ages.

3.5 Na+-K+-ATPase Cytochemistry

3.5.1 Light microscopy

Under the incubation conditions used in this study, the sites of activity of the K+-dependent component (K+-NPPase) of the Na+-K+-ATPase enzyme complex were not adequately demonstrated by the light microscope. In sections which had been sequentially treated with lead

Figure 3.25. Electron micrographs of the the epithelium taken from the forestomachs of 45- and 56-day old lambs.

A. Tenuous and fenestrated endothelium of a subepithelial blood vessel. The arrow indicates a basal cell. x 25,200.

B. Intercellular junctions in the outer limit of the stratum granulosum. Gap junctions, desmosomes and close membrane appositions. x 56,166.

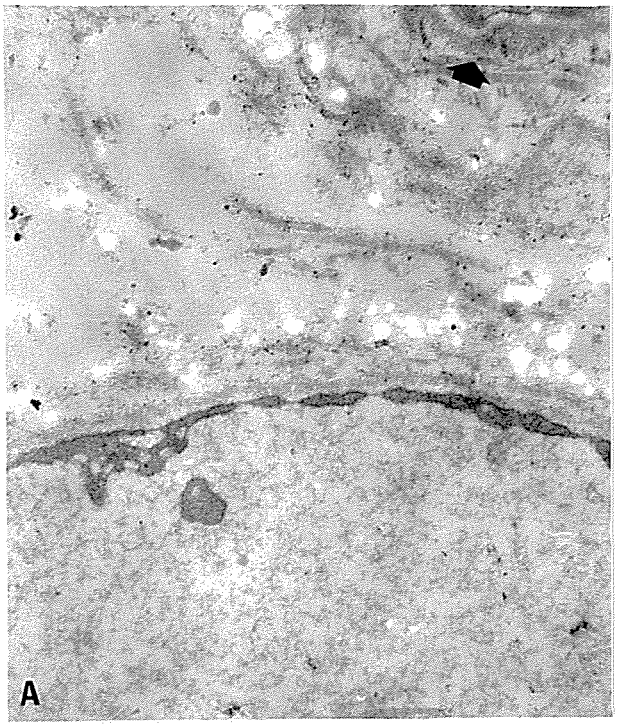
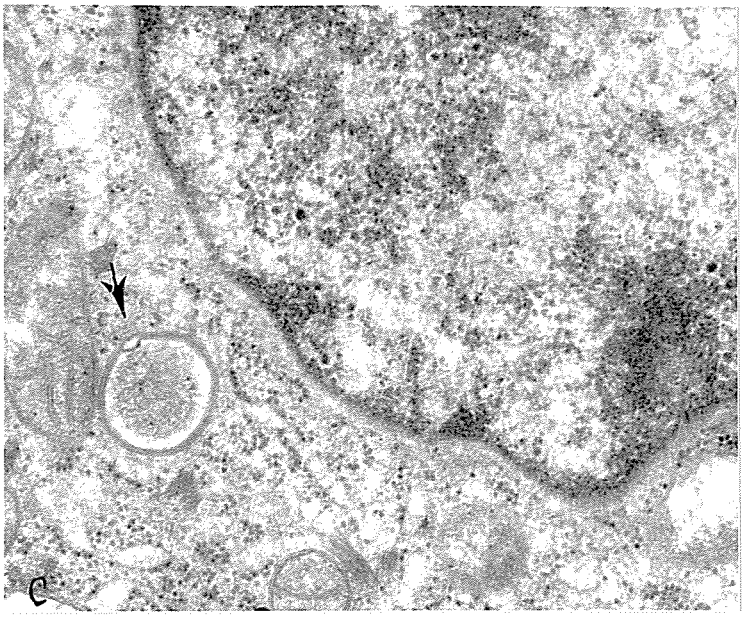

A

Figure 3.25 (Continued) Electron micrographs of the epithelium taken from the forestomachs of 45- and 56-day-old lambs.

C. Internalised gap junction (Albertini and Anderson, 1974) (arrow). x 26,200.

D. Internalised gap junction with the appearance of being invaginated. x 24,750.


C


D

Figure 3.26 Na^+-K^+-ATPase cytochemistry of the ruminal epithelium.

Complete Medium.

A. Depostis are found both on the cytoplasmic and intercellular space side of the cell membranes, basal cell. Unstained, x 11,700.

B. Deposits on the intercellular space side of the cell membranes, spinous cell. Unstained, x 11,700.

C. Deposits in the stratum corneum due to alkaline phosphatase. Unstained, x 13,600.

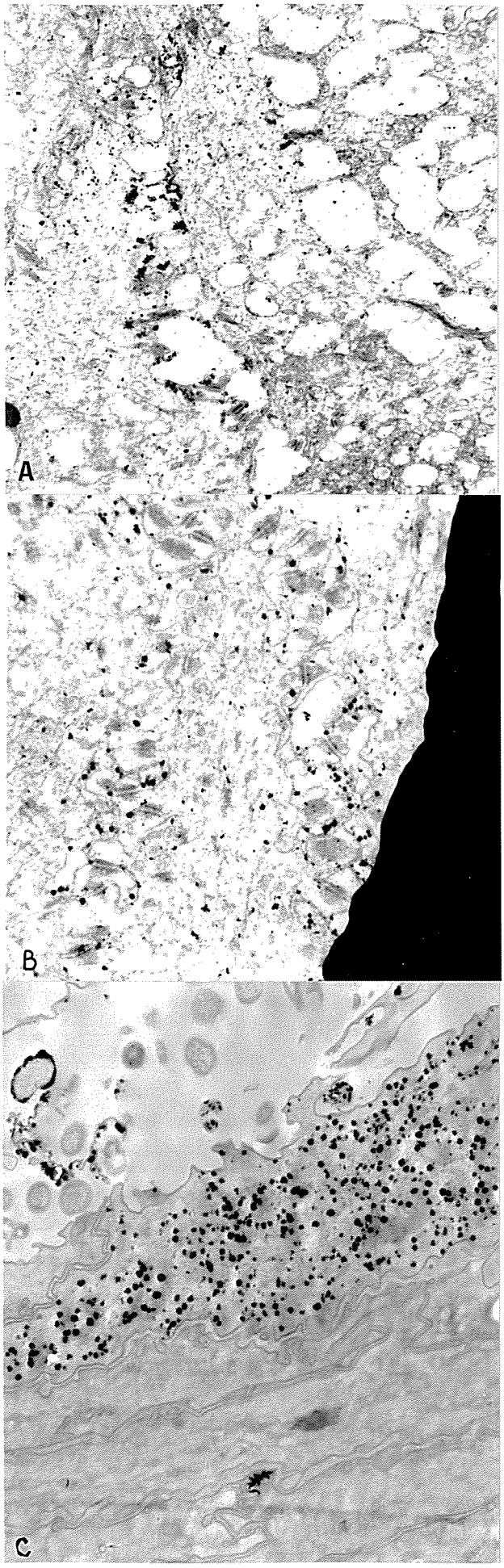

A
B
C

Figure 3.27. Na^+-K^+-ATPase cytochemistry of the ruminal epithelium.

Controls.

A. Medium in which p-NPP was replaced by 10 mM inorganic phosphate. Deposits are in the intercellular spaces between the stratum transitionale and the stratum corneum. Unstained, x 13,600.

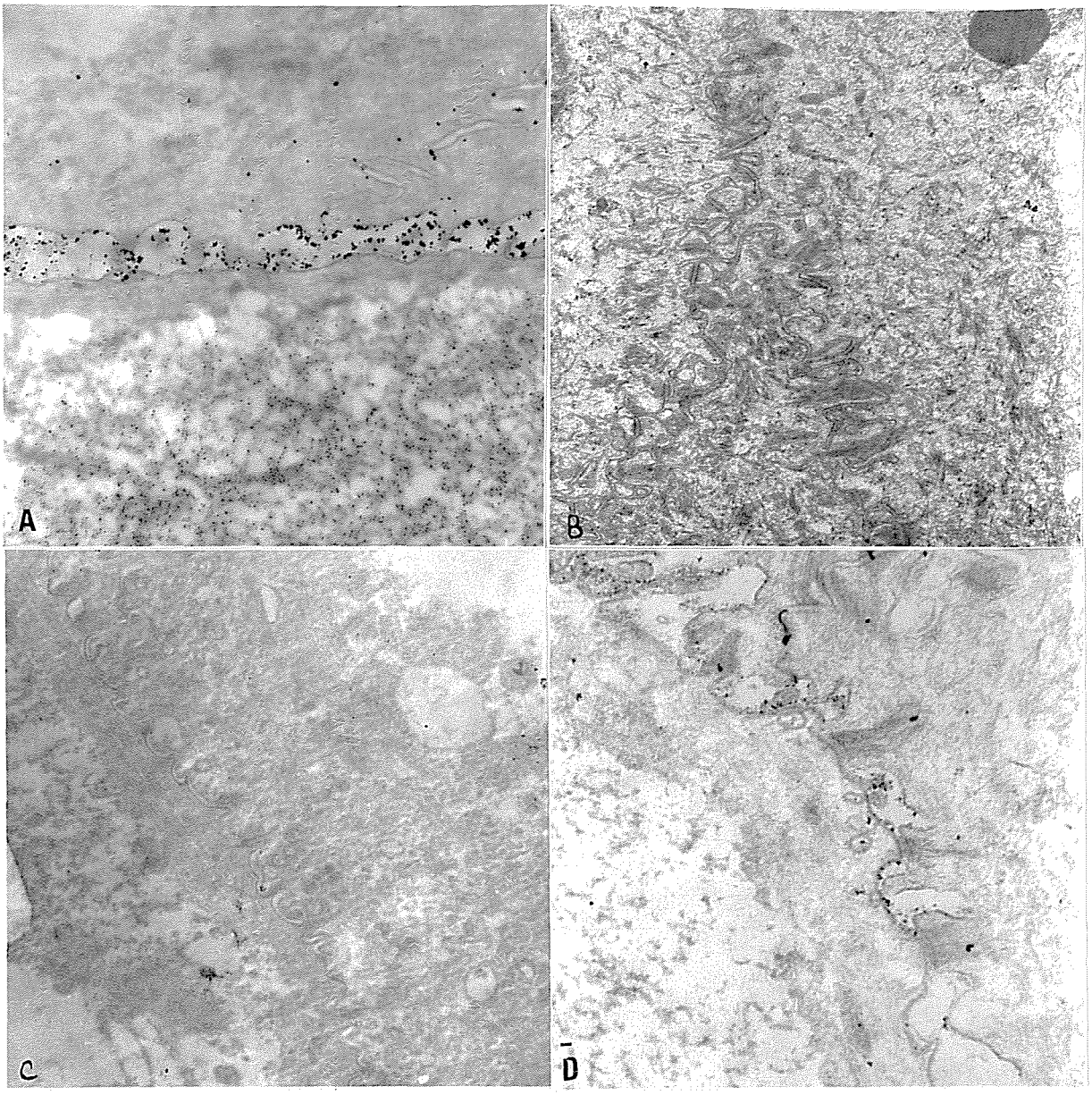
B. K^+-free medium. No deposits are found, stratum spinosum. Stained with lead citrate, x 11,700.

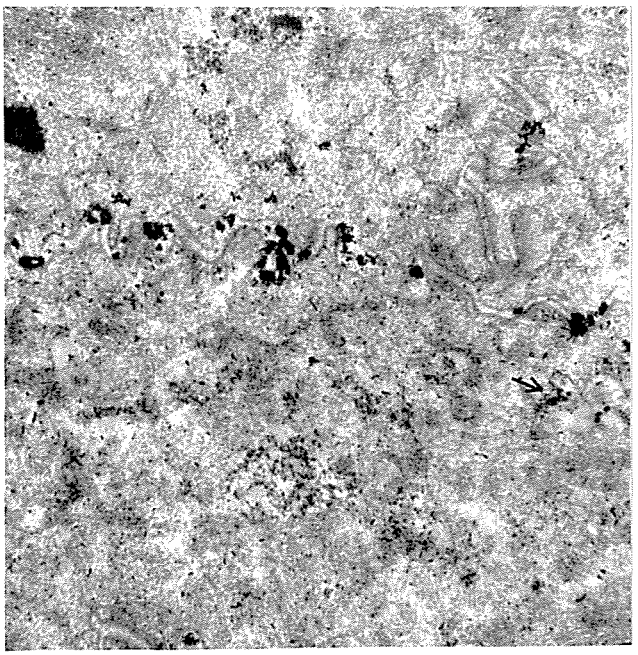
C. Mg^{++}-free medium. No deposits are found, stratum granulosum. Unstained, x 11.700.

D. Complete medium to which 10 mM ouabain wad added. Deposits on the cytoplasmic side of the membrane are absent, stratum granulosum. Unstained, x 11.700.

Figure 3.28. Mg^{++}-ATPase cytochemistry of the ruminal epithelium.

Deposits are found in the intercellular spaces in the stratum granulosum and in a mitochondrion (arrow). x 13,600.


A
B
C
D



nitrate and ammonium sulphide solutions and stained with crystal violet, only a slight reaction in the stratum corneum was observed, and this was inconsistent. Because of this, evaluation has been based solely on what has been seen in the electron micrographs.

3.5.2 Electron microscopy (Figures 3.26 - 3.28)

With the brief fixation used in the technique, the preservation of cytoplasmic structures was inadequate. However, the cytoplasmic membranes were well preserved. When blocks of papillary tissues (50 - 90 mg wet mass) were incubated in the complete medium capable of revealing the K+-dependent component of the enzyme Na+-K+-ATPase, reaction product was localized in all cell layers of the epithelium. In the stratum corneum, phosphate deposits were distributed in the cytoplasm of keratinized cells. In the remainder of the layers, reaction product was found on the plasma membrane on both outer (facing the intercellular space) and inner (cytoplasmic) surfaces, the deposits on the outer surface being of greater density than those on the inner surface. Some deposits were seen in the intercellular spaces. No deposits were seen in the gap junctions in the stratum granulosum. Figure 3.26 A, B and C shows a typical pattern of deposition of reaction product in the ruminal epithelium.

To clarify whether the sites of phosphate deposits indicated the location of phosphatases or simpley the "trapping" of liberated phosphate, samples of papillae were incubated in a medium in which p-nitrophenyl phosphate was replaced by inorganic phosphate. Under these conditions, phosphate deposits were seen in the cytoplasm and along the surface of plasma membranes facing the intercellular spaces, particularly in cells of the stratum transitionale and upper stratum granulosum (Figure 3.27 A).

To distinguish whether the deposits were either due to K+-NPPase or alkaline phosphatase activity, papillary samples were incubated in complete medium to which L-cysteine had been added. This resulted in the abolition of deposits

in the cytoplasm of cells in the stratum corneum but not on the cytoplasmic membrane surfaces of cells in the deeper layers. This indicated that alkaline phosphatase was active in the stratum corneum and K+-NPPase in the lower levels of the epithelium. When incubations were carried out in the absence of K+ in the medium, deposition in the lower layers in both sides of the cytoplasmic membranes was greatly reduced or absent, indicating the K+-dependence of the reaction (Figure 3.27 B). Similar results were obtained when Mg++ was deleted from the incubation medium (Figure 3.27 C) or when the substrate p-NPP was absent in the medium indicating the Mg++ and substrate dependence of the reaction respectively. When ouabain was added to the complete medium, the deposits in the intercellular space side of the cytoplasmic membrane were reduced and abolished on the cytoplasmic side , as shown in Figure 3.27 D. It was also noted that deposits due to alkaline phosphatase activity were not affected by KCl omission or by ouabain treatment.

Reaction product was found in the epithelium in all ages except in the epithelium taken from one-day-old lambs.

In samples incubated in a modified Wachstein-Meisel method using the same mild fixation schedule but disodium adenosine triphosphate (disodium-ATP) as substrate and Pb++ as the capture ion, reaction product was localised in the stratum corneum, in the intercellular spaces in the stratum transitionale and stratum granulosum and in the mitochondria (Figure 3.28).

CHAPTER FOUR

DISCUSSION

In the first part of this chapter, the classification of the epithelium is reconsidered in the light of the results in the present study. The remainder of the chapter focuses on the possible functions of various structures described in the results.

4.1 Classification and Nomenclature

The epithelium lining the ruminant forestomach has been classified in several ways, based on its histologic and electron microscopic appearance and its histochemical reactions.

Histologically, the forestomach epithelium is generally classified as a stratified squamous keratinizing epithelium. However, this classification has been considered by Phillipson (1970) as misleading in that the basal layer is composed of columnar cells. Stinson and Calhoun (1976 a) dropped the term 'squamous' and described the epithelium simply as stratified keratinizing. The present author favours the designation 'stratified squamous keratinizing' because the shape of the basal layer of cells is not usually relevant in the classification of stratified epithelia[¢].

However, there is still no agreed nomenclature for the cellular layers which compose the epithelium. It is usual to describe the cellular strata according to the classic differentiation of cells of the epidermis: stratum basale, stratum spinosum, stratum granulosum and stratum corneum. This has been used by many authors including Ellenberger (1911); Barcroft et al. (1944 b); Dobson et al. (1956); Schnorr and Vollmerhaus (1967); Lavker, Chalupa and Dickey (1969); Henriksen (1970 c); Steven and Marshall (1970) and Stinson and Calhoun (1976 a). This convention however, according to Fell and Weekes (1975), has the disadvantage of naming the structures of the rumen epithelium in terms of the human skin, despite the differences between the two epithelia, e.g., the cells of the stratum corneum in the rumen epithelium may be

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¢ Results from the present study (P. 62, paragraph 2, and Figs. 3.8 and 3.14 have shown squamous cells in the superficial layers of the epithelium.

vesiculated and do not display the typical "keratin pattern" (unstained filaments embedded in a dense matrix) of the skin but have a horny periphery with a central core of fine granular material. The convention also ignores the presence of a distinct layer of swollen cells beneath the stratum corneum of the rumen epithelium which forms the so-called area of primary swelling, as opposed to the area of secondary swollen cells in the stratum corneum (Hauser, 1928; Hofmann, 1973) and is regarded by Henriksson and Habel (1961) as the stratum transitionale in which the second phase of keratinization takes place (Lavker and Matoltsky, 1970). The extent of this layer is directly proportional to the degree of keratinization of the epithelium and to the rate of epithelial turn-over (Janacek, Borik and Holman, 1972). Dobson et al. (1956) and Stinson and Calhoun (1976 a) have included this layer in the stratum granulosum, while others including Steven and Marshall (1970) and Henrikson (1970 c) failed to mention it at all.

In the present study, five cellular layers in the epithelium are described, viz., stratum basale, stratum spinosum, stratum granulosum, stratum transitionale and stratum corneum. The stratum granulosum is treated in the present study as a separate layer; Henrikson and Habel (1961) regarded it as a sublayer of the stratum transitionale because it is sometimes absent in the epithelium (Dobson et al., 1956). The discontinuity of the stratum granulosum in some stretches of the epithelium rather than its complete absence was observed in the present study; this is in agreement with the observations of Barcroft et al. (1944 a).

The discontinuity of the stratum granulosum in the epithelium is probably because differentiation of cells does not occur simultaneously so as to create a uniform layering: cell division in the basal layers of the epithelium occurs in bursts and waves (Moon and Campbell, 1973) and follows a circadian rhythm (Sakata and Tamate, 1978 a). It is also probable that migration of

differentiating cells does not always follow a vertical pattern but may migrate horizontally . This could especially operate in the forestomach epithelium in which infoldings of the deeper layers are common, The epithelial cells are not stacked directly on top of one another to form columns like those found in the epidermis of the mouse ear and dorsum (Allen and Potten, 1974).

In the present study, the cells of the epithelium are also classified as keratinocytes and non-keratinocytes. This is based on the proposal of Breathnach (1980) that the term "clear cells" to describe the light-staining non-keratinizing cells be dropped from usage since cells which undergo keratinization may also appear histologically as "clear cells" in certain conditions, e.g., embryonic, fetal and neonatal cells with high glycogen content.

Keratinocytes are those cells which undergo differentiation towards keratinization - i.e., basal, spinous, granular and transitional cells. In the basal layer, the keratinocytes may also be grouped into cycling and non-cycling cells; cycling cells are those cells which continue to divide to produce keratinocytes while non-cycling (or post-mitotically mature) cells are those which have permanently left the proliferative pool and are maturing while awaiting their turn to migrate towards the upper levels (Potten, 1981). Although cycling and non-cycling cells are difficult to identify without special staining - e.g., FITC staining - post-mitotically mature cells in the epithelium may sometimes be recognised in the light microscope as those cells that have a shape suggesting that they are in the process of leaving the basal layer (see Figure 3.6 A), and in electron micrographs, as those basal cells which show high concentrations of differentiation products such as tonofilaments, occasional mucous granules and Er-protein (see Figure 3.24 A).

Non-keratinocytes are those cells which do not differentiate into keratinocytes. In the forestomach epithelium, Langerhans cells (Gemmel, 1973; Nagatani

et al., 1974; Gerneke, 1977) and intraepithelial lymphocytes (Steven and Marshall, 1970; Nagatani et al., 1974) have so far been identified which may be considered as non-keratinocytes. In the present study, cells similar to mast cells/globule leukocytes as described by Murray (1972) and to Merkel cells have been observed (see Figure 3.17 D and E and Section 4.4) but further work is needed to confirm their identifacation. Although these cells do not share the same ontogeny as keratinocytes, Breathnach (1980) considers them to compose a permanent subpopulation in keratinizing epithelia.

Church (1976) also classified the epithelium as non-mucus producing and non-glandular.

Consequently, he preferred to classify the mucosal lining of the forestomach simply as epithelium instead of mucosa. This classification is ambiguous and misleading.

Although no glands were found in the epithelium or in adjacent tissues, either in the present study or in previous studies starting with Ellenberger (1911), based on the results of P.A.S. and toluidine blue stainings, the epithelium appeared to be capable of producing mucus. P.A.S.-positivity and toluidine blue metachromasia in the stratum corneum have been interpreted, in accord with the observations of many authors including those of Habel (1963); Lavker, Chalupa and Dickey (1969); Lavker, Chalupa and Opliger (1969) and Schnorr and Hild (1974) who observed neutral and both sulphated and non-sulphated acidic mucosubstances in the same location.

As seen in the present electron microscopic study, the P.A.S.-positivity and toluidine blue metachromasia in the stratum corneum which Henrikson (1970 a) and Henrikson and Stacy (1971) considered as glycocalyx, appeared to be due to the dense material discharged from membrane-coating granules (MCGs) and degraded cellular organelles before membrane thickening had occurred during keratinization. Other authors including Lavker, Chalupa and Dickey (1969) and Schnorr and Hild (1974) have observed P.A.S.-positivity in spinous and granular cells

where MCGs were numerous in the cytoplasm.

In addition to MCGs, in the present study, large mucus granules in the epithelium, like those found in the frog skin (Lavker, 1974) were also seen, confirming the earlier observations of Lavker and Matoltsky (1970). It appears that mucus-containing granules are so numerous in the forestomach epithelium as compared to those in other keratinizing epithelia that, according to Lavker, Chalupa and Dickey (1969), the epithelium may undergo differentiation towards mucus production. This differentiation however, is not similar to mucous metaplasia observed in embryonic avian skin treated with vitamin A (Elias and Friend, 1976) in which post-mitotically mature basal cells abandon their pathway towards keratinization in favour of differentiation towards a glandular mucus-secreting epithelium; there is an attendant 90^{o} shift in the polarity of the cells forming microscopic acini, from squamous to columnar arrangement. In the rumen epithelium, spinous and granular cells abundant in MCGs remained flattened with their long axes parallel to the luminal surface and were seen to keratinize in higher levels.

The presence of retained nuclei which give a positive Fuelgen reaction in the stratum corneum has led Henriksson and Habel (1961) and Lavker, Chalupa and Opliger (1969) to classify the epithelium as being parakeratotic. In the present study, nuclei in the stratum corneum were also observed, although in electron micrographs, they appeared to be in advanced state of degradation. Fell and Weekes (1975) disagreed with this classification as this attempts to define the epithelium in terms of the normal epidermis. It appears that the characteristics of the forestomach epithelium - i.e., thin or discontinuous stratum granulosum and retention of nuclei in the stratum corneum fulfill the classic definition of parakeratosis. However, parakeratosis, as it manifests in the epidermis of zinc deficient swine, is usually accompanied by the thickening

of the epidermis due to marked acceleration of epithelial turn-over (Smith, Jones and Hunt, 1972; Fell and Weekes, 1975). The rumen epithelium however, has usually a low mitotic index not exceeding 1 % (see Section 3.3) which is lower than the index of normal parakeratotic epithelium such as that of the esophagus (Fell and Weekes, 1975). Furthermore, a thinning of the epithelium actually occurred during development of the rumen (see Section 3.2.3 and Table IV) rather than a thickening which should have been observed had parakeratosis actually ensued.

A true parakeratotic condition appears commonly in the rumen epithelium of animals fed with grain or pelleted diet (Ørskov, 1973) and the histological and electron microscopic appearance of the epithelium taken from these animals is quite different from the normal epithelium (Tamate and Kikuchi, 1978).

Finally, the forestomach epithelium has also been classified as psoriatic because of the presence of lipid droplets which can be demonstrated histochemically, especially in the cells of the stratum corneum (Lavker, Chalupa and Opliger, 1969). This is another classification which should be dropped from usage. As with parakeratosis, the ruminal epithelium also shares a number of features with psoriatic epidermis, one of them being the presence of lipid droplets in the stratum corneum. However, this is only one sign and may not be pathognomonic.

Psoriasis is usually associated with inflammatory lesions such as cytolysis and necrosis (Nagy-Vezekenyi and Zs.-Nagy, 1971), features which are not associated with the normal rumen epithelium. Although the epithelium may show lysis of cell organelles in the stratum transitionale, this lysis is associated with keratinization and not with inflammatory reactions. Vacuoles in the ruminal basal epithelial cells which may appear similar to lytic vacuoles in psoriasis are not actually lytic vacuoles but in fact cell processes of Langerhans cells (Nagatani et al., 1974). Widened intercellular spaces and the presence of cytoplasmic processes in epithelial

cells can be explained in the rumen epithelium as structural modifications because of its absorptive functions (Keynes, 1969). In psoriasis, widening of the intercellular spaces appears to be due to the lessening of desmosomal attachments between cells (Nagy-Vezekenyi and Zs.-Nagy, 1971). The basement membrane is not double in the rumen epithelium, as it is in a psoriatic epidermis. The mitotic index in the rumen epithelium is low compared to that of the psoriatic skin which usually exhibit elevated mitotic activity (Cox and Watson, 1972).

4.2 Organisation of the Forestomach Mucosa and Epithelium as Related to their Functions

The architecture of the forestomach mucosa as observed in the gross, histologic and electron microscopic examinations in the present study, except for some ultrastructural details which will be discussed in later sections, were consistent with the findings of many workers including those of Lavker, Chalupa and Dickey (1969); Henrikson (1970 a, b and c); Steven and Marshall (1970); Gemmel (1973); Hofmann (1973) and many others, and presented nuances of structure that implied absorptive and metabolic functions.

The presence of papillae, particularly in the rumen, increases the absorptive area of the mucosa: in the goat, this increase has been calculated to be as much as seven-fold (Schnorr and Vollmerhaus, 1967). This amplification of the surface area for absorption is repeated in the histological structure in the extensive infoldings of the basal area into epithelial bulbs. It has been pointed out by Klein-Szanto and Schroeder (1977) and Tamate et al. (1979) that active interaction - e.g., absorption - between the epithelium and the underlying connective tissue takes place between the connective tissue and the basal surface. Extensive convolutions of this interface, together with papillation in the luminal surface, undoubtedly increase the surface area through which absorption could take place. It is

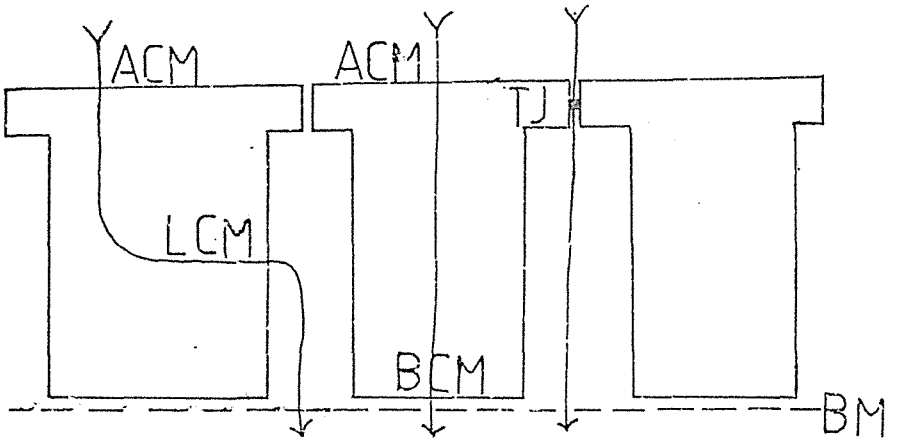

Figure 4.1 Schematic representation of three models for transepithelial volume flow. The epithelium is depicted as a single layer. Arrows indicate direction of flow (after Welling and Welling, 1980).

Resistance to flow could be encountered in the:

ACM = apical cell membrane
LCM = lateral cell membrane
TJ = tight junction
BCM = basal cell membrane

Flow resistance in the basement membrane (BM) and intercellular spaces is small.

noted that individual papillary processes are invested with blood vessels which could further facilitate absorption. In electron micrographs, the surface area was seen to be further increased by the microvillus-like array of processes in the basal surface of basal cells.

The prominent intercellular spaces and numerous intercellular connections in the form of tight and gap junctions and the cytoplasmic processes in the basal cells as seen in the electron micrographs in the present study are consistent with the well-developed absorptive and transporting capabilities of the epithelium. In accordance with Welling and Welling (1980), three possible pathways for the flow of solutes could be envisaged in the epithelium based on the morphology, as seen in the present electron microscopic examination (Figure 4.1).

At the centre, is the simplest, where absorbed fluid is shown to move in a direct fashion across the apical cell membrane of keratinized cells, to traverse the epithelium via transcellular pathways - e.g., gap junctions, and to exit in the basal cell membranes.

On the right, the solute moves across the tight junctions between the cells of the stratum corneum and through the intercellular spaces.

On the left is the most complicated scheme in which fluid passes through the apical cell membrane of the stratum corneum, traverses a portion of the epithelium through transcellular pathways, and then gains access to intercellular channels by crossing the lateral cell membranes.

In these models, resistance to flow of fluids could be encountered in the apical cell membranes, the lateral cell membranes, tight junctions and basal cell membranes. Flow resistance in the intercellular spaces and basement membrane is small (Welling and Welling, 1980).

The relative importance of these pathways in the rumen epithelium cannot be ascertained without measurements

of the apical surface area, the dimension of tight junctions, the area of lateral and basal membranes and the dimensions of the intercellular spaces.

However, evidence from tracer studies of Schnorr and Wille (1972 a) using Myofer as the opaque marker indicates that the model in the middle of Figure 4.1 could operate in the rumen epithelium. It was shown in these studies that the tracer was absorbed through the epithelium via transcellular pathways and was not encountered in the intercellular spaces. A similar model has been proposed by Stevens (1970) for the diffusion of VFAs across the epithelium. In this model, Stevens (loc. cit.) postulated that the apical cell membrane is permeable to dissociated as well as undissociated fatty acids, while the blood-facing membrane, the basal cell membrane, is permeable only to the undissociated acid. The passage of VFAs through the epithelium could be modified by their metabolism in the epithelial cells which could occur more readily in the spinous and basal layers because of the large numbers of mitochondria in the cells of these layers.

A study on the ultrastructural localisation of sodium by Henrikson (1970 c) and tracer studies using lanthanum, ferritin and horseradish peroxidase by Henrikson and Stacy (1971) have shown that the model illustrated on the left in Figure 4.1 is favoured, at least for the transport of sodium across the epithelium. This is in accordance with the model proposed for the frog skin (Koefoed-Johnsen and Ussing, 1958). For this model to work however, it was pointed out that Na^+ pumps must exist in the lateral cell membranes in the middle layers as well as a barrier in the more superficial layers to the back diffusion of sodium into the lumen.

The present ultrastructural study supports the idea that the barrier is located in the keratinised layer as suggested by Houpt (1970). Zonulae occludentes were observed in the present study usually between the cornified cells below the cells undergoing degradation. Similar observations have been made in studies on the goat epithelium (Schnorr and Wille, 1972 b) and in wild ruminants (Hofmann,

1973) where these junctions were postulated to consist the permeability barrier.

However, the suggestion of Henrikson and Stacy (1971) based on the results of their marker studies on the rumen epithelium, that the barrier in the keratinized layer is not due to zonulae occludentes but to the prominent glycocalyx, tortuous intercellular spaces and the nature of the keratinized cell cannot be excluded altogether. It was noted in the present study that the glycocalyx so described was seen in the electron micrographs as dense-staining, sometimes lamellated material which originated from MCGs and filled the interstices in the stratum corneum. Marker studies in the skin, esophagus and vaginal epithelium have shown that such lamellated material from MCGs in the intercellular spaces functions as the diffusion barrier, especially in moist epithelia (Elias, Goerke and Friend, 1977) where the tight junctions are focal and incomplete as seen in freeze fracture preparations (Elias, McNutt and Friend, 1977).

The zonulae occludentes described by Steven and Marshall (1970) and Gemmel and Stacy (1973) in the stratum granulosum were also observed in the present study in the locations that they described. However, their appearance in the present study suggests that they are gap junctions rather than tight junctions in that they are not particularly situated in the lateral apical portions of the cells. Supportive evidence that these pentalaminar junctions are gap junctions has already been given by Henrikson and Stacy (1971), who showed that the junctions in the same locations which they termed as maculae occludentes were readily permeated by lanthanum: electron micrographs of tangentially section lanthanum-permeated junctions revealed the closely packed hexagonal particles which are characteristics of gap junctions as defined by Peracchia (1980). The term "tight junction" is now reserved for the true tight junction which forms a belt-like seal around the apical end of epithelial cells which are not permeable to markers (Staehelin, 1974).

In the present study, both tight and gap junctions were seen together, with the tight junctional strands

present in the margins of gap junctions, especially in the outer limits of the stratum granulosum. This association is thought to represent a stage in the development of tight junctions, and is also seen in mammalian liver and ovary and in avian skin (Elias and Friend, 1976).

Gap junctions were also found in the stratum basale and the stratum spinosum in the present study as well as in the studies of Steven and Marshall (1970) and Scott et al. (1970) although these previous studies termed them as tight junctions. The positions of gap junctions in the epithelium suggest that they are pathways for transcellular exchange of materials. Gap junctions are particularly considered as low resistance communications between cells and are widely implicated in cell-to-cell transfer of ions (ionic or electrotonic coupling) and in cell-to-cell transfer of metabolites (metabolic coupling) (Gilula, Reeves and Steinbach, 1972). In the rumen epithelium, they are particularly consistent with the models proposed in the middle and on the left in Figure 4.1 for transport pathways in the epithelium.

4.3 Keratinization

The findings reported in this study on the ultrastructure of the two types of keratinized cells, namely, the vesicular and flattened cells were similar to the descriptions of Steven and Marshall (1970), Lavker and Matoltsky (1970), Gardner and Scott (1972) and Fell and Weekes (1975). It is quite surprising though that in the only detailed study on the formation of the keratinized cell in the rumen epithelium of which the present writer is aware, Lavker and Matoltsky (1970) did not mention the presence of the vesicular keratinized cell and only focused their description on the formation of the flattened keratinized cell.

The sequence of events which occur during keratinization can be traced by examining the ultrastructure of the cells in the stratum granulosum, stratum transitionale and stratum corneum. The ultrastructure of the cells undergoing transformation as reported in the present

study is consistent with the descriptions of Lavker and Matoltsky (1970) in that transformation appeared to have been initiated by selective lysosomal degradation of formed cell organelles and nuclei, the products of which were then later discharged into the intercellular spaces; that the disposition of Er-protein and keratohyalin granules involved dispersion and mixing of the two components; and that subsequent infiltration into the tonofilaments in the cell periphery by the Er-protein-keratohyalin mixture formed the keratin matrix, particularly in the flattened cell and in the periphery of the vesicular cell. It appears that the same mechanism operates in the epithelium of the reticulum in spite of the relative paucity of swollen transitional cells in that organ.

However, in Figure 3.18 E, it can be seen that Er-protein has been discharged into the cell cytoplasm long before transformation has taken place. This is contrary to the finding of Lavker and Matoltsky (1970) who stated that Er-protein is only released during degradation of endoplasmic reticulum by lysosomes.

There is also evidence in the present study that MCGs are not degraded by lysosomes as Lavker and Matoltsky (1970) have described; they were seen to be discharged into the intercellular spaces (Figure 3.20 C). This is consistent with the later finding of Lavker (1976) and the finding of other authors including Elias, Goerke and Friend (1977) and Hayward (1979) in other keratinizing epithelia. It is possible that during the discharge of MCGs into the intercellular spaces, a part of their contents are imparted to the cell membrane resulting in the thickening of the inner leaflet of the trilaminar membrane in the keratinized cell (Martinez and Peters, 1970). Histochemical studies by Schnorr and Hild (1974) have demonstrated similar mucosubstances in MCGs, intercellular spaces, and in the thickened inner leaflets of keratinized cells.

The present study has also thrown more light on the structure and function of MCGs in the rumen epithelium. Consistent with the finding of Lavker (1969), the lamellated internal structure of MCGs in the rumen epithelium was observed in the present study. Hayward (1979)

has stated that MCG with lamellated internal structure are common to all keratinizing epithelia. However, the tubular or ring-like granules in MCGs have not been reported in the literature so far as the present writer is aware, although Farbman (1964) as cited by Hayward (1979), saw evidence of tubules penetrating lamellations.

It is tempting to believe that the two kinds of granules observed in the present study represent two different products. The lamellar organisation of the internal structure is similar to that observed in artificially prepared phospholipids (Hayward, 1979). It is possible that they are phospholipids as these compounds have been demonstrated histochemically in the epithelium particularly in the stratum granulosum and in the intercellular spaces (Habel, 1959; Cerny, 1977). (In turn, the lipid droplets in the stratum corneum could have been undischarged MCGs.). However, MCGs have also been demonstrated to be P.A.S. positive due to glycoproteins (Lavker, Chalupa and Dickey, 1969; Schnorr and Hild, 1974) and, in other keratinizing epithelia, due to glycolipids (Elias, Goerke and Friend, 1977). It is probable that one form of organisation is a stage of development of the other as intermediate forms with both tubular granules and lamellations were observed in the present study. In other keratining epithelia, the shift from polar to neutral lipids in MCG contents have been shown during keratinization (Elias, Goerke and Friend, 1977).

There is also a possibility however, that the lamellations and granulations represent artifacts of fixation.

Several functions have been attributed to MCGs. Their role in membrane thickening has been described earlier. In the present study, granular material was seen between closely apposed cell membranes in non-junctional associations found at the level where MCGs are discharged. This material which joined the closely apposed cells could have originated from MCGs. This observation supports the hypothesis of Hashimoto

(1971) that MCGs provide the material for cementing adjoining cells together. However, in widened intercellular spaces in the stratum transitionale and stratum corneum, material from discharged MCGs (and degraded cell organelles) was also seen not to participate in cell-to-cell adhesion (as in Figure 3.20 B and 3.21 D). In these layers, the discharged contents could form the barrier to diffusion in the epithelium.

4.4 Epithelial Non-keratinocytes

The non-keratinocytes present in the epithelium could include Langerhans cells, indeterminate cells, lymphocytes, mast cells or globule leukocytes and cells similar to Merkel cells. In particular, the results of the present study can be considered in relation to recent reports on other epithelia.

4.4.1 Langerhans cells

The identification of Langerhans cells in the present study is consistent with the reports of others including those of Gemmel (1973); Nagatani et al. (1974) and Gerneke (1977) who have identified this type of branching cell in earlier studies on the forestomach epithelium. Their functional significance in the forestomach is not well known, although Gemmel (1973) and Tamate and Fell (1978) have observed the phagocytosis of necrotic epithelial cells by Langerhans cells in the rumen of sheep subjected to sudden changes in ruminal osmolality and in diet. It may be possible that the lysosomal bodies observed in Langerhans cells in the present study were phagosomes, however no signs of phagocytosis were observed.

In the epidermis, it has been established that the Langerhans cells are related to cells from the monocyte-macrophage-histiocyte series and serve as front-line elements in immune reactions of the skin (Breathnach, 1980). Langerhans cells are the only epidermal cells

which express surface Fc-IgG and C3 receptors (Stingl, Wolf-Schreiner, Pichler, Gshnait, Knapp and Wolff, 1977) and those antigens (Ia antigens) of the major histocompatibility complex known to be involved in genetically determined immune responsiveness (Rowden, Lewis and Sullivan, 1977; Klareskog, Tjerlund, Forsum and Peterson, 1977). Epidermal Langerhans cells pulsed with antigen were found to induce proliferative responses in immune T cells that are of the same magnitude as those induced by antigen-pulsed macrophages (Toews, Bergstresser and Streilein, 1980). Langerhans cells were also found to replace Ia bearing macrophages in their capacity to induce antigen-specific and allogeneic T cell activation (Stingl, Katz, Green and Shevach, 1980). Because of these, Langerhans cells have been implicated as sensitizing factors in contact hypersensitivity (Baer, 1980). The phagocytic nature of epidermal Langerhans cells although much less than macrophages, has also been reported (Thorbecke, Silberberg-Sinakin and Flotte, 1980).

It has also been reported that Langerhans cells exert some control over certain aspects of keratinization. Allen and Potten (1974) and Potten (1981) identified an "epidermal proliferative unit" (EPU) in the mouse skin consisting of layers of cells arranged in columns with 9 - 10 basal keratinocytes and 1 Langerhans cell functioning together to provide controlled replacement of the keratinized cell layer. Within each EPU, occupying a central position, is a single Langerhans cell ideally placed to provide a controlling role in the EPU (Allen and Potten, 1974).

Although it has been pointed out that there is no certainty that Langerhans cells of some tubular organs are of the same species as that of the epidermis (Billingham and Silvers, 1965), based on their morphologic similarity to epidermal Langerhans cells and to macrophages, and their phagocytic activity, it is likely that Langerhans cells in the forestomach also form the animal's first line of immunologic defense, whether from simple ingestion

of particulate matter to the presentation of antigen to immuno-competent lymphoid cells.

The ruminant forestomach, because of the nature of the diet of the animal, is continually exposed to potentially antigenic agents. Under normal conditions, small molecules are the only ones which have the capacity to penetrate its epithelial lining (Henrikson and Stacy, 1971). The location and morphology of Langerhans cells in the forestomach suggest that they are ideally placed to intercept antigenic materials of small molecular weight which have passed through the upper levels before they are disseminated into the subepithelial space. It has been shown that, in contrast to monocytic phagocytes, at least in the epidermis, Langerhans cells selectively take up small molecules (Shelley and Juhlin, 1976).

It was noted by Nagatani et al. (1974) and in the present study that apposition of lymphocyte-like cell to Langerhans cell is not uncommon in the epithelium (see Figure 3.8 B and C). This association, although it could be entirely fortuitous, could indicate the presentation of antigen taken up by the Langerhans cell to T-lymphocyte as has been demonstrated by Silberberg (1973) in contact allergic reactions in the epidermis.

The capacity of a Langerhans cell to participate in immunologic function in the epithelium can be enhanced by (1) the presence of dendrites which would increase its immunologically reactive surface, and (2) its apparent migratory character, as the Langerhans cell is also sometimes observed in the upper levels of the epithelium (see Figure 3.8 C). In addition, the Langerhans cell, because of its phagocytic activity, may also engulf bacteria and other foreign bodies which have escaped the barrier in the keratinized layer of the epithelium (Steven and Marshall, 1973).

The role of the Langerhans cell in keratinization in the forestomach epithelium cannot be ascertained in

the present morphologic study. It is noted though that even if the epithelial cells are not arranged in columns, a Langerhans cell is frequently found associated with an epithelial bulb, usually on the tip or center of the bulb (Figure 3.12 A). This relationship could suggest a "proliferative unit" similar to the EPU of Allen and Potten (1974). However, this could be entirely fortuitous as Langerhans cells were also found not associated with an epithelial bulb, or not centrally located in the epithelial bulb. It is conceivable though, that aside from its primary immunologic function, a Langerhans cell could have additional functions such as control over keratinization.

4.4.2 Indeterminate cells

There were dendritic or branching cells in the epithelium without recognisable Birbek granules, and which appeared similar to "indeterminate dendritic cells", Type 3 cells or α-dendritic cells found in the oral mucosal epithelium, dermis and lymph nodes (Breathnach, 1980: Thorbecke et al., 1980). However, since Birbek granules in Langerhans cells identified in the present study were usually few, it is possible that these "indeterminate cells" are in fact Langerhans cells, only that their Birbek granules have been missed during sectioning. It is also possible that, because of their morphological similarity to Langerhans cells, these cells represent immature or developing Langerhans cells, or even effete Langerhans cells. Evidence to support this comes from studies of other epithelia (Rowden, Phillips and Lewis, 1979; Tamaki, Stingl, Gullino, Sachs and Katz, 1979) which showed that these indeterminate cells also express surface antigens and receptors associated with Langerhans cells. Morphological studies of Breathnach (1977) also showed that they and other similar cells are precursor Langerhans cells.

4.4.3 Intraepithelial lymphocytes

The finding of cells which were similar in histological appearance and ultrastructure to lymphocytes (Figures 3.8

B, C and 3.17 C) is consistent with the findings of Steven and Marshall (1970) and Nagatani et al. (1974) who also described lymphoid cells in the rumen epithelium. As noted in Section 4.4.1, lymphocyte-like cells are frequently associated with Langerhans cells and may represent T-cells being stimulated by Langerhans cells for antibody production. Since no lymphoid centres were found in the epithelium or the underlying connective tissue, it appears that these lymphocytes, if they are indeed lymphocytes, are migratory or "graduates" from other lymphoid tissues (Seelig and Billingham, 1980) presumably from the abomasum where lymphoid centres are abundant.

4.4.4 Mast cells and globule leukocytes

The cells with metachromatic granules in their cytoplasm which, in electron micrographs, appeared as branching cells without apparent intercellular junctions and with pale cytoplasm containing globular granules of differing densities limited by single membranes (Figure 3.17 D), are ultrastructurally identical to both mast cells (Kent, 1966; Stinson and Calhoun, 1976 b) and globule leukocytes (Toner, 1965; Kent, 1966; Takeuchi, Jervis and Sprinz, 1969; Murray, 1972; Jeffrey and Reid, 1975). Since the granular inclusions in both mast cells and globule leukocytes stain metachromatically with toluidine blue (Toner, 1965), their separate identification cannot be ascertained without differential staining. In electron micrographs, the appearance of granules in both cells is often similar (Kent, 1966; Murray, 1972) thus precluding their separate ultrastructural identification. According to Murray (1972) and Gregory (1979), globule leukocytes in fact originate from sub-epithelial mast cells. Murray, Miller and Jarret (1968) have demonstrated conclusively the progressive transformation of mast cells to globule leukocytes during amine discharge reactions. It is likely then that these cells in the forestomach epithelium, as found in the present study, are mast cells undergoing transformation into globule leukocytes.

The appearance of the globule granules of mast cell/globule leukocyte found in the present study is particularly consistent with the findings of Murray (1972) in that there may be, in one cell (Figure 3.17 D):
(1) granules with homogeneous electron-dense matrix;
(2) granules with rims of less electron-dense matrix;
(3) granules which have paracrystalline matrix; and
(4) granules with a vacuolated appearance. This great variation in morphologic appearance of the granules is thought to reflect the different stages in the process of accretion and utilisation of their contents (Takeuchi et al., 1969)

Globule leukocytes are found in the epithelia of the alimentary, urinary and respiratory tracts of various mammals and birds (Toner, 1965). Sub-epithelial mast cells, under certain stimuli such as parasitic infections, undergo changes which give the cells the appearance of globule leukocytes (Gregory, 1979). There is evidence that sub-epithelial mast cells and globule leukocytes can contain antibody (Dobson, 1966), but their roles in immune reaction to parasites are not well-understood. It is known though that in the presence of parasites in the lumen, mast cell/globule leukocytes migrate towards the luminal surface (probably through pseudopodial movement of their cytoplasmic processes) and secrete their granules outside. In the absence of parasites, the granules are internally degraded (Murray, 1972).

In the ruminant, globule leukocytes are usually found in the intestines (Dobson, 1966) and have been studied in responses to parasitic infestations. So far as the present writer is aware, the presence of globule leukocytes/mast cells in the forestomach epithelium is first reported in the present study.

4.4.5 Other non-keratinocytes

The light-staining cells which formed desmosomal connections with their neighbouring cells shared similar characteristics with Merkel cells of the epidermis and other keratinizing epithelia (Breathnach, 1971) in that formed cell organelles and tonofilaments were

few and small dense-cored granules smaller than MCGs were situated to one side of the cell near the cell surface.

Merkel cells are cells intimately associated with neurites and are thought to be specialised slowly adapting mechanoreceptors, but the exact mechanism of stimulus transduction is not well established (Munger, 1977). The most commonly held view is that the Merkel cell is the prime transducer element that detects deformities in the epithelium via its processes and desmosomal attachments and to thereby give rise to a receptor potential that activates the neurite by a synaptic mechanism involving the release of a transmitter substance from the dense-cored cytoplasmic granules (Breathnach, 1980).

Because of the failure to locate nerve fibres in the epithelium in many studies, including the present one, despite diligent searching, it is quite tempting to conclude that the light-staining cells which formed desmosomal connections with neighbouring cells are indeed Merkel cells and that they represent the sensory receptors in the epithelium that have been consistently detected by electrodes (Leek and Harding, 1975) but not by histologic methods. This temptation is further strengthened by the fact that these cells were usually observed in the reticulum where the sensory receptors were mostly detected.

The electron micrographs taken of these cells however, provided an equivocal picture for even a presumptive identification of these cells as Merkel cells can be drawn. Their similarity may just be accidental. First, no defined nerve fibres were found adjacent to these cells, although a synapse-like structure associated with the dense-cored granules was observed, and second, no rod-shaped inclusions were found in their nuclei. It may be that these cells were just post-mitotically maturing cells which were forming cell constituents which made older cells

dense-staining. The dense-cored granules may just be developing MCGs. Actively dividing cells or cells that have just undergone mitosis have been observed in this study to be light-staining and showed a few MCGs. Furthermore, evidence from Leek and Harding (1975) has shown that sensory receptors in the epithelium are rapidly adapting; Merkel cells in mammalian skin have so far been demonstrated to be slowly adapting (Breathnach, 1980).

However, other findings on Merkel cells from other sources also give some descriptions that fit the light-staining cells in question. Breathnach (1971) has shown that nerve fibres are not necessarily associated with Merkel cells as in non-digital skin. Tachibana and Nawa (1980) have also shown that intermediate cells may not show intranuclear rodlet inclusions. Breathnach (1980) also stated that Merkel cells in birds and reptiles are rapidly adapting mechanoreceptors suggesting that species differences may occur. Lastly, the dense-cored granules in these cells as found in the present study were smaller than MCGs and did not show any tubular granules or internal lamellations implying that they may be a different class of granules. As shown in Figure 3.17 E2, a few of the granules were situated near a synapse-like structure.

The finding of these light-staining cells in the present study which showed similar features to Merkel cells opens the possibility that specialised sensory cells may exist in the forestomach epithelium, explaining why nerve fibres have not been found in the epithelium in electron microscopic examinations, despite physiological evidence of the presence of sensory receptors. However, careful electron microscopic study - i.e., serial sectioning - has to be undertaken to confirm the Merkel cells in the forestomach epithelium, particularly in the areas (reticulum, cranial sac, longitudinal pillars) where sensory receptors have been detected by Leek and Harding (1975).

4.5 Structural Changes from Birth to 56 Days of Age

4.5.1 Gross anatomical development of the forestomach

In agreement with the descriptions of Wardrop and Coombe (1960), Leat (1970), Ralston (1971), Church (1976) and Phillipson (1977), the present study has shown that at birth the rumen and reticulum are smaller in relation to the abomasum than in the adult, but these organs soon enlarge and by 56 days of age the various compartments assume relative proportions as in the adult.

The development of the forestomach is influenced by the diet of the animal (Wardrop, 1960; Phillipson, 1977). Growth of the forestomach proceeds normally in milk-fed lambs until about three weeks of age, after which development becomes retarded until solids (roughage) are given (Wardrop, 1960). Numerous experiments involving dietary manipulation (Brownlee, 1956; Warner et al., 1956; Sander et al., 1959; Tamate et al., 1962 and 1963) have shown that inert bulk of the diet is largely responsible for the muscular development of the stomach wall.

Under free-ranging conditions, lambs start to nibble grass within 10 - 14 days of birth and are eating considerable amounts by the time they are 4 weeks old (Leat, 1970). The development of the rumen and omasum under these circumstances is more rapid that in milk-fed animals (Phillipson, 1977) and, as confirmed in the present study, by the 8th week the rumen has reached its adult proportion relative to liveweight and volume of abomasum contents (Wardrop and Coombe, 1960).

4.5.2 Gross and histological development of the forestomach mucosa

Growth of papillae appears to follow the general development of the forestomach. The papillae, as seen in the present study, particularly in the rumen, were small and indistinct at birth and increased in length thereafter, thereby changing the appearance of the

ruminal mucosa from a smooth velvety appearance at birth to a coarse, towel-like appearance at 56 days. In grazing lambs, it has been shown that papillae develop quickly once pasture is ingested (Wardrop, 1961 b) and active fermentation is developed (Comline et al., 1968). Papillary development has been shown to be due to the stimulatory effect of volatile fatty acids (Brownlee, 1956; Warner et al., 1956; 1958; Sander et al., 1959; Tamate et al., 1962; 1962) probably as a consequence of an increase in blood flow and/or increased metabolism of VFA's by the rumen wall (Warner et al., 1956; Sander et al., 1959; Fell and Weekes, 1975). The lengthening of the papillary connective tissue core could also be due to the increased production of collagen fibres by the numerous fibroblasts in the subepithelial layer.

Enlargement of the rumen papillae and hence the absorptive surface during development not only increases the absorptive capacity of the mucosa but can also be regarded as an adaptation to decreasing pH values and, consequently, as a further means of stabilising pH conditions for the development of microflora, particularly of the ciliates, to be established (Eadie and Mann, 1970; Kroman and Meyer, 1972; Kaufman et al., 1980).

The increase in papillary length, as seen in the light microscope in the present study, was accompanied by the development of papillary process from the connective tissue core. This is in agreement with the observations of Hofmann (1973) on the development of papillae in the rumen of wild ruminants. In the present study, individual papillary processes were seen to be invested with blood vessels suggestive of proliferation of the subepithelial microvasculature. The papillary processes grew towards the free surface of the epithelium thus establishing an intimate contact between the basal layers of the epithelium and the blood vessels. The proliferation and subsequent dilatation of these blood vessels in older animals could be result from increased blood flow in the capillaries due to increasing

concentrations of VFAs in the rumen. Butyrate in particular, has been shown to be effective in increasing blood flow to the rumen (Phillipson, 1977). Increasing osmotic gradients across the rumen epithelium and high concentrations of carbon dioxide in the rumen contents have also been shown to stimulate mucosal blood flow (Dobson, Sellers and Gatewood, 1976). Dobson, Sellers and Shaw (1970) have given convincing evidence that increased vascularisation of the ruminal wall is the reason for a faster absorption of solutes.

The finding of four orders of length of laminae in the omasum at birth and in the adult accords well with the findings of Lubis and O'Shea (1978). Wardrop (1961 a) observed a 5th order of laminae developing in the omasum. No evidence of this was found in the present study; there was however, a wide variation of lengths of laminae in the omasum, but generally, they could be classified into four orders. Lengthening of the omasal laminae during development could also increase the absorptive area in this organ.

Although it is well known that the abnormal development of the mucosa - i.e., papillary clumping, mucosal erosion leading to rumenitis, results from feeding the animal with high concentrate or pelleted grain rations (Ørskov, 1973; Fell and Weekes, 1975; McGavin and Morrill, 1976 a), it is apparent that the abnormal development of the reticular mucosa observed in a one-day-old lamb in the present study could not be due to dietary intake. This could have been due to a hormonal imbalance in the fetal lamb for it has been shown (Comline and Edwards, 1965) that abnormal mucosal and submucosal development occurs in hypophysectomised and thyroidectomised young calves and goats. Abnormal mucosal development in the form of isolated rose-shaped cysts have also been reported in normally functioning rumens (Shiomura and Tamate, 1980).

Histological examination allowed more detailed study of the epithelium lining the forestomach. In summary, it appears that the histological differentiation

of the epithelium into its adult form, particularly in the rumen, involves: (1) an increase in the number of transitional cells; (2) the development of epithelial bulbs in the deeper layers of the epithelium; (3) a decrease in epithelial thickness; (4) the disappearance of glycogen in the epithelium; (5) an increase in mucopolysaccharides in the intercellular spaces in the stratum corneum; and (6) an increase in the number of non-keratinocytes in the epithelium. The first five of these observations are in agreement with the combined findings of many authors including those of Wardrop (1961 a); Tamate et al. (1962); Henrikson (1970 c); Arias et al. (1978); and Lubis and O'Shea (1978). The first three observations paralled the gross development of the stomach, in agreement with the findings of Tamate et al. (1963). So far as the writer is aware, an increase in the number of non-keratinocytes in the epithelium (observation 6) has not been reported before.

The formation of a distinct stratum transitionale as seen in the present study, did not occur until about 45 days of age. Although keratinization was evident at birth and five cellular layers were already recognisable in the epithelium at 12 days, the transitional layers were indistinct. Recognising that the number of transitional cells is indicative of the degree of keratinization in the rumen epithelium in particular (Janacek, Borik and Holman, 1972), the appearance of a complete stratum transitionale at 45 days is consistent with the epithelium reaching its mature form at this age.

The influence of VFAs on the maturation of the epithelium can now be considered. Wardrop and Coombe (1961) have shown the peak of VFA production in the developing rumen at about 7 - 8 weeks. Tamate et al. (1962) showed that such increase of VFAs in the rumen (as in calves fed with large amounts of VFAs) resulted in the increase in the number of cells in the stratum transitionale, while calves fed with milk alone showed poor development of this layer. Hamada (1975) showed that 1,2-propanediol, like volatile fatty acids, is capable of stimulating mucosal growth and

suggested that chemicals might penetrate regions of the cell boundaries and increase the osmotic pressure around the basal cells. This could then affect the local control of mechanisms for the rate of cell division including stimulation of DNA synthesis.

The development of extensive epithelial bulbs observed in the present study is in agreement with the observations of Wardrop (1961 a) while the decrease in epithelial thickness during development has also been reported by Tamate et al. (1962); Tiwari and Jamdar (1970 a, b and c) and Arias et al. (1978). The formation of epithelial bulbs increases the efficiency of absorption by reducing the distance between the mucosal surface and the blood vessel in the papillary processes (Wardrop, 1961 a) thus ensuring the rapidity of absorption. Furthermore, it increases the absorptive area to a greater extent than would be accounted for by an increase in the external surface of the epithelium (Wardrop, 1961 a; Hofmann, 1973): Figure 3.10 A in the present study shows that the epithelial bulb-papillary interaction establishes intimate contact between a large number of basal keratinocytes and the endothelial wall of the blood vessels in the papillary process - basal cells practically surround the blood vessel. This arrangement was also found to a lesser extent in the omasum. It occurs to the present writer that the decrease in the thickness of the epithelium could also be due to the stretching of the epithelium due to papillary lengthening and enlargement of the stomach as a whole.

Single cell death - i.e. apoptosis - could also regulate epithelial thickness. A few apoptotic bodies were observed in 45 and 56 day epithelium. Marked apoptosis was also observed in normal sheep (Fell and Weekes, 1975) and in sheep subjected to rapid changes in diet (Tamate and Fell, 1978). Single cell death would reduce the number of basal cells available for differentiation. Since apoptosis was mainly observed in 45 and 56 day-old lambs, that is, at the stage when the ruminal mitotic indices were decreasing,

it could be expected that their combined effects would result in a remodelling of the epithelium. This has been observed in sheep subjected to abrupt dietary changes (Tamate and Fell, 1978).

Desquamation of the keratinized cell also plays an important role in regulating epithelial thickness. In the rumen epithelium, this could have well been speeded up by adherent bacteria on the epithelium. (see Figure 3.21 E; see also Bauchop, Clarke and Newhook, 1975). Studies by Cheng and Costerton (1980) have shown that adherent bacteria on the rumen epithelium are capable of digesting epithelial cells.

The accumulation of large quantities of glycogen in almost all epithelial tissues during development is well-documented. Its role as energy store in muscle and liver cells is well accepted but its presence in non-muscle and non-hepatic cells has not been well-explained. It is believed that carbohydrates including non-hepatic glycogen are the source of energy in the developing fetus (Lev and Weisberg, 1969) and in areas that rely on anaerobic metabolism in the adult such as in thick skin (Meller and Barton, 1979).

The presence of glycogen in neonatal epithelium and its absence from older epithelium as observed in the present study is comparable to the observations of Habel (1963); Henrikson (1970 a); Lubis and O'Shea (1978); Sheyanova, Demidova, Davletova and Kruglyakov (1978) and Arias, Fernandez and Cabrera (1980). The large quantities of glycogen in the developing epithelium could represent a store of energy of the developing animal. Sheyanova et al. (1978) believe that the forestomach epithelium functions as a glycogen depot of the body up to 30 days of age post-natally, similar to the function of the liver. However, since the epithelium is avascular, it is probable that the glycogen is metabolised locally. Lubis and O'Shea (1978) believe that metabolism of glycogen in the epithelium is related to the general anaerobic metabolism of cells,

not necessarily for keratinization as glycogen is found long before keratinization has taken place.

The absence of glycogen in basal cells (see Figure 3.7) could be explained by the proximity of these cells to the vasculature in the underlying connective tissue; this implies that respiration of basal cells, at least in the late fetal and neonatal animals, is aerobic and that their energy source comes from somewhere else, e.g., maternal circulation. The disappearance of glycogen in the epithelium during post-natal development could be correlated with a shift from carbohydrates to volatile fatty acids as the main energy source of the young ruminant.

The increase in the mucopolysaccharide deposits in the intercellular spaces in the stratum corneum during postnatal development as observed in the present study was also reported by Henrikson (1970a) and has been related to the shift of the pathway of carbohydrate metabolism in the epithelium of the young animal - i.e., from glycogen formation and storage to formation of glycocalyx. The development of the prominent glycocalyx could be related to the increase in the number of MCGs which have been discharged into the intercellular spaces during the process of keratinization. This could reflect the increased development of the diffusion barrier in this layer. (The importance of the glycocalyx coating of cornified cells in the formation of the diffusion barrier has been discussed in Section 4.2.) The increased development of the diffusion barrier could be related to the increasing absorption of ions, particularly of sodium, associated with the increased salivary secretion during eating (Carr and Titchen, 1978) as more than half of the sodium secreted in the saliva is reabsorbed by the rumen epithelium (Keynes, 1969).

The development of the glycocalyx could also provide temporary attachment sites for bacteria.

Since all of the non-keratinocytes with the exception of Merkel cell-like cells identified in the epithelium in the present study (see Section 4.4) were cells

commonly regarded as having immunologic functions, it seems reasonable to conclude that their proliferation in the epithelium during post-natal development reflects the increasing immuno-competence of the epithelium. This conclusion however, is drawn with caution as the accuracy of the measurement (based on the proportion of light-staining cells to the number of basal cells) has not been established. A more accurate measurement could be obtained by counting the number of individual non-keratinocytes per given area of the epithelium, using specific stains for the non-keratinocyte cell concerned - e.g., ATPase for Langerhans cells. In an adjunct to the present study, a preliminary preparation of epithelial sheets from the rumen separated by EDTA digestion and stained with either ATPase or heavy metal ion (zinc) method for Langerhans cells was successful in demonstrating Langerhans cells but the epithelial sheet prepared was not flat because of papillations and epithelial bulb formation in the epithelium. Although consequent measurements of Langerhans cells in any given area did not give satisfactory results, the method could well prove more successful for tissue samples from the dorsal sac of the bovine rumen, where there is an absence of papillation and the epithelial sheet prepared could be expected to remain flat.

Figure 3.8 C shows several Langerhans cells with the appearance of migrating into the epithelium from the underlying connective tissue. This suggests that they may have originated from extra-epithelial sources. Gerneke (1977) also showed electron micrographs of Langerhans cells breaking the basement membrane suggesting that they are not of epithelial origin. Evidence from other sources have shown that Langerhans cells are of mesenchymal origin as they have also been identified in mesodermal tissues such as the dermis (Breathnach, 1977), lymph nodes (Kondo, 1968) and thymus (Hoshino, Kukita and Sato, 1970). More recently, Tamaki, Stingl and Katz (1980) have conclusively demonstrated that epidermal Langerhans cells are derived from and are

continuously replenished by a mobile pool of precursor cells which for the most part, originate in the bone marrow.

As for the other non-keratinocytes, as previously stated in 4.4.3, lymphocytes could have migrated from lymphoid centres from other tissues presumambly from the abomasum. Seelig and Billingham (1980) believe that migration of lymphocytes into any epithelium is tissue drawn rather than antigen drawn. However, there is evidence as originally supplied by Silberberg (1973) that migration of lymphocytes into the epithelium could also be antigen-drawn especially that they were seen associated with presumably, antigen presenting Langerhans cells.

Globule leukocytes have been shown to migrate across the epithelium from the subepithelial tissues in the intestines, in the presence of parasites in the lumen (Murray, 1972).

Merkel cells have been postulated to originate from the neural crest (Breathnach, 1980).

4.6 Mitotic Index

It was found in the present study that up to 23 days of age, during the period corresponding to the monogastric phase of digestion and during the first week of the transition phase after the lambs have been assumed to consume grass, there occurred a decrease in the rate of basal cell mitosis. This decrease was associated with the decrease in the thickness of the epithelium. The rate of mitosis then increased at about the middle of the transition phase (at 34 days) although not exceeding 1 % which is thought to be the physiological limit (Sakata and Tamate, 1979). The proliferation of basal cells at this age did not result in the thickening of the epithelium, contrary to the expectations of Sakata and Tamate (1974). Instead, there was a proliferation of epithelial bulbs which effectively maintained epithelial thickness. This suggests that the cells produced from mitosis did not migrate right away to

the next upper layer, thus creating lateral pressure in the basal layer and causing it to fold. This is in accord with the theory of Bullough (1973) on epidermal kinetics (cited by Sakata and Tamate, 1974). After this increase in basal cell division at 34 days, the rate became low in the succeeding ages (45 and 56 days) and was lowest in the adult.

The changing rates of cell division during development is shown in Figure 3.13. The shape of the graph is quite similar to those obtained from sheep which have been intraruminally administered with volatile fatty acids (Sakata and Tamate, 1978 b; Sakata and Tamate, 1979). It is possible that the proliferation of basal cells at 34 days was due to the stimulation of volatile fatty acids being produced from the developing fermentation. It has been demonstrated that the mitogenic effect of butyrate is greater than either propionate or acetate (Sakata and Tamate, 1979) and as stated previously in earlier sections, butyrate could exert its effect on cell differentiation because (1) it is preferentially metabolised in the tissue (Warner et al., 1956; Sander et al., 1959); (2) it increases blood flow to the tissue (Fell and Weekes, 1975); and (3) along with propionate and acetate, it may alter the microenvironment of the cell that could then induce cell division (Hamada, 1974). However, these factors do not explain the delay in the increase of basal cell division during the transitional phase when VFAs are already present (Wardrop and Coombe, 1960) and also the decrease in mitotic rate at 45 and 56 days when VFA concentrations are rising and comparable to that found in the adult (Wardrop and Coombe, 1960). Also, they do not explain the low rate of mitosis in the adult. It appears then that there are other factors that could either modify the influence of VFAs on cell division or influence cell division itself, and these factors may include, besides individual variations: (a) circadian rhythm; (b) food intake of the animal; (c) VFA concentrations; (d) tissue-specific chalone; and (e) other hormones.

It has been demonstrated that cell division in the rumen epithelium follows a circadian rhythm, being low in the morning, high in the afternoon and in the evening, and declining at about midnight (Sakata and Tamate, 1978 a). This however, may be ruled out in the present study as all the lambs were killed at about the same time of the day.

It has also been shown that fasting and intermittent feeding markedly influence the rate of mitotic division in the rumen epithelium (Sakata and Tamate, 1974; Tamate et al., 1974). Unfortunately, the effect of food intake on cell divsion could not be determined in the present study as the time and rate of food intake of the lambs were not monitored. However, since the time intervals between removal from pasture and tissue sampling were about the same in all animals, the effect of food intake on the rate of mitosis in the present study would have been equalised to a considerable extent.

The lag in the stimulatory effect of VFAs on cell division observed in the present study during the transition phase is similar to that observed in sheep rapidly administered with propionate and acetate into the rumen (Sakata and Tamate, 1979). This lag is not observed in sheep rapidly administered with butyrate (Sakata and Tamate, 1978 b). Since the in vivo concentrations of propionate andacetate are higher than butyrate (Phillipson, 1977), it is probable that the the cell division observed was due to the stimulation by propionate and acetate. However, it is also possible that the lag in the increase in cell division during the transition phase is due to the low concentrations of VFAs in the rumen during this period, and that VFAs were not produced rapidly to attain the critical concentrations to have an effect on cell division. The decrease in mitosis during the monogastric or preruminant phase could be attributed to the withdrawal of VFAs from maternal circulation (Tamate et al., 1963).

The decrease in the rate of cell division at 45 and 56 days and in the adult despite high concentrations of VFAs in the rumen at these ages, suggests that a mechanism which inhibits mitosis to maintain the cell population constant exists in the rumen epithelium. This mechanism could be a tissue-specific chalone widely implicated in most mammalian tissues (Fell and Weekes, 1975). Characterisation of the chalone has been slow in keratinizing epithelia because an adequate system for its assay is lacking (Dueli, Kelsey and Voorhees, 1975). In the skin however, it has been established that adrenalin is a cofactor for its full inhibitory activity to take effect (Dueli et al., 1975).

The stimulatory effect of the volatile fatty acids could also be mediated in part by insulin whose release is stimulated by volatile fatty acids (Thivend et al., 1980).

4.7 Ultrastructural Features of the Epithelium during Development

The ultrastructural changes in the epithelium as observed in the present study during the post-natal development of the lamb could be related almost exclusively to the developing function of the forestomach. Several of these changes could be considered here. It was interesting to find though, that as early as 12 days of age, when the lambs were presumably still in the preruminant phase of digestion, the ultrastructure of the epithelium was already comparable to that found in the adult. This could indicate that at this age, the epithelium is already capable of performing functions that are attributed to it in the adult as has been demonstrated by Khouri (1969); Giesecke et al. (1979) and Scharrer et al. (1981).

The observation in the present study that keratinization has already occurred at birth is contrary to the finding of Henriksen (1970 a) that the epithelium remains non-keratinized before three days of birth. The present finding however, is in agreement with Pancharukhi and

Mudholkar (1978) and Arias et al. (1978) who have conclusively shown that keratinization in the rumen epithelium occurs during the pre-natal stage.

The type of keratinized cell in the neonatal epithelium found in the present study is said to be partially keratinized (Breathnach, 1971), presumably because the keratohyalin granules and the Er-protein which have been observed in the intermediate cells were not sufficient to allow full keratinization. Transformation into partially keratinized cells appeared to have also been initiated by lysosomes as in the the fully keratinized cell. The electron-lucent areas in the cells could have been due to the loss of glycogen during keratinization.

The appearance of a fully keratinized cell occurred at about 12 days of age at the same time that differentiation products - e.g., Er-protein, thick tonofilament bundles, large keratohyalin granules and membrane-coating granules - became abundant, confirming that full development of these products is important in producing a fully keratinized cell.

Examination of electron micrographs from developing epithelium throws more light on the origin of some of these differentiation products. In one-day-old epithelium for example, small areas of increased density due to accumulation of amorphous substance were observed within some tonofilaments in the intermediate cell layers (Figure 3.22 E). These then enlarged and became more electron dense as they incorporated more tonofilaments to become keratohyalin granules. No ribosomes were particularly associated with these keratohyalin granules. These observations are in agreement with those made on the developing human skin (Breathnach, 1971). Although the electron micrographs provided no other information on the primary source of the keratohyalin granules, they did suggest that ribosomes may not be a contributing factor, at least in their initial formation. It is presently thought that keratohyalin granules are self-aggregating (Matoltsky, 1975).

Electron micrographs of the epithelium from the developing rumen also showed the increasing abundance of MCGs and their close association to the highly dilated cisternae of the Golgi apparatus. This association suggests that MCGs are synthesized in this organelle. This relationship could not have been entirely by chance as this was consistently found in many micrographs including those of basal cells where a few MCGs were already seen. However, Takaki (1974) on the basis of enzyme histochemical observations in other keratinizing epithelia believes that MCGs originate directly from the rough endoplasmic reticulum. In the rumen epithelium, since the rough endoplasmic reticulum were usually filled with Er-protein at the same time when MCGs were near the Golgi apparatus, it is more likely that MCGs originate in the latter.

Another synthetic product which was observed in the young epithelium was the large mucous granules (Figure 3.22 B) similar to those described in the frog skin (Lavker, 1974). The fate of these granules in the rumen epithelium could not be ascertained in the present study. In the frog skin, they contribute to the formation of the horny matrix in the keratinized cell (Lavker, 1974).

Basal cells starting from 12 day-old epithelium were observed in the present study to display a large number of mitochondria in their cytoplasm. Some of these mitochondria were completely partitioned similar to those found in Figure 3.22 B, or in irregular shapes (Figure 3.23 A), all of which suggest impending division (Ghadially, 1975). This mitochondrial proliferation indicates high metabolic activity in basal cells. High metabolic activity is further reflected in the presence of membranous whorled and lysosomal bodies containing myelin figures (Figure 3.22 A) which are interpreted as mitochondria being transformed or incorporated into cytolysosomes. Such transformation is the most common method of eliminating exhausted mitochondria (Ghadially, 1975).

The concentric lamellar bodies (Figure 3.23 B) which were found in the 12 day old epithelium appeared to be composed of smooth endoplasmic reticulum. This conformation is interpreted to represent new formation of endoplasmic reticulum - i.e., a regenerative change leading to a specialised type of hypertrophy (Ghadially, 1975) which in the rumen epithelium could be associated with its function in the increased formation of Er-protein necessary for full keratinization. Thus, by 12 days of age, the epithelium displays high metabolic and synthetic activity as seen in the adult.

Amplification and complexity of the basolateral membrane surfaces of basal cells in the form of finger-like cytoplasmic processes in the lateral surfaces and the microvillus-like array of processes in the basal surfaces were observed in the epithelium to increase with age and particularly with the establishment of fermentation. The development of microvillus-like array of processes increased the basal sinus (the distance between the original cell surface and the basement membrane) which is considered to be the basal extension of the intercellular spaces (Tamate et al., 1974).

Such increase in the basolateral membrane surface area and basal sinus volume could be considered as structural adaptations to increasing VFA concentrations and alterations in the ionic environment in the epithelium brought about by fermentative digestion, and could be correlated to increasing absorption of volatile fatty acids and transport of ions across the epithelium. These structural changes could also be mediated by hormones.

Similar increases in the basolateral membrane surface area and intercellular space and basal sinus volume were observed in the epithelium from fasted and

subsequently re-fed sheep (Tamate et al., 1974), from intermittently fed sheep (Tamate and Sakata, 1974) and from intensively grain-fed cattle (Tamate and Kikuchi, 1978). These changes were attributed to the sudden changes in the amount of VFAs in the rumen contents. It has also been shown that the increase in basolateral membrane surfaces could be induced in the cells of the colon and kidneys of rats by feeding the animals with excess potassium chloride or treating them with dexamethasone (Kashgarian, 1980).

These structural changes could have significant physiological implications in the rumen in that: (1) they may be related not only to an increase in the absorptive surface of the basal cells but most especially to an increase in the number of available transport sites for ions, and (2) as this increase is accompanied by an increase in length and complexity in the intercellular spaces, these structural changes could alter the intercellular osmotic gradients proposed by Diamond and Bossert (1967) or the paracellular conductance, which would in turn, have major effects on the net transepithelial movements of ions and fluids (Kashgarian, 1980; Ernst et al., 1980). For example, it has been shown that the potential difference in the epithelium in 1-week-old lambs is less than in 3-week-old lambs (Scharrer et al., 1981). This could have been partly due the differences in the ultrastructure of the basolateral membrane surfaces between these ages.

Accompanying the increase in basolateral membrane surfaces was the progressive thinning of the endothelial wall of the subepithelial blood vessels. This would facilitate the entry of absorbed fluids into the blood stream.

One of the most striking findings of this study was the proliferation of gap junctions in the intermediate layers of the epithelium, particularly in the stratum granulosum, which coincided with the development of fermentative digestion. Gap junctions in the chick embryo shank skin show similar proliferation in response

to vitamin A (Elias and Friend, 1974) and in mammalian ovary in response to hormonal stimulation (Albertini and Anderson, 1974). Although no physiological evidence is available in the present study, because of the role of gap junctions in ionic coupling between cells, this proliferation in the rumen epithelium could be related to the increasing transport of ions by the epithelium as observed by Scharrer et al. (1931) during development.

Similar observations on the presence of 'internalised gap junctions' or 'annular gap junctions' have been made in granulosa cells in the ovary (Albertini and Anderson, 1974) especially during ovulation (Coons and Especy, 1977). Annular gap junctions are likely to represent an intermediate step in junction degradation following internalisation of gap junctions by a process of invagination (Albertini and Anderson, 1974) (see also Figure 3.25 C). A similar mechanism has also been suggested for desmosome and tight junction degradation (Staehelin, 1974).

The presence of tight junctions between the uppermost cells of partially keratinized neonatal epithelium can not be adequately explained. It could be that transport of ions is already occurring in the fetal lamb, especially since swallowing of saliva is already established in the fetus (Comline et al., 1968), thus necessitating a permeability barrier. However, since it has been demonstrated by Elias and Friend (1976) that gap junctions (which generally appeared later in the present study, except in the cranial sac where short strands were already present in neonatal epithelium) serve as sites of origin for tight junctional formation, it may be that their presence merely reflects the predetermined differentiation of the epithelium that would appear later in the adult. Their presence could also mean that other mechanisms for tight junction formation could exist.

4.8 Na+-K+-ATPase Cytochemistry

The rumen epithelium actively transport sodium from

the luminal side towards the blood (Keynes, 1969). An important component of the active transport mechanism is an Na^+-K^+-activated Mg^{++} dependent adenosine triphosphatase or transport ATPase which is now generally thought to be the enzymatic expression of the Na^+-K^+ pump responsible for the transepithelial movement of Na^+ (see reviews by Bonting, 1970; Albers, 1976; Ernst et al., 1980).

Biochemical studies (Schnorr, Hegner and Eckermann, 1969; Currell and Munn, 1970; Schnorr, 1971; Hegner and Tellhelm, 1974) have demonstrated the presence of Na^+-K^+-ATPase in the rumen epithelium in the microsomal fractions, particularly in the basal cell membranes (Hegner and Tellhelm, 1974). However, its histochemical and cytochemical localisations have not been entirely successful. Previous localisations (Schnorr, 1971; Henrikson, 1971) of Na^+-K^+-ATPase activity in the rumen epithelium have used modifications of the technique originally developed by Wachstein and Meisel (1957). The validity of these methods has been seriously questioned on the basis that the enzyme complex is strongly inhibited by both the lead ion (Pb^{++}) and glutaraldehyde and formaldehyde fixation that are employed in these methods and that these methods are not specific for this enzyme - e.g., variants are also used for the localisation of mitochondrial proton-translocating ATPase, sarcoplasmic reticular Ca^{++}-transporting ATPase and myosin ATPase. Furthermore, localisation of Mg^{++}-ATPase activity by these methods has often been interpreted as revealing the distribution of Na^+ transport sites, although this enzyme does not bear relationship to Na^+-K^+-ATPase as Mg^{++}-ATPase does not co-purify with Na^+-K^+-ATPase. (For a detailed review of these points, see Ernst, 1972 a and b; Ernst, 1975; Firth, 1980).

A recent localisation (Gray and Habel, 1979) using a modified Wachstein-Meisel technique employing mild fixation and low concentration of Pb^{++} failed to localise Na^+-K^+-ATPase in the rumen epithelium.

Recently, localisation of Na+-K+-ATPase activity among various reabsorptive and secretory epithelia has been accomplished by a technique which employs strontium (Sr^{++}) as capture ion and p-nitrophenyl phosphate (p-NPP) as substrate developed by Ernst (1972 a and b) to locate the activity of the ouabain sensitive, K+-dependent phosphatase component (K+-NPPase) of the Na+-K+-ATPase enzyme complex. Detailed consideration of the method including discussion of criteria establishing its validity and description of its application to a wide variety of tissues from various species has been the subject of several recent reviews (Ernst and Mills, 1980; Ernst et al., 1980; Firth, 1980). This method was employed in the present localisation of Na+-K+-ATPase activity and the results were compared to the results obtained from the method using the modified Wachstein-Meisel method devised by Gray and Habel (1979).

The results of the present localisation revealed K-NPPase activity on the lateral membrane surfaces of the lower granular, spinous and basal cells of the epithelium on both their cytoplasmic sides and the sides facing the intercellular spaces (Figure 3.26 A and B). Activity of a small magnitude was also detected in the basal processes of basal cells. Reaction product was also seen in the cytoplasm of the stratum corneum and the intercellular spaces in the stratum transitionale and the upper stratum granulosum. However, the deposition in the stratum corneum was interpreted as due to alkaline phosphatase activity because it was inhibited by cysteine, its specific inhibitor; its presence in the stratum corneum has been related to the transport process (Galfi et al., 1982). The deposits in the stratum transitionale and the upper stratum granulosum could have been due to K-NPPase activity, but were interpreted as non-specific deposits because similar deposits were also found in epithelium incubated with inorganic phosphate (Figure 3.27 A).

The presence of K-NPPase activity on both sides of the cell membranes in the deeper layers is difficult

to explain. These deposits on both sides of the membrane were both dependent on the presence of K^+ and Mg^{++} in the medium (Figures 3.27 B and C) and insensitive to cysteine, indicating that these deposits resulted from K^+-NPPase activity. However, reaction product of K^+-NPPase activity has been consistently localised in the cytoplasmic side of the cell membrane in other transporting epithelia such as in mammalian kidney, avian salt gland and teleost gill (Ernst et al., 1980). The reaction product on the cell membrane surface facing the intercellular spaces observed in the present study could have been due to the diffusion of the reaction product into nonenzymatic sites. This may occur due to inefficient capture of hydrolysed phosphate (Ernst, 1972 a and b; 1975; Leuenberger and Novikoff, 1974). A possibility could exist though that enzymatic sites in all transporting epithelia are not always the same, as reaction products using the same method in other transporting tissues such as the rat cornea (Leuenberger and Novikoff, 1974) and human blood platelets (Cutler, Feinstein and Christian, 1980) have been localised outside the membrane.

Inhibition of K^+-NPPase activity by ouabain could not be unequivocally demonstrated in the present study. This is due to the fact that Na^+-K^+-ATPase of the rumen epithelium has been shown as relatively insensitive to the glycoside (Currell and Munn, 1970). In the present study, deposits on the cytoplasmic side of the membrane were ouabain sensitive while the deposits on the opposite side were insensitive, similar to that shown in Figure 3.26 G. The ouabain insensitivity of the enzyme complex has been explained as being due to the fact that ouabain has not reached enzymatic sites because of the tortuous intercellular channels and that therefore the enzyme stays uninhibited, and to the presence of an additional ouabain-insensitive meachnism also responsible for transepithelial movement of ions (de la Manna, Proverbio and Whittembury, 1980). There is a strong possibility that the second mechanism

could exist in the rumen epithelium. Biochemical studies by Hegner and Tellhelm (1974) have revealed two distinct maxima of Na+-K+-ATPase activity in the rumen epithelium, implying that two different enzyme systems could be present in the tissue. Ion flux studies by Harrison et al. (1975) have shown an ouabain insensitive K+ pump for the movement of potassium from blood to rumen direction separate from the classical Na+-K+ pump. These studies could explain the 'duality' of K+-NPPase deposits in the present study. It is probable that the ouabain-sensitive reaction product on the cytoplasmic side of the membrane is due to Na+-K+-ATPase activity (equivalent to activity of the classical Na+-K+ pump) while the deposits on the outer surface are due to the separate K+-dependent, ouabain insensitive pumps which may also require ATPase as a catalyst.

The localisation of K+-NPPase activity, particularly on the cytoplasmic side of the membrane in the lower living layers of the epithelium is similar to the findings ov Mills, Ernst and Dißona (1977) in the frog skin using autoradiography of ouabain attachment sites. They proposed a model of Na+ transport which could operate in the rumen epihtelium and which, although in general agreement with the model proposed by Koefoed-Johnsen and Ussing (1958) for the frog skin on which the the model for the rumen epithelium was based (Steven and Marshall, 1970; Henrikson, 1970 c; 1971; Gemmel and Stacy, 1973) and illustrated on the left of Figure 4.1, has the following additional features (Figure 4.2):

> All of the Na+ to be actively transported enters the epithelium across the outer membrane of the outermost living layer from the stratum corneum; a portion of this is actively extruded into the intercelluar space by the first layer. A considerable larger portion of the Na+ passes through the extensive gap junctions to the cells of the deeper layers - i.e., lower cells of the granular, spinous and basal layers. Most of the Na+ that enters the epithelium is then

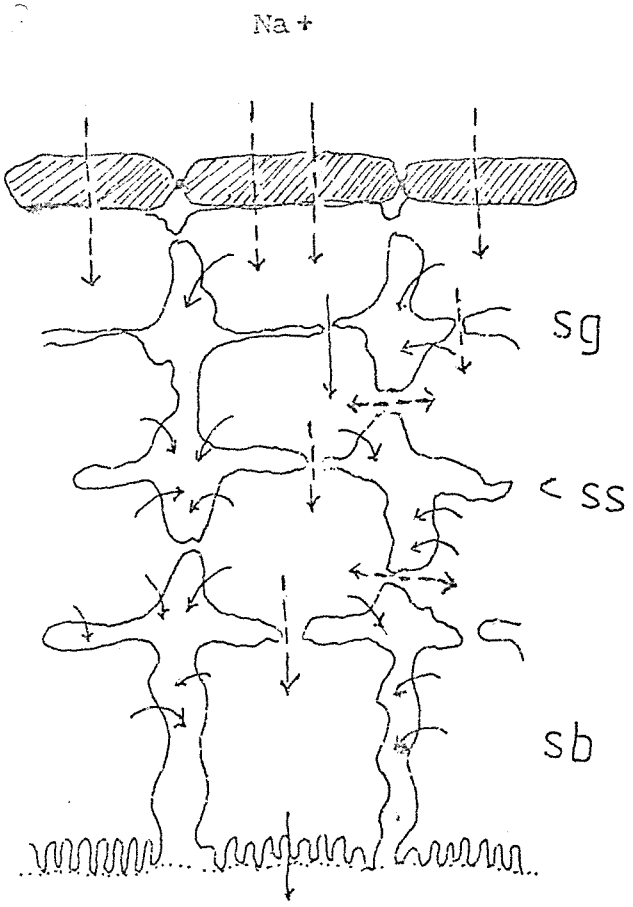

Figure 4.2 Schematic representation of the transport model for Na+ based on the results of the present study and in accord with the model proposed by Mills, Ernst and DiBona (1977). See text for explanations. Solid arrows = Na+ pump; dashed arrow = passive movement of Na+; sg = stratum granulosum; ss = stratum spinosum; sb = stratum basale.

actively extruded by the cells in the spinous and basal layers (Mills et al., 1977).

This proposed model is compatible with the protective function of the keratinizing epithelium in that most of the Na^+ pumps are located deep in the epithelium. It assumes continuity of the Na^+ reabsorptive activity of the epithelium even when a whole layer of granular cells is being transformed into cornified cells and their cell membranes lose their ability to act as selective permeability barriers. Since transport of Cl^- is also partly coupled to Na^+ transport (Chien and Stevens, 1972), part of the Cl^- transported is expected to follow the same routes. Localisation of K^+-NPPase in the epithelium from 12 day lambs suggests that at this age, the active transport of Na^+ across the epithelium is already in operation.

It was interesting to note that Mg^{++}-ATPase localised by a modified Wachstein-Meisel method developed by Cray and Habel (1979) was observed in the present study in the stratum corneum, in the intercellular spaces in the stratum transitionale and the upper stratum granulosum (Figure 3.28). This indicates that the Mg^{++}-ATPase localised may be related to keratinization.

CHAPTER FIVE

CONCLUSIONS

The findings in the present study are generally consistent with previous reports on the structure of the forestomach epithelium in ruminants.

The increase in papillary length, the development of extensive papillary process-epithelial bulb interactions and the decrease in epithelial thickness accompanied by proliferation of blood vessels in the papillary processes are consistent with an increase in absorptive function of the mucosa and the facilitation of rapid absorption of solutes from the rumen contents by increasing the absorptive area of the epithelium and decreasing the distance between lumen and blood. The appearance of a complete stratum transitionale at 45 days reflects the fact that at this age the epithelium has reached its mature form.

Similarly, the increase in the number of non-keratinocytes (other than those similar to Merkel cells) identified in the present study, namely, Langerhans cells, 'indeterminate cells' similar to Langerhans cells, mast cells/globule leukocytes and lymphocyte-like cells is consistent with a progressive increase in immunological activity in the epithelium. The cells similar to Merkel cells could represent the sensory receptors in the epithelium which have been demonstrated by using electrode recordings (Leek and Harding, 1975) but not yet by ultrastructural means.

The disappearance of glycogen from the intermediate layers in the epithelium and the increase in mucopoly-saccharide deposits in the keratinized layer could reflect changing pathways of carbohydrate metabolism. The disappearance is consistent with the change in the animal's source of energy from carbohydrates to VFAs during development. The increase in the mucopolysaccharide coating seems to result in the formation of the prominent

glycocalyx in the stratum corneum.

The barrier to diffusion in the epithelium could be associated with the tight junctions between the cells of the stratum corneum. However, it could also be related to the prominent glycocalyx, that is, to the presence of dense-staining, sometimes lamellated, materials which have been discharged from membrane-coating granules. The extensive network of gap junctions in the living layers of the epithelium, particularly in the stratum granulosum, is consistent with the transport function of the epithelium, especially since gap junctions are reported in the literature to be particularly associated with cell-to-cell transfer of metabolites and ions and to permit the tissue to function as a syncitium.

The appearance of completely keratinized cells by about 12 days of age, together with the large numbers of mitochondria in the epithelium at this time, indicates the beginning of increased metabolic and synthetic activity. Whether this actually reflects VFA absorption and metabolism could not be ascertained in the present study, although other physiological studies indicate that the epithelium is capable of VFA absorption and metabolism from the first week of age.

The proliferation of gap junctions from birth and the increased folding of basolateral membranes of basal cells during development could be adaptations to increasing levels of VFA and ionic concentrations in the forestomach associated with the development of fermentative digestion. Basolateral-membrane amplification could increase the absorptive area and ion transport sites and alter the intercellular osmotic gradients in the epithelium.

The localisation of Na^+-K^+-ATPase sites in the cell membranes in the granular, spinous and basal layers and the presence of gap junctions between the cells in these layers are consistent with the transport model for sodium ions, in which sodium ions from the rumen contents would diffuse through the stratum corneum and through

the deeper layers via low resistance transcellular pathways and then be pumped into the intercellular spaces by membrane pumps in the cells in the deeper layers of the epithelium. The localisation of the Na+-K+-ATPase enzyme and the appearance of gap junctions in the epithelium at 12 days both indicate that at this age, Na+ transport could be appreciable.

Thus, as early as 12 days of age, the ultrastructural features and Na+-K+-ATPase cytochemistry of the epithelium appear similar to those found in the adult and could indicate maturity at this age. However, the appearance of a complete stratum transitionale and the stability of the mitotic index and of the epithelial thickness after 45 days of age suggest that structural (and presumably functional) maturity had been attained at this stage.

The results of the study on the mitotic index during development also strongly suggest that the regulation of epithelial cell populations is influenced by VFAs. However, the results also imply that a mechanism which regulatesmitosis, probably a tissue-specific chalone, could operate in the epithelium. Single cell death (apoptosis) could also be implicated in this regulation.

Future studies on the structure of the forestomach epithelium during development or during dietary change should incorporate freeze fracture examinations and goniometric measurements and parallel studies of the abomasum and the intestines. These future studies could also include the examination of the effects on the epithelium of hormones and drugs such as insulin (which has been implicated to promote cell proliferation - Thivend et al., 1980); dexamethasone (which has been shown to increase basolateral membranes in rat colonic cells - Kashgarian, 1980) and monensin (an antibiotic used in ruminants to enhance digestibility and which has been shown to induct Na+ transport sites in the avian colon - Bindslev, 1979). The histochemical

localisation of Epidermal Growth Factor, a hormone-like mitogen for a wide variety of cells including those of non-epidermal origin and which have been shown to activate Na+-K+-ATPase sites (Das, 1979) could be significant. Further studies could be geared toward better characterisation of epithelial non-keratinocytes by immuno-histochemical methods. On the other hand, future studies could also focus on events in the petri dish, for procedures for culturing ruminal epithelial cells in vitro are now available (Galfi, Veresegyhazy, Neogrady and Kutas, 1981). It is in the combination of these several possible approaches that our best hope lies for the greater understanding of the significance of the progression of changes in the epithelium of the ruminant forestomach.

APPENDIX I

BIRTHDATES AND DATES OF REMOVAL OF LAMBS

1981 Season - flock kept at Massey University No. 1 Sheep Farm

Lamb No.	Date of Birth	Date of Removal	Age
1	22 July	16 September	56 days
2	23 July	17 September	56 days
3	23 July	17 September	56 days
4	22 August	3 September	12 days
5	22 August	3 September	12 days
6	23 August	7 October	45 days
7	23 August	7 October	45 days
8	23 August	7 October	45 days
9	23 August	5 September	23 days
10	24 August	6 September	23 days
11	24 August	6 September	23 days
12	25 August	26 August	1 day
13	25 August	26 August	1 day
14	26 August	7 September	12 days
15	27 August	30 September	34 days
16	27 August	30 September	34 days
17	28 August	1 October	34 days
18	28 August	29 August	1 days

1982 Season - flock kept at Best's Farm (Massey University)

3	5 August	30 September	56 days
5	5 August	30 September	56 days
6	5 August	8 September	34 days
7	5 August	8 September	34 days
9	11 August	23 August	12 days
10	11 August	23 August	12 days
12	13 August	27 September	45 days
13	14 August	28 September	45 days
14	15 August	7 September	34 days
16	16 August	8 September	34 days
25	22 August	23 August	1 day
unmarked	22 August	23 August	1 day

APPENDIX 2

METHODS FOR HISTOLOGY

A. Fixative

Bouin's Fluid (Culling, 1974)

Picric acid - saturated solution	75 ml
Formalin (40 %)	25 ml
Glacial acetic acid	5 ml

Tissues were fixed for 24 - 48 hours.

B. Ethanol-Chloroform procedure for paraffin wax processing

Process	Reagent	Time
Dehydration	70 % ethanol	1 hour
	95 % ethanol	1 hour
	100 % ethanol	1 hour
	100 % ethanol	1 hour
	100 % ethanol	2 hours
Clearing	chloroform	1 hour
	xylene	1 hour
	xylene	1 hour
Impregnation	paraffin wax 56°C M.P.	2 hours
	paraffin wax 56°C M.P.	2 hours

After processing, tissue blocks were embedded in 56°C melting point paraffin wax.

C. Staining procedures

Haematoxylin and Eosin (H & E) Staining for routine Histology (Culling, 1974)

1. De-wax in two changes of xylene at five minutes each.
2. Rinse in absolute alcohol.
3. Rinse in 70 % alcohol.
4. Wash in tap water.
5. Stain in Mayer's Haemalum for 10 minutes.

6. Wash in tap water.
7. Blue in Scott's tap water for two minutes.
8. Wash in tap water.
9. Stain in 1 % Eosin for two minutes.
10. Rinse rapidly in tap water.
11. Dehydrate through 70 % alcohol and two changes of absolute alcohol at 5 minutes each.
12. Clear in 2 changes of xylene.
13. Mount in DPX mountant.

Results:

Nuclei and cytoplasmic basophilia - blue/black
Cytoplasm and other tissue components - pink to red
Red Blood Cells - bright red

Masson's Green Tri-chrome staining for connective tissue

1. De-wax in two changes of xylene at 5 minutes each.
2. Rinse in absolute alcohol.
3. Rinse in 70 % alcohol.
4. Wash in tap water.
5. Mordant if necessary in Bouin's fluid or Potassium dichromate.
6. Wash in running tap water until colourless.
7. Stain in Celestine Blue for 10 minutes.
8. Rinse in tap water.
9. Stain in Mayer's Haemalum for 10 minutes.
10. Rinse in tap water.
11. Blue in Scott's tap water for two minutes.
12. Rinse in tap water.
13. Stain in acid fuchsin for 1 minute.
14. Rinse in 0.2 % acetic acid.
15. Differentiate in 3 % phosphotungstic acid until the collagen is almost colourless.
16. Stain in Acetic Light Green for 1 minute.
17. Dehydrate through 70 % alcohol and two changes of absolute alcohol at 5 minutes each.
18. Clear in xylene and mount.

Results:

Nuclei - blue/black
Muscle - pink
Keratin and RBCs - bright red
Collagen - green

Source: Lillie, 1965

Toluidine blue staining for mucopolysaccharides

1. De-wax in two changes of xylene at five minutes each.
2. Rinse in absolute alcohol.
3. Rinse in 70 % alcohol.
4. Wash in tap water.
5. Stain in aqueous solution of 1 % toluidine blue for 30 - 60 seconds.
6. Dehydrate in acetone.
7. Clear in xylene and mount.

Results:

Nuclei - deep blue
Cytoplasm - light blue
Red Blood Cells - green
Mucus and cartilage matrix - red purple to violet
Mast cell granules - deep violet

Source: Lillie, 1965.

APPENDIX 3

A FIXATION AND EMBEDDING SCHEDULE FOR ELECTRON MICROSCOPY

Primary Fixation	Time	Temperature
4 % glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.4	2 - 4 hours	4°C
or		
Karnovsky's fixative		
Buffer wash (pH 7.4)	3 washes in 30 minutes	4°C
Secondary Fixation		
1 % OsO_4 in buffer, pH 7.4	1 hour	4°C
Buffer wash (or distilled water)	2 quick changes	R. T.
Dehydration - Ethyl alcohol (ethanol, absolute alcohol) as follows:		
50 %	20 mins	R. T.
70 %	20 mins or overnight	R. T. 4°C.
95 %	20 mins	R. T.
100 %	20 mins x 2	R. T.
Infiltration		
Propylene oxide	20 mins x 2	R. T.
50% resin/50% propylene oxide	overnight	R. T.

Embedding

Fresh 100% resin in embedding boats, 48 O 72 hours, 60°C.

Embedding Media

Epon 812 (Hayat, 1972)

Mixture A

Epon 812	5 ml
DDSA	8 ml

Mixture B

Epon 812	8 ml
NMA	7 ml

Final embedding mixture

Mixture A	13 ml
Mixture B	15 ml
DMP-30	16 drops

Staining of thick sections

Basic fuchsin-methylene blue (Hayat, 1972)

Solutions

A.	basic fuchsin	2 gm
	distilled water	50 ml
B.	methylene blue	1 gm
	distilled water	50 ml

Method

1. Cut 0.5-1.0 micron thick sections with glass knife.
2. Transfer the sections with the aid of a very fine camel's hair brush to a drop of distilled water on a clean microscope slide and dry on a hot plate for 1 min at 70°C.
3. Flood the sections with freshly prepared Solution A for 1 min at 70°C.
4. Rinse with distilled water to remove excess stain and then destain with a few drops of distilled water for 1 min at 70°C.
5. Dry the sections with a filter paper at room temperature.
6. Apply 2 drops of 0.1 N aqueous NaOH to provide a pH of 12.5 and 1 drop of Solution B, and agitate the slide for 15 sec to mix the solutions. Stain for about 2 min at 25°C.
7. Rinse the slide with distilled water and allow the sections to air dry.
8. Seal the coverslip with a small amount of household epoxy cement after placing a drop

of immersion oil on the section, and allow to harden overnight.

Quick Methylene blue or toluidine blue stain (Weakley, 1972)

1. Transfer sections to a drop of water on a microscope slide.
2. Adhere sections to slide by heating slides on a hot plate (80°C).
3. Flood sections with 1 % aqueous solution of methylene blue or toluidine blue for 3 - 6 minutes.
4. Rinse slide with distilled water and allow the sections to air dry.
5. Mount with DPX mountant.

P.A.S.-staining for resin sections (Weakley, 1972)

1. Dry sections to slide by flaming.
2. Soak in xylene at least 1 hour to facilitate penetration of stain.
3. Hydrate sections by passing them through decreasing concentrations of ethanol to water.
4. Oxidise for 15 mins in 1 per cent periodic acid.
5. Rinse in distilled water.
6. Stain for 45 mins in Schiff's reagent.
7. Rinse in two changes (2 mins each) of freshly prepared bisulphite solution.
   (5 ml of 10 per cent $K_2S_2O_5$, 5 ml HCl, water to make 100 ml)
8. Rinse in two changes of distilled water.
9. Conterstain lightly with equal amounts of 1 per cent methylene blue and 1 per cent borax mixed on the slide and flamed. Rinse in hot water.
10. Dry in air at room temperature, protected from light.
11. Mount in DPX.

APPENDIX 4

METHODS FOR Na+-K+-ATPase CYTOCHEMISTRY

Strontium capture technique for the localisation of K+-NPPase (Ernst, 1972)

1. Fix tissue by immersion for 2.5 minutes in 1 % paraformaldehyde-0.25 % glutaraldehyde in 0.1 M cacodylate buffer, pH 7.5 at room temperature.
2. Rinse in cacodylate or tris HCl buffer, pH 7.5.
3. Incubate in the complete medium containing: (30 - 90 mins.)
   20 mM diTris or di-sodium-p-nitrophenyl phosphate
   20 mM $MgCl_2$
   20 mM $SrCl_2$
   250 mM tris HCl buffer, pH 9.0
   30 mM KCl

   Controls:

   a. Addition of 10 mM L-cysteine to inhibit alkaline phosphatase.

   b. Omission of KCl to demonstrate K+ dependence of the reaction.

   c. Omission of $MgCl_2$ to demonstrate Mg++ dependence of the reaction.

   d. Addition of 10 mM ouabain to demonstrate sensitivity of the enzyme to cardiac glycosides.

   e. Omission of p-nitrophenyl phosphate or addition of 10 mM inorganic phosphate to detect unspecific phosphate deposits.
4. Rinse in 100 mM, pH 9.0.
5. Treat with 2 % $Pb(NO_3)_2$ for twenty minutes to convert $Sr_3(PO_4)_2$ to $Pb_3(PO_4)_2$ which is electron opaque and visible in the electron microscope.
6. Rinse with 0.1 M cacodylate buffer, pH 7.5.
7. Postfix in 1 per cent osmium tetroxide in 0.1 M cacodylate buffer, pH 7.5 for 15 minutes.
8. For light microscopic evaluation, treat with 1 % $(NH_4)_2S$ to convert lead phosphate to lead sulphide which is visible under the light microscope.

9. Dehydrate in a graded series of ethanol and embed in Epon 812 resin.
10. Sections may be examined under the electron microscope with or without staining with 1 % lead citrate.

Histochemical localization of Mg++-ATPase (Gray and Habel, 1979)

1. Fix tissue by immersion in cold solution containing 2 % glutaraldehyde, 7 % sucrose and 0.055 M cacodylate buffer, pH 7.1 - 7.5 (2 minutes).
2. Rinse in 0.055 M cacodylate containing 7 % sucrose at 1 - 4°C.
3. Incubate at 23 to 24°C for 45 minutes: (incubation medium):

   200 mM sucrose
   20 mM tris maleate buffer
   10 mM KCl
   100 mM NaCl
   1.5 mM lead nitrate
   2.0 mM $MgCl_2$
   2.0 mM tris-adenosine triphosphate

   Controls:
   a. Omission of tris-ATP
   b. Omission of $MgCl_2$
   c. omission of KCl and NaCl
   d. Addition of 1 mM ouabain
   e. substitution of tris-ATP with B-glycerophosphate; adenosine diphosphate, adenosine-5'-monophosphate or inosine triphosphate.
4. Rinse twice in 7% sucrose-0.080 M tris-maleate solution for 15 minutes each after incubation at 4°C.
5. Rinse in 7% sucrose-0.075 M Sorensen's buffer solution for 15 - 25 minutes.
6. Fix in 1 % osmium tetroxide-0.075 M Sorensen's buffer at 4°C for 1 hour.
7. Dehydrate in methyl alcohol and embed in an epon-araldite mixture.
8. The sections may be examined in the elecron microscope after staining with uranyl acetate and lead citrate.

APPENDIX 5

STATISTICAL ANALYSES

A. A sample of a computer print-out for the analysis of variance for epithelial thickness in different ages

```
COLUMN     NAME       COUNT
C1         ADULT         35
C2         1DAY          35
C3         12DAYS        35
C4         23DAYS        35
C5         34DAYS        35
C6         45DAYS        35
C7         56DAYS        35

CONSTANTS USED: K1

-- print c1-c7
COLUMN     ADULT        1DAY       12DAYS       23DAYS       34DAYS       45DAYS
COUNT         35          35           35           35           35           35
ROW
  1          11.          11.          11.           9.           7.           7.
  2           7.          10.           9.           6.           5.           5.
  3           9.          12.           6.           5.           4.           6.
  4           5.           9.           9.           7.           6.           5.
  5           6.           9.           8.           5.           7.          15.
  6           8.          11.           8.           7.           6.           5.
  7           6.          13.           9.           4.           9.           5.
  8           7.          11.          10.           9.          10.           8.
  9           9.          10.          10.           7.           7.           7.
 10           5.          25.           7.           9.           9.           7.
 11           4.          21.           8.           5.           7.           7.
 12           6.          23.          13.           6.          16.          10.
 13           9.          25.           9.           7.           7.           8.
 14           5.          21.          10.           9.           7.          10.
 15           6.          20.          11.           6.           6.           8.
 16           5.          21.          11.          11.          14.          10.
 17           9.           9.           6.           4.           8.           9.
 18           6.          18.           8.          12.           8.           8.
 19           8.          10.           9.           8.           8.           7.
 20           6.          18.           9.           9.           9.           5.
 21           7.           8.          11.          13.           7.          12.
 22           8.           8.          12.           8.          13.          12.
 23          16.           6.          12.          15.           8.           8.
 24          10.          10.          12.          11.           8.           8.
 25          12.          26.          15.           7.           8.           7.


OK, minitab


 26           4.          10.          12.           7.           9.           7.
 27           8.          25.          11.          11.           8.           8.
 28           6.           8.          12.           8.           9.           8.
 29           8.           9.          11.          12.           6.           6.
 30           7.           6.          14.           7.           8.          14.
 31           9.          24.           8.          10.           7.           7.
 32          10.           8.          11.           9.           6.           8.
 33           8.           9.           7.           5.           9.           8.
 34           6.          24.           9.           6.           9.           6.
 35           7.           8.           6.           6.           8.           8.

COLUMN     56DAYS
COUNT         35
              8.           7.           8.           5.           7.           7.
              5.           7.           7.           9.           8.           9.
             10.           6.           8.           6.           8.           7.
              6.           7.           6.           5.           8.           8.
              6.           6.           7.           6.           9.           6.
              6.           6.           8.           7.           7.

-- aovo c1 c2

ANALYSIS OF VARIANCE

DUE TO          DF          SS       MS=SS/DF      F-RATIO
FACTOR           1       775.6          775.6        30.21
ERROR           68      1745.7           25.7
TOTAL           69      2521.3


LEVEL            N          MEAN        ST. DEV.
ADULT           35          7.51           2.42
1DAY            35         14.17           6.75

POOLED ST. DEV. =          5.07

INDIVIDUAL 95 PERCENT C. I. FOR LEVEL MEANS
(BASED ON POOLED STANDARD DEVIATION)
                +---------+---------+---------+---------+---------+---------+
ADULT           I********I********I
1DAY                                          I********I********I
                +---------+---------+---------+---------+---------+---------+
              4.0       6.0       8.0      10.0      12.0      14.0      16.0
```

B. Mitotic Index

-- INFO

COLUMN	NAME	COUNT
C1	ADULT	2
C2	A1DAY	2
C3	B1DAY	2
C4	A12DAYS	2
C5	B12DAYS	2
C6	A23DAYS	2
C7	B23DAYS	2
C8	A34DAYS	2
C9	B34DAYS	2
C10	45DAYS	2
C11	A56DAYS	2
C12	B56DAYS	2

CONSTANTS USED: NONE

-- CHIS C2 C3

EXPECTED FREQUENCIES ARE PRINTED BELOW OBSERVED FREQUENCIES

	A1DAY	B1DAY	TOTALS
1	81	82	163
	81.5	81.5	
2	11321	11321	22642
	11320.5	11321.5	
TOTALS	11402	11403	22805

TOTAL CHI SQUARE =
0.00 + 0.00 +
0.00 + 0.00 +

= 0.01

DEGREES OF FREEDOM = (2-1) X (2-1) = 1

-- CHIS C4 C5

EXPECTED FREQUENCIES ARE PRINTED BELOW OBSERVED FREQUENCIES

	C4	C5	TOTALS
1	88	42	130
	70.41	59.6	
2	11124	9458	20582
	11141.6	9440.4	
TOTALS	11212	9500	20712

TOTAL CHI SQUARE =
4.42 + 5.21 +
0.03 + 0.03 +

= 9.69

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