4 Discussion

4.1 Macroscopic and SEM examination

Shape and colour of papillae

The present study showed that both type and duration of concentrate feeding affected size and shape of the papillae. The variations in shape of papillae were significant among the different experimental sheep-groups, as demonstrated by scanning electron microscopy. Examination showed a complete sequence of transitional forms under the influence of diet ranging from small, smooth tongue-shaped papillae presented in the rumen of hay-fed group to large, heavily keratinized, finger-, foliate- or mushroom-shaped papillae presented in the rumen of 4, 6 and 12 weeks concentrate-fed sheep. The various forms of papillae are possibly initially shaped by mechanical forces which act on the mucosa and shaped papillae, which arise from epithelium expansion on the one hand and from muscular contraction on the other hand (Wardrop, 1961a). Nevertheless, subsequent development of papillae has been shown to depend on the nature of the feed stuffs (Brownlee, 1956); in particular SCFA, mainly butyric and to lesser extend propionic acids (Sander et al., 1959; Kauffold et al., 1977). McGilliard et al. (1965) suggested that the enhancement of metabolism in the rumen mucosa, which is stimulated by the SCFA, leads independently to both structural changes, and to increased absorptive ability. Scott and Gardner (1973) found that the papillae are largest and most dense in the ventral and cranial sacs of the rumen, areas where the papillae are exposed to the highest concentrations of soluble nutrients. In this context, the sequential changes from tongue to finger, foliate, or mushroom as the duration of concentrate feeding gradually increased, was seen in the recessus ruminis of the ventral sac of the rumen, which is an area that shows large alteration in the absorptive surface area of the papillae in relation to the feeding regimes (Gäbel et al., 1987). In addition to the changes in shape this study also revealed diet-dependent changes in colour of the papillae. Papillae from hay-fed sheep or sheep fed concentrate for 4 weeks had light brown colour. However, dark brown coloured papillae were observed in 6 and 12 concentrate-fed groups. The dark brown colour of papillae appears to be a combination of a supply of keratinized tissue, resulted from rapid growth and limited abrasion, high supply of iron, and an acid pH (Nockels et al., 1966). On the other hand, a preliminary observation on the histology of rumen tissues from calves fed hay, starter, and milk revealed a dark green pigment present in the outermost layer of desquamating horn

cells of the rumen epithelium, which is in direct contact with the rumen contents (Nockels et al., 1966). Existence of this pigment in the same locus of the rumen mucosa has been also reported in lamb by Sinclair and Kunkel (1959), who suggested that the pigment represents products of microbial activity. In contrast, Nockels et al. (1966) investigated rumen mucosa from calves which received higher amounts of SCFA and they found that the papillae remain light in colour.

Surface of papillae

In hay-fed sheep S.E.M revealed that surface of papillae was not smooth; it possessed shallow grooves, which gradually became deeper and increased in number with duration of concentrate feeding (more obvious in 4-12 weeks concentrate-fed groups). These deep grooves may increase the surface of papillae and offer place for the ingesta to be settled for long time, hence, increase the absorptive capacity of the epithelium. High magnification of these ridges and grooves revealed highly keratinized squamous cells on the surface of the epithelium. Examination of the surface epithelium revealed the presence of cellular protrusions (microvilli-like processes) of the superficial horn cells which vary in their arrangement and degree of development in the different experimental sheep groups. Cellular protrusions were well developed and had different shape and arrangement (foliate structure or more elongated structure and forming an arrangement of complex cytoplasmic flaps or folds) in sheep fed concentrate for 4-12 weeks compared to the less developed nipple-like projections characterized the hay-fed sheep and sheep fed concentrate for 2 weeks. These cellular protrusions may increase the total absorptive surface of the rumen-epithelium. It is important to recognise that these cellular protrusions do not represent the complex cellular protrusions which are commonly found in the cells forming the stratum basale of rumen epithelium (Steven and Marshall, 1970). Surface cellular protrusions have been reported in a number of regions of the alimentary tract; including the intestine (Fujita et al., 1971) and abomasum (Scott and Gardner, 1973). These, however, are true microvilli or microvillus-like structures and differ, in general form and in ultrastructure, from the microstructures present in the rumen epithelium. Furthermore, nipple-like projections on the horn surface of skin were noted by Kligman (1964). Evidence was presented that these cellular protrusions represent attachment sites of desmosomes (Kligman, 1964; Scott and Gardner, 1973). Dietary effects on the forestomachs were also observed using S.E.M (McGavin and Morrill, 1976; Yamamoto et al., 1994). These authors claimed that ruminal papillae of milk-fed calves show a simpler structure compared to calves fed roughages.

Dimensions of papillae

The results of this study clearly demonstrate the effect of the type of diet and the duration of the concentrate feeding on the development of the ruminal papillae. The morphometrical evaluation revealed significant differences in the development of papillae between hay- and concentrate-fed groups and even among concentrate-fed groups as a consequence of increasing the duration of concentrate feeding. The hay-fed group had the narrowest and shortest papillae among all sheep-groups. Among concentrate-fed groups, 4-12 weeks concentrate-fed groups showed a significant increase in the dimensions and the total surface area of the ruminal papillae. Among all concentrate-fed groups, 4 weeks concentrate-fed group showed the maximum increase in the papillae-dimensions and the highest total papillary surface of the papillae (the length, width and total surface of papillae were 2, 1.5 and 4 fold (4, 6 and 12 weeks, correct?), respectively, that of hay-fed sheep). These observations are in accordance with those of Zitnan et al. (1999) who found that the nutritional regimes affect the morphometrical and histological development of the mucosa. Although, papillae serve as absorptive structures the total ruminal volume and surface area have a significant influence on nutrient transport (James et al, 1983), so changes in papillary size indicating a marked increase of relative rumen epithelial absorptive surface. The intake of high levels of protein and carbohydrate appears to increase papillary size and density via butyrate and propionate regulation of IGF-1 production (Shen et al., 2004) and is partially due to SCFA dependent increase in the mitotic index of the rumen epithelium (Mentschel et al., 2001).

The number of papillae increased significantly in concentrate-fed groups, except in 4 weeks concentrate-fed group, compared to that of hay-fed ones. Among concentrate-fed groups, the decrease in the number of papillae per cm^2 mucosa with increasing the duration of concentrate feeding was observed, but there was no indication of fusion of several papillae into one papilla. The reduction in the number might be due to increase in thickness of individual papillae, thus, a accommodating fewer papillae per unit area (Tiwari and Jamdar, 1970a). Aafjes (1967) demonstrated that areas of the rumen wall with large numbers of papillae absorb more volatile fatty acids than do areas with few papillae.

On the other hand, the present study showed that adaptation of the rumen to concentrate feeding occurred rapidly within two days, where the length, density and total surface of papillae increased significantly compared to hay-fed sheep. This finding is clearly in accordance with Goodlad (1981), who stated that differences, which attributed to the SCFA, can be only observed for a couple of days after onset of feeding and it is explained by a

transient decrease in the cell cycle duration. Thereafter, the mitotic index decreases again (Sakata and Tamate, 1979).

Unfortunately, it is difficult to compare findings obtained by different researchers because of the lack of uniform nomenclature and illustrations. On the other hand, different abnormalities have been observed in the papillary forms due to the feed regimen. Oltjen and Davis (1965) observed papillae, which were cub shaped and clumped by starch like ruminal ingesta in animal fed only concentrate rations. Hentges et al. (1966) found that by adding greater amounts of dried citrus meal to the ration, ruminal papillae became smaller and darker, with deeply serrated, irregular perimeters. By feeding weathers a ration containing ground roughage Nockels et al. (1966) observed that the papillae on the ventral floor of the atrium ruminis become black and covered by layers of swollen, vacuolated cells.

4.2 Histology

Shape and size of papillae

The present study showed that both type of diet and duration of concentrate feeding affected the morphometric and histological development of the rumen mucosa. Although, ruminal papillae basically showed a considerable degree of similarity in their basic micro-architecture, apparent differences in appearance among the different experimental sheep-groups occurred. The papillae showed a gradual development in both shape and size with more irregular or waved sides and inter-papillae mucosa in response to the intake and increasing the duration of the concentrate feeding. These irregular sides of the papillae may increase its length and the total absorptive surface of the rumen-epithelium. However, branching of the lamina propria, with the resultant formation of mushroom-shaped papilla, were best developed in the recessus ruminis of 12 weeks concentrate-fed group. This was accompanied by less or more parakeratosis (horn cells form a cap over the tip of the papilla). McGavin and Morrill (1976) stated that when papillae became parakeratotic at one point (usually on the tip at first); this possibly induced branching of the lamina propria. Thus, it could be postulated that the branching of the papillae was an example of compensatory hyperplasia to increase surface area in response to loss of absorptive capacity because of the thick layers of parakeratotic cells. Previous studies revealed the presence of secondary papillae at the bases of primary papillae and caps cover the papillary tips in cows (Bastain and Menon, 1963) and the presence of only caps without secondary papillae in buffalo (Tiwari and Jamdar, 1970a).

Papillary pegs

The present findings showed that epithelial pegs were more developed at the tips of the papillae than at the base and inter-papillae area, which could be due to the fact that tips of the papillae were the most parts of the rumen directly in contact with the milieu.

Compared to hay-fed sheep, concentrate-fed ones exhibited hypertrophic changes in the ruminal papillae and showed well developed epithelial pegs (more obvious in 2-6 weeks concentrate-fed groups). The tips of some epithelial pegs divided into two small ones in 4-6 weeks concentrate-fed groups. Development of epithelial pegs may either due to increasing number or size of the rumen epithelial cells. It may offer large contact surface of the basal cells with the blood capillaries located in the papillary bodies and increase the absorptive capacity of the epithelium. Previous studies showed that the dietary transition from hay to diet of barley was associated with a marked increase in the mitotic index of the basal epithelial cells and accompanied by the development of the epithelial pegs, which grew down into the lamina propria (Tamate and Fell, 1977; Sakata and Tamate 1978a, 1979). Then, the rate of mitosis declines and the incidence of apoptosis rise, despite the continuation of SCFA infusion. This causes regression of epithelial pegs because of the extensive spontaneous cell loss, particularly from the region of epithelial pegs (Tamate and Fell, 1977; Sakata and Tamate 1978a, 1979). The present findings in 12 weeks concentrate-fed sheep are in the same line.

Number of papillae

In hay- fed sheep, the number of papillae varied from 3-4 papillae / field to 5-6 papillae / field in 2 days to one week concentrate-fed groups. Thereafter, it decreased to 4 and 2-4 papillae / field in 2-6 and 12 weeks concentrate-fed groups, respectively. As it has been mentioned before, the decrease in the number of papillae per field mucosa may be due to increase size of individual papillae which was more obvious at the base of the papillae (Tiwari and Jamdar, 1970a). Additionally, other factors such as the distance between papillae, folds of interpapillae-mucosa or interposed papillae, and secondary papillae may also affect number of papillae per field mucosa.

Core of the papillae

The lamina mucosa was composed of dense connective tissue, mainly collagenous fibers. There was a gradual increase in the lamina propria-connective tissues in concomitant to increasing duration of concentrate feeding and the size of papillae. Although, the papillae in 1-2 weeks concentrate-fed groups were shorter compared to those of 2-4 days concentrate-fed groups, they have wider bases and a wider connective tissue core. This is in the line of the

findings made by Dozsa et al. (1964) who stated that the short papillae usually have wider connective tissue core than do long ones. After 12 weeks of concentrate feeding, the lamina propria branched giving off secondary papillae.

Irregular or unusual branching of ruminal papillae has been observed in calves fed a ration containing ground roughage or inadequate roughage (Nockels et al., 1966). The present study showed that papillary bodies were longer at the tip of the papillae than at the base or at the inter-papillary mucosa. Compared to hay-fed sheep, the mean length of the papillary bodies was increased significantly at the tip of the papillae with increasing the duration of concentrate to 2-12 weeks. In the concentrate-fed groups, the mean length of the papillary bodies increased significantly after 2-4 weeks compared to that of animals fed concentrate up to 1 week. It reached the maximum value in 2 weeks concentrate-fed group. Noteworthy, Galfi et al. (1988) reported that the undulation of the basement membrane (papillary bodies) is influenced by the intraruminal concentration of volatile fatty acids, especially butyrate, by increasing the number of the protrusions.

The core of the papilla is an important structure because large blood and lymphatic vessels are found there. Due to the intake of concentrate as well as increasing the duration of concentrate feeding the number of blood vessels and capillaries increased gradually with increasing length of papillary bodies. In 4-6 weeks concentrate-fed groups, large number of blood vessels and capillaries extended into the core of the papillae and they were found between the epithelial pegs (papillary bodies). At papillary bodies, these capillaries became wider and took the form of sinusoids.

Increasing in the thickness of lamina propria is correlated with the increase in the number of blood capillaries, which also aid absorption (McGavin and Morrill, 1976). Since it became known that the volatile fatty acids are absorbed in the rumen and reticulum it has been regarded as an adaptation to increase the epithelial surface. Papillary development is stimulated by these acids, especially butyric, and their absorption is facilitated by the very rich subepithelial capillary plexus (Dyce et al., 2002). Moreover, distended capillaries with no marked congestion are readily seen in the papillary bodies (Dozsa et al., 1964). Furthermore, the morphology of the subepithelial connective tissue has been examined using different techniques (Tamate et al., 1979; Weyrauch and Schnorr, 1979; Scheurmann and Weyrauch, 1983; Yamamoto et al., 1993; 1996). Scheurmann and Weyrauch (1983) noted the possible effects of feeding on the organization of the subepithelial connective tissue. On the other hand, Yamamoto et al. (1993) described the species-specific subepithelial networks of collagen in cattle and sheep.

Epithelial strata (shape, size and thickness)

The present study showed that ruminal mucosa of hay-fed sheep was lined by a keratinized, stratified, squamous epithelium and a lamina propria of connective tissue. The rumen epithelium was composed of five to six layers of nucleated cells and relatively thin stratum corneum formed by two layers of horn cells. Generally, the intake of concentrate-diet for various intervals of time revealed a gradual increase in both size and number of cells comprising the different epithelial strata. Moreover, other distinct morphological changes in the rumen epithelium occurred in response to concentrate feeding and they can be summarized as follow:

1. Granular cells developed both aggregates of keratohyaline granules and vacuoles or lipid droplets in 4-12 weeks concentrate-fed groups. The presence of vacuoles in concentrate-fed animals has been reported by Nocek et al. (1984) who stated that the concentrate-fed animal has thicker ruminal epithelial lining and shows more vacoulization in the stratum granulosum and more mucosa to muscle compared to rumen of calves fed ground or chopped hay. Vacuolization of epithelial cells may be due to unphysiological osmolarities caused by high concentrate intake.

2. The stratum corneum was compact on the tips of papillae. Generally, vacuolated horn cells were absent in the tips of the papillae, probably because of the abrasion of the surface by ingesta. Nevertheless, vacuolated horn cells were regularly found along the more protected lateral sides or surface of the papillae and on the interpapillary epithelium. Thus, the thickness of stratum corneum on the papillary base of various sheep groups was higher than those on tip and interpapillary epithelium, which agrees with findings of Henrikson, (1970b). Desquamation could be suggested that the abrasive action of the digesta might be such as to polish the exposed horn cell surfaces (Scott and Gardner, 1973). Furthermore, friction and desquamation may result in the loss of the outermost layer of keratinized cells in the fully differentiated rumen epithelium and decrease the total thickness of the epithelium as seen in 12 weeks concentrate fed-group. These observation and explanation are in the line of those of Scott and Gardner, (1973). It has been described that defects in the ruminal mucosa such as vacuolization of epithelial cells and desquamation may be caused by unphysiological osmolarities, which has further consequences like hyperkeratinization and even atrophy of papillae (Gäbel et al., 1987). On the other hand, Fell and Weekes (1975) found that the rumen adapts to a more than 3-fold increase in the food intake in lactating sheep. This adaptation is achieved by hyperplasia of the epithelial cells morphologically. However, if the rates of cell division and turnover are disturbed, pathological conditions such as parakeratosis and

ruminitis may occur. Present observation in 12 weeks concentrate fed-group are in the line of those of Fell and Weekes (1975), where evidence of inflammation or parakeratosis such as heavily keratinized papillae with the ingesta impacted between them was observed. Moreover, isolated microabscesses were also observed in the stratum corneum.

3. The stratum corneum increased significantly in 4-12 weeks concentrate fed-groups compared to that of hay-fed sheep. Feeding concentrate for 12 weeks resulted in the highest significant increase in the thickness of the stratum corneum among all the concentrate fed-groups (maximum value). Thickness of the epithelium, particularly the stratum corneum (barrier layer) and potential effects on transport of nutrient have been linked to diet (Fell et al., 1968; Weigand et al., 1975; Hofmann and Schnorr, 1982). The efficiency of nutrient transport across the epithelium depends to a large extend on the integrity and degree of keratinization of the stratum corneum (Dirksen and Garry, 1987). Marked differences in the thickeness of the stratum corneum on the tip and on the sides of the papillae were observed in the present study. In this context the study of Jensen et al. (1958) on parakeratosis in sheep is of interest; in lambs with parakeratosis, the stratum corneum and the stratum germinativum was thickened significantly compared to those of hay-fed sheep. On the other hand, the lack of effective fiber may predispose the epithelium to develop a thick stratum corneum due to the reduction of sloughing of necrotic cells, which may impede transport (McGavin and Morrill, 1976).

4. Feeding concentrate for various intervals of time (up to 12 weeks) affected the size of the epithelial cells more than their number. In 1 week concentrate-fed group, the number of cells constitute each stratum remained constant compared to that of hay-fed sheep, however, their size increased. In 2-6 weeks concentrate-fed groups, both size and number of granular and horn cells were increased to 3 cells, meanwhile, the number of spiny cells decreased to 1-2 cells. In 12 weeks concentrate-fed group, the thickness of the stratum corneum markedly increased (2-5 cell-thick), whereas, the thickness of the stratum spinosum decreased to one cell-thick. Cell number in the epithelium depends on the transit time of the post-mitotic cells which migrate to the outer, exfoliating layer. The duration of cell cycle is about 24 hr but decreased to 17 hr when the diet of sheep is changed from roughage to a concentrate-based diet. This decrease in cell cycle time is mainly due to the reduction in the duration of the phase DNA synthesis (Goodlad, 1981). This suggests that the rumen epithelium is subjected to a self-regulatory mechanism, which decreases the rate of production of new cells once a new tissue mass is reached. This decrease may be due to mitotic inhibitors produced by the maturing cells or by a reduction in local or systemic stimuli (Goodlad, 1981). Presumably

such changes represent aspects of the physiological process of adaptation, or indeed indicate that adaptation to dietary change has already taken place (Goodlad, 1981). The decrease in number of spiny cells with competence increase of both granular and horn cells, which was most obvious in 12 weeks concentrate fed-group, means a rapid transition to dead horn cells. These findings are in accordance with those of Goodlad (1981).

5. The mean thickness of the epithelium (μm) increased significantly, firstly with intake of concentrate diet for 4 days-12 weeks when compared to that of hay fed animals. Secondly, with increase the duration of concentrate feeding up to the 6 weeks (maximum value), thereafter, it decreased significantly in 12 weeks concentrate-fed group from that of 6 weeks concentrate fed-group. The thickness of the epithelium was affected by both total number and size of the cells that constitute all strata of the rumen epithelium in each location. Generally, the mean thickness of the epithelium covering the tip of the papillae was larger than that covering the base and inter-papillar regions, which may be due to the fact that the tips of the papillae are the most parts of the rumen in contact with the fermenting ruminal contents.

Concentrate feeding resulted in a significant increase in the thickness of the strata germinativum (basale + spinosum) and granulosum (living cells) compared to hay, except in 4 and 12 weeks concentrate-fed groups, where the differences were not significant. Feeding concentrate for 6 weeks resulted in the maximum value of this increase among all concentrate-fed groups.

The relation between the living cells (strata germinativum (basale + spinosum) + granulosum) and dead cells (stratum corneum) increased significantly in animals fed concentrate for 2-4 days compared to that of the hay-fed group and 2-12 weeks concentrate fed-groups. Importantly, the relation depends on the thickness of stratum corneum. The lowest value in 12 weeks concentrate-fed sheep was due to the fact that the thickness of the stratum corneum was the highest among all concentrate fed-groups (maximum value).

The present results are in line with the previous observation that intake of easily fermentable carbohydrates increases the proliferation of the rumen epithelium (Bull et al., 1965; Dirksen et al., 1984). The reason for the stimulatory effect of diets rich in carbohydrates could be due to increase in production of SCFA, which are considered to be physiologic trophic factors and promoters of the rumen epithelial cell proliferation in vivo (Qrskov, 1976; Sakata and Tamate, 1978 b, 1979; Sakata and Yajima, 1984). Among the SCFA, it is assumed that mainly butyric acid but also propionic acid, though to lesser extent, cause the proliferation in vivo (Kauffold et al., 1977; Sakata and Tamate, 1978 c; Sakata and Tamate, 1979). SCFA, butyrate in particular, act locally by increasing the blood supply to the papillae, indicating the

possibility of increasing the transfer of promoting factors, which may participate in rumen development (Thorlacius, 1972). Additionally, a diet rich in concentrate increases the transport of SCFA through the ruminal wall (Dirksen et al., 1984; Gäbel et al., 1987, 1991) than observed with a diet based on forage (Weigand et al., 1975; McGavin and Morril, 1976; Goodlad, 1981; Lane and Jesse, 1997; Zitnan et al., 1999; Swan and Groenewald, 2000). On the other hand, structural changes of the rumen epithelium are associated with the corresponding effects on its function such as absorptive capacity and SCFA metabolism (McGilliard et al., 1965). The presumptive absorptive function of the papillae is emphasized by the full thickness of the papillae relative to its length (Graham and Simmons, 2005). It is likely that the SCFA in turn have a regulatory effect on the absorptive cell population (Fell and Weekes, 1975). Dirksen et al. (1985) described a faster absorption of acetate, propionate and butyrate in animals with a thick ruminal mucosa. So, mucosal proliferation and parakeratosis are indications for physiological adaptations to the increased SCFA production (Dirksen and Garry, 1987). The proliferating activity of the rumen epithelium is controlled by the basal cells, which are considered as a renewing population (Messier and Leblond, 1960). This population is regulated by the proportion of cells that in the cell renewal cycle, and also by the duration of the phase of the cell cycle, namely mitosis, the post-mitotic pre-synthetic gap, the period of DNA synthesis and the post-mitotic gap. On the other hand, the epithelial cell mass is determined by the cell production rate and the rate of the cell death (Bullough, 1975). These factors are related to the food intake (Fell and Weekes, 1975), the nutritional level (Tamate and Fell, 1977; Goodlad, 1981) and the ruminal SCFA concentrations (Sakata and Tamate, 1978 b, 1979; Dinsdale et al., 1980). Thus, rumen epithelium can alter its tissue mass in a variety of ways, namely changing the proportion of basal cells that are proliferating, changing the time taken for cell division and adjusting cell transit time (Goodlad, 1981)

The mitotic index has been used for measuring the proliferating activity of the rumen epithelium (Fell and Weekes, 1975; Tamate and Fell, 1977; Sakata and Tamate, 1978 a, b, 1979; Dinsdale et al., 1980; Sakata et al., 1980; Goodlad, 1981; Ohwada and Tamate, 1983). It has been shown that under standard conditions, the mitotic index of the epithelial basal cells of the rumen does not exceed 1.0% and it increases significantly after intraruminal administration of butyrate (Sakata and Tamate, 1978a, b, 1976), propionate and acetate (Sakata and Tamate, 1979). Insulin was found to stimulate epithelial cell proliferation in the rumen and it may, at least partly, mediate mitotic stimulation by SCFA in sheep (Sakata et al., 1980). On the other hand, the mitotic index of the rumen epithelium increases rapidly when cell proliferation is stimulated by changing the diet of sheep from roughage to concentrate.

The increased mitotic index, however, soon declines to a new level (Goodlad, 1981). Additionally, one possible mechanism may be that the stimulus for cell proliferation declines as soon as the cell population increases sufficiently to cope with new functional demands (Goodlad, 1981). The decrease in the thickness of the epithelium in 12 weeks concentrate-fed group is in accordance with the findings of Goodlad (1981).

Nockels et al. (1966) attributed changes in growth pattern, histologic features, and mineral content of ruminal papillae to the following 5 factors: (1) differences in kind or amount of SCFA, (2) pH of ruminal fluid, (3) possible change in the ability of the epithelium to use acids, (4) growth-factor production in the rumen, and (5) mineral content of the ration. These authors suggested that rapid acids production could irritate the ruminal wall, and depending on the severity of the irritation, papillae are lost by desquamation or grow in unusual directions or sizes which could be seen in the present study, especially in 12 weeks concentrate-fed group.

6. Material stained by the PAS reaction, presumably polysaccharides, sharply outlined the horn cells. The degree of PAS- reaction was weak in hay-fed sheep, 2-4 days or 12 weeks concentrate-fed sheep, moderate in 1 and 2 weeks concentrate-fed sheep and strong in 4 and 6 concentrate-fed sheep. The present findings agree to some extent with those of Henrikson (1970b) who found, material stained by the PAS-reaction, sharply outlines the horn cells, but is absent from the more proximal layers of epithelium. In contrast to Henrikson (1970b), the present study found that, in addition to the thick red lines outlined the cells of the stratum corneum, both inter and intra-cellular PAS-reaction was found in the stratum granulosum and the superficial layers of the stratum spinosum (in 4 and 6 weeks concentrate-fed sheep). These findings could be explained by Lavker et al. (1969); a PAS-positive reaction has been noted in the area of the rumen epithelium, where membrane-coated granules occurred. This indicates that these granules contain mucous and the cells of the stratum spinosum are involved in the synthesis of mucopolysaccharides. A similar PAS-positive reaction has been observed in a keratinizing epithelium like the esophageal epithelium of the mouse (Parakkal, 1967), where spiny cells contains numerous mucous granules and filaments. The periodic Acid-Schiff-reactive material, which was removed by digestion with diastase, was glycogen and it was not observed in the mucosa of all sheep groups.

4.3 Ultrastructure

In response to the type of feeding regime and its duration, the present study showed ultrastructural differences between the different experimental groups of sheep.

Lamina propria: In 4-12 weeks concentrate-fed groups, the collagenous fibres establishing the lamina propria markedly increased compared to that of hay-fed sheep. As reported before (McGavin and Morrill, 1976), the increase in the collagenous fibres is correlated with the increase in the blood supply. Added to that, the vascular system has been found to be located in close proximity to the basal epithelial cells (0.04-0.80 μm) in 4-12 weeks concentrate-fed groups when compared to that of hay-fed sheep (0.72-2.0 μm). Decreasing distance between basal epithelial cells and capillaries may lead to increase ion exchange and the transport capacity of nutrients.

Stratum basale: The stratum basale of the rumen epithelium revealed distinct morphological changes in the basal cells at various time intervals after the addition of concentrate to the hay diet. These changes include transforming of the shape of the basal cells from cuboidal or short columnar in hay-fed sheep and 2 weeks concentrate-fed groups to large pyramidal or columnar in 4-12 weeks concentrate-fed groups. This transforming of the shape of the basal cells was found to be accompanied by increase in the size of nucleolus, density of mitochondria and other metabolic organelles. They contain high number of mitochondria compared to cells of the stratum spinosum or stratum granulosum, this may be due to the energy requirements of primary active Na^+-K^+-ATPase (Graham and Simmons, 2005). Basal cells contained abundant metabolic organelles and numerous large vesicles, which are involved in the assimilation and metabolism of products absorbed from the rumen (Lavker et al., 1969). Moreover, it has been indicated that the high intake of concentrate stimulates increase cell metabolism in stratum basale (Tamate and Kikuchi, 1978). Thus, the increase in the mitochondria and other organelles in response to feeding concentrate diet or increase the duration of concentrate feeding, may serve to provide sufficient energy, which is required to enhance the active (Na^+-K^+-ATPase) or secondary active transport mechanisms of different nutrients.

On the other hand, Warner and Stacy (1972) reported that due to the increase in the osmolarity of the ruminal contents, water is no longer absorbed from the rumen, but in fact moves from the blood to the lumen down an osmotic gradient. This movement of water from the basal side to the luminal side of the rumen could explain the transitory swelling of the basal cells of 12 weeks concentrate-fed sheep.

The present study showed that the convoluted proximal membranes of the basal cells of hay-fed sheep converted to numerous basal labyrinths in 4-6 weeks concentrate-fed groups and in 12 weeks concentrate-fed group, these basal labyrinths were less developed. This transformation of the basal membrane was found to be accompanied by deep folded basal

lamina. The increase in the surface area of the proximal portion of the basal cells is suggested in sheep rumen (Henrikson, 1970b), goat rumen epithelium (Hyden and Sperber, 1965) and in other tissues (Pease, 1956). These authors speculated that this modification in structure increases the passage of molecules through the basal cell layer. Additionally, Lavker et al (1969) reported that the basal lamina, proximal portion of the basal cells and close proximity of the vascular system to the epithelium tissue are important in the absorptive function of the rumen. The micropinocytotic vesicles along the proximal portion of basal cell membrane, and the tenuous endothelium containing micropinocytotic vesicles too, provide further evidence for an active nutrient exchange between the basal cells and the circulatory system (Hyden and Sperber, 1965). Moreover, the increase of the microvillus-like projections of the basal cells cytoplasm is found to be linked to feeding concentrate; return to hay after feeding barley diet causes shrinkage and regression of these projections (Tamate and Fell, 1977). Thus, the development of extensive proximal projections of the basal cells accompanied by a deeply folded basal lamina and the presence of the blood capillaries located near the basal lamina, as it has been seen in 4 and 6 weeks concentrate-fed groups, may increase the surface area of the proximal portion of the basal cells and improve nutrient exchange capability between epithelial tissue and the circulatory system.

In the present study, very thick tonofibril bundles present in the proximal part of the basal cells were found in 6-12 weeks concentrate-fed groups. In addition to that, lipid droplets of various sizes were found in the cytoplasm of all epithelial strata of 12 weeks concentrate-fed group. These findings are consistent to what the finding reported by Tamate and Kikuchi (1978), who concluded that it could be a characteristic feature of rumen parakeratosis.

Stratum spinosum: Cells in the stratum spinosum of the concentrate-fed groups contained more abundant metabolic organelles and membrane coating granules compared to that of hay-fed sheep. The membrane coating granules, which were clearly found in 4 weeks concentrate-fed group, were more concentrated at the upper surface of the most superficial spiny cells and lower part of deep granular cells. The presence of abundant metabolic organelles and membrane coating granules may be due to the increase in transport capacity and cell metabolism as a result of high intake of concentrate.

Furthermore, thin tonofibril bundles (0.08-0.10 μm thick) present in hay-fed sheep was markedly increased in thickness (0.08-0.32 μm thick) with strong attachment to desmosomes in 6 and 12 weeks concentrate-fed groups. In 12 weeks concentrate-fed group, spiny cells present lipid droplets and small sized-keratohyaline granules. Additionally, foreign body lesions (microabscess) were also found between spiny cells.

Importantly, T.E.M study on parakeratotic rumen epithelium in cattle revealed the presence of tonofibrillar bundles in stratum basale and stratum spinosum (more pronounced) and lipid droplets throughout the epithelium, which are larger and more abundant in stratum corneum (Tamate and Kikuchi, 1978). It is well established that bundles of filaments are characteristic of keratinizing cells and eventually become a main constituent of the terminal horny cells (Rhodin and Reith, 1962). These filaments are present in the stratum basale, their aggregation into tonofilaments occurs mainly in the stratum spinosum (intre-mediate layer) (Hyden and Sperber, 1965; Lindhe and Sperber, 1959; Rhodin and Reith, 1962). The presence of both thick tonofibrillar bundles (in stratum basale and stratum spinosum), and small sized-keratohyaline granules rich stratum spinosum as it has been revealed in 12 weeks concentrate-fed sheep can be explained by the suggestion made by Bullough (1967) and Tamate and Kikuchi (1978), that the keratin synthesis might be complete in these strata and the rate of post-mitotic aging was much faster in parakeratotic rumen epithelium.

Stratum granulosum: Feeding concentrate for 4-12 weeks, revealed granular cells, which developed aggregates of large keratohyalin granules (0.40-5.50 μm in diameter) and cytoplasmic keratin aggregates compared to those present in hay-fed sheep (0.5-0.8 μm in diameter). Moreover, the appearance of the granular cells rich in degenerating mitochondria, thick tonofilament bundles which were peripherally arranged and in close association with desmosomes and keratohyalin granules and accumulation of small lipid droplets more pronounced in 1-6 weeks concentrate-fed groups.

In electron microscopic studies on the stratum granulosum of goat rumen epithelium, opaque bodies in close contact with bundles of filaments were observed by Hyden and Sperber (1965). These keratohyalin granules are specific differentiation product of the keratinizing cells and eventually constitute a major part of the horny product (Matoltsy, 1962). Thus, increased size of keratohyalin granules, thickness of tonofilament bundles and cytoplasmic keratin aggregates, which were observed in 4-12 weeks concentrate-fed groups, could mean a rapid transformation of granular cells into horn ones.

In hay-fed sheep, the membrane-coated vesicles appeared similar to those described in the spiny cells and were numerous and regularly observed in the peripheral cytoplasm of granular cells. In all living strata (stratum basale, spinosum and granulosum), the membrane coating granules or vesicles (MCG) increased in numbers with the intake of concentrate feeding. Hence, these MCG could possibly serve in storage and transport of metabolites between the lumen of the rumen and the circulatory system.

In 12 weeks concentrate-fed group, swelling of the area of the cytoplasm of the most superficial granular cells by large lipid droplets and the presence of very thick tonifilaments bundles appears to be characteristic of parakeratosis as mentioned before by Tamate and Kikuchi (1978).

Stratum corneum: It was characterized by several layers of electron dense cells. Some of which were plate-shaped and electron-opaque (horn cells type A and B) and others were distended by a central vacuole (voluminous balloon-shaped type C). The outer lamina that present between horn cells was covered by fine projections (cellular like-processes) which were covered by amorphous fuzzy coatings (glycocalyx). In response to concentrate diet, these fine projections were increased gradually in size and modified to form finger-like projections, reaching its maximum size within 4 to 6 weeks of concentrate feeding.

The fuzzy-coated finger-like projections of horn cells which appears similar to those seen in keratinizing epithelium of the mouse esophagous (Parakkal, 1967) and frog epidermal horn cells (Parakkal and Matoltsy, 1964) represent attachment sites of desmosomes (Scott and Gardner, 1973). Histochemical test suggested that the outer coat of the horny cells of rumen mucosa contains mucopolysaccharides (Lavker et al., 1969). It is likely that this fuzzy coat is an aid in the protective mechanism of horn cells (Lavker et al., 1969) or might influence the movement of substances through the intercellular space (Henrikson, 1970b). It has been suggested that partial occlusion of the intercellular space by the glycocalyx is important for the formation of a diffusion barrier in the epithelium, which could be a necessary component in the mechanisms of active transport of sodium across the epithelium (Henrikson and Stacy, 1971). Differentiation of glycocalyx, as observed in 4-6 weeks concentrate-fed groups, is indicative of the developing transport function of the epithelium (Henrikson, 1970a).

Moreover, in 6 weeks concentrate-fed group, horn cells showed lysosome-like structures. These cavities were lined by a membrane and often covered a complex system of projections. This may be due to bacteria embedded the horn cells surface.

In 12 weeks concentrate-fed group, lipid droplets were present throughout the epithelium and they were large and abundant in stratum corneum. Thick layer of flattened, nucleated horn cells also existed and many areas of the stratum corneum were detached from the underlying tissue. Moreover, the luminal surface of this stratum showed regression of the cytoplasmic projections. The presence of nuclei and the large lipid droplets throughout the horn cells of the rumen epithelium, as it has been seen in 12 weeks concentrate-fed group, appears to be a characteristic of parakeratosis (Lavker et al., 1969). Thus, regression of the cytoplasmic

projections covered the horn cells may be due to the regression of the transport function of the epithelium.

Intercellular relationships: In hay-fed sheep, the intercellular space surrounding the basal cells and the deep layer of the spiny cells were dilated. However, the width of the intercellular space was very much reduced at the luminal level of the epithelium. In stratum granulosum, the intercellular "bridges" of stratum spinosum disappeared and the cell surface became increased many times in size by the deep interdigitations. The formed deep interdigitations between the cells ultimately interlock the outer part of the epithelium into a cohesive and protective stratum corneum (Lavker and Matoltsy, 1970). Points of membrane fusion or tight-junctions are found throughout the epithelium from the basal layer to the superficial horn cells. Generally, the tight-junctions between horn cells are smaller and less numerous than those found deeper in the epithelium (Henrikson, 1970b), which is consistent with the present findings.

Concentrate-feeding for 4-12 weeks caused widening of the intercellular spaces in all epithelium-strata which was more obvious at both stratum basale and spinosum. The intercellular "bridges" of stratum spinosum were branched and carrying large number of desmosomes. In 12 weeks concentrate-fed group, the intercellular space was very wide compared to that of all concentrate-fed groups. Moreover, the cell junctions between the stratum granulosum and the stratum corneum and within horn cells were disrupted.

Gemmel and Stacy (1973) reported that the intercellular spaces of the stratum basale become larger due to a moderate increase in ruminal osmolarity. The change in appearance of the spaces suggests that sodium is being pumped out of the cells in the stratum basale at a faster rate than usual; this increase in the transport rate could also account for the rise in potential difference. Thus, it is possible that the intake of concentrate at different time intervals caused hypertonicity of the ruminal contents, which could lead to the widening of the intercellular spaces. On the other hand, the increased number of gap-junctions and desmosomes lined the wide intercellular spaces, which was more observed in the stratum spinosum due to increasing the duration of concentrate feeding, may function to facilitate absorption from the intercellular spaces (Hyden and Sperber, 1965; Lavker et al., 1969). Desmosomes have been reported in many epithelia, as exceedingly important in relation to the adhesion to the different cell layers and the cellular differentiation in keratinizing epithelium (Staehelin, 1974). They eventually break down to allow the surface layer of stratum corneum to slough off leaving the former attachment sites as processes scattered over the surface of the cells (Scott and Gardner, 1973).

On the other hand, a barrier to the back diffusion and free movement of solutes along the intercellular spaces from the lumen to the blood may be formed by the narrow tortuous intercellular space, the polysaccharide coating on horn cells and the tight junctions (maculae occludentes) (Henrikson, 1970a) or the proximal horn cells and distal granular cells (Schnorr and Vollmerhaus, 1967b), or by the outermost layer of the stratum granulosum (Henrikson and Stacy, 1971; Gemmel and Stacy, 1973).

In the stratum corneum, the intercellular spaces between upper and lower horn cells adjacent to stratum granulosum (apical-basal intercellular spaces) showed gradual dilatation with more dense granular material in response to increasing the duration of concentrate feeding for 2 to 6 weeks compared to hay-fed group. As it has been reported by Schnorr et al. (1975), the amorphous materials occupied the intercellular spaces come from the degraded organelles of the granular cells, which undergo transformation process to horn cells. So, the increased density of these materials with the increasing the duration of the intake of concentrate, which was more pronounced after 6 weeks, may be due to increase number of lysosomes present in the stratum granulosum.

4.4 Immunohistochemistry

The muscularis mucosa in the rumen

Different studies reported the absence of muscularis mucosa in the rumen of domestic ruminants; however, they described the presence of smooth muscle cells and the presence of condensed fibrous layer (Krölling and Grau, 1960; Dellmann, 1971; Dellmann and Brown, 1987; Banks, 1981, 1993). Nevertheless, Taluja and Saigal (1987) reported the presence of distinct layer containing smooth muscle cells in the ruminal mucosa of the Buffalo, which has been referred to as lamina muscularis mucosa. Recently, the condensed fibrous layer in the bovine ruminal mucosa has been detected immunohistochemically and ultrastructurally by Ikemizu et al. (1994) and Kitamura et al. (2003).

Using immunohistochemistry, the present study confirmed the existence of a specialized layer in the rumen mucosa of sheep, which composed of α smooth muscle actin-immunoreactive cells. A strong antibody reaction (thick layer) was found in 2 and 4 weeks concentrate-fed sheep. These cells were distributed as a condensed accumulation in the layer at the position equivalent to the muscularis mucosae.

Ikemizu et al. (1994) reported that, more abundant actin-immunoreactive cells can be detected in the ruminal papillae than in the interpapillar mucosae of cow, which is in the line with the present study. Actin is known for its contractile and cytoskeletal functions in various types of

cells (Vandekerckhov and Weber, 1978; Joyce et al., 1987). Thus, the condensed layer comprising these cells may play an important functional role in some physiological conditions. For example, the tension generated by the contraction of this layer may provide the supportive force for the ruminal papillae and acts as skeleton for it and helps to increase the efficiency of absorption (Ikemizu et al., 1994).

Numerous blood vessels, which showed intensive immunoreactivity for αSMA, were observed in 4 weeks concentrate-fed sheep compared to hay-fed as well as other groups of concentrate-fed animals. Increase number of blood capillaries could also aid absorption (McGavin and Morrill, 1976). αSMA and γSMA are the major actin isoforms of vascular and enteric smooth muscle cells, respectively (Vandekerckhov and Weber, 1978).

Connexin 43 (Cx43)

The present study showed that, plasma membrane connexin 43 immunostaining was most intense in the stratum basale and stratum spinosum (deep layers) and decreased in intensity through stratum spinosum (superficial layers) to stratum granulosum. Meanwhile, the stratum corneum was negative. The reaction around the cells gave a syncitial appearance with more apical-immunostaining concentration. The degree of antibody reaction was weak in hay-fed sheep and 2 days concentrate-fed sheep, moderate in 4 days and 1 week concentrate-fed sheep, strong in 2 weeks concentrate-fed sheep and very strong reaction in 4 to 12 weeks concentrate-fed sheep.

Gap junction communication mediated by the connexin gene family allows both cell-cell ionic coupling, that is, the intercellular passage of small ions such as potassium, and cell-cell biochemical coupling facilitating the sharing of small molecules (metabolites, sugars, lactate, butyrate, etc.) or intercellular signaling molecules (cyclic nucleotides) (Saez, et al, 2003; White, 2003). The key concept of the arrangement for multicellular-stratified epithelia capable of active sodium transport (such as in the frog skin) is that the epithelial barrier layers communicate with lower basal cell layers via low-resistance intercellular gap-junctional pathways (Mills et al, 1977). Graham and Simmons (2005) showed that connexin-43 is immunolocalised to the stratum granulosum, stratum spinosum and stratum basale of the bovine rumen epithelium and is consistent with the formation of a functional syncytium between these cells. Furthermore, Shahin and Blankemeyer (1989) studied the morphology and distribution of the intercellular junctions in isolated skin of Rana pipiens using various electron-microscopic techniques. They demonstrated the presence of gap junctions and suggest that the distribution of gap junctions is not homogeneous among the epithelial strata. They are less frequent in the stratum corneum and stratum granulosum than in the stratum

spinosum and stratum germinativum. The present findings are consistent with these finding; and a model of wide spread intercellular coupling, although the lower number of gap junctions in the stratum granulosum, suggested a possible deficiency in intercellular coupling. Gap junction channels are responsible for direct intercellular transfer of ions and small molecules including propagation of inositol trisphosphate-dependent calcium waves (Saez et al., 2003). Thus the increase in gap junctions, which were more pronounced in both stratum basale and deep stratum spinosum of 2-12 weeks concentrate-fed sheep, could be considered as adaptive mechanism for increasing the absorptive capacity of the rumen epithelium.

NHE3

Feeding concentrate diet causes excessive production of SCFA and as a consequence of the transport of these products the intracellular pH (pHi) could be sharply reduced. Therefore, efficient pH regulatory mechanism(s) are required to keep the intracellular pH within the physiological value; one of these mechanisms could be mediated by NHE. Recently, RT-PCR demonstrated expression of multiple NHE family members in the rumen tissue, including NHE1, NHE2, NHE3, and NHE8 (Graham et al., 2007). According to these authors, the immunostaining technique showed that NHE1 is predominantly localized to the stratum granulosum, with a progressive decrease toward the stratum basale. NHE2 immunostaining is observed mainly at an intracellular location in the stratum basale, stratum spinosum, and stratum granulose (Graham et al., 2007). Nevertheless, these authors did not mention the localization of NHE3. Schweigel et al., (2005) demonstrated the presence of mRNA for both NHE1 and NHE3 in the rumen.

The present findings showed that NHE3-immunostaining exists in almost all strata of the epithelium except stratum corneum, with more intense at both stratum granulosum (deep layer) and stratum spinosum (superficial layer), predominantly at the apical surface of the cells. This is consistent with many previous studies in other tissues, which reported the localization of NHE3 to be restricted to the apical membrane (Hoogerwerf et al., 1996; Biemesderfer et al., 1997). Because the stratum corneum is a dead layer, it showed negative reaction for this isoform. Nevertheless, the degree of antibody reaction was weak in hay fed-sheep and in all concentrate-fed groups, with exception of 2 and 4 weeks concentrate-fed groups, in which the degree of the antibody reaction was strong. The trafficking capability of NHE3 from the intracellular vesicles to the plasma membrane in response to challenge caused by reduction in the intracellular pH is well known (Kurashima et al., 1998; Chow et al., 1999). Furthermore, the enhancement of Na transport across the ruminal epithelia due to the presence of SCFA has been also reported, probably takes place by NHE (Gäbel et al., 1991;

Sehested et al., 1996). SCFA, in particularly butyrate, induces NHE3 activity and its protein and mRNA expression both in vivo and in vitro (Kiela et al., 2007). Therefore, the increase in the expression of NHE3 with increasing the duration of feeding concentrate could be due to the high concentration of SCFA. The transport capacity of the ruminal epithelia could reach its maximum and the continuous accumulation of SCFA could increase the osmolarity of the ruminal contents. Wakabayashi et al. (1997) found that NHE3 activity is decreased by the hyperosmolarity. Since, the hyperosmolarity decreases intracellular pH and inhibits Na^+ transport in the rumen, probably by inhibition of NHE3 (Schweigel et al., 2005), the weak antibody reaction observed in 6 and 12 weeks concentrate-fed animal can be justified in this context. The delay in response to feeding concentrate until 1 week (low antibody reaction against NHE3) could show that the expression of this isoform may require this period to be strongly expressed. This study is the first one which identifies NHE3 in the rumen using immuno-histochemistry technique.

Conclusion

As a conclusion, this study showed that the sheep rumen epithelium undergoes morphological (and functional) adaptation in response to feeding concentrate diet. According to the duration of feeding concentrate, some of adaptation events started in 2 days e. g. the increase in length and number of papillae. Most of the adaptation events were significantly established in 4-6 weeks. Some of these events have been already reported in the previous studies e. g. the increase in both number and size of papillae. However, the present study showed some new findings and the most significant ones can be summarized in:

1. Changes of the shape of papillae from small, tongue shaped papillae (hay-fed group) to large, heavily cornified, finger and foliate shaped papillae (4 and 6 weeks concentrate-fed groups, respectively).
2. The total surface of papillae increased in 2 days concentrate-fed sheep to 2 folds of that of hay-fed sheep and reached the maximum value (4 folds) within 4 weeks of concentrate feeding.
3. The mean thickness of the epithelium (μm) increased significantly after 1 week of concentrate feeding and reached the maximum value after 6 weeks of concentrate feeding.
4. The total number of the epithelial cells increased and showed well developed epithelial pegs within 2-6 weeks of concentrate feeding.
5. The increase of the intercellular spaces and the presence of a well developed desmosomes rich-stratum spinosum occurred within 4-12 weeks of concentrate feeding.
6. Well developed cytoplasmic protrusions covering the horn cells were observed in 4 and 6 weeks concentrate-fed groups.
7. Change of the shape of basal cells from short columnar (hay-fed group) to large pyramidal and the development of extensive proximal projections of the basal cells accompanied by deeply folded basal lamina and short distances-located blood capillaries were well observed within 4-6 weeks of feeding concentrate.
8. α-smooth muscle actin-immunoreactive cells were detected as condensed layer at the position equivalent to the Lamina muscularis mucosae in the rumen mucosa. Strong degree of antibody reaction (thick layer) was seen in 2-6 weeks concentrate-fed sheep.
9. Plasma membrane connexin 43 immunostaining was most intense at the stratum basale and stratum spinosum (deep layers) and the intensity of the staining decreased through stratum spinosum (superficial layers) to stratum granulosum. The degree of antibody reaction was weak in hay fed-sheep and in 2 days concentrate-fed sheep, moderate in 4 days and 1 week

concentrate-fed sheep, strong in 2 weeks concentrate-fed sheep and very strong in 4 to 12 weeks concentrate-fed sheep.

10. NHE3-immunostaining exists in all strata of the epithelium except stratum corneum. Its intensity increased at both stratum granulosum (deep layer) and stratum spinosum (superficial layer), predominantly at the apical surface of the cells. The degree of antibody reaction was weak in hay-fed sheep and in all concentrate-fed groups, except in 2 and 4 weeks concentrate-fed groups, in which the degree of the antibody reaction was strong.