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Scanning Electron Microscopical and Morphometrical Studies on Ruminal Papillae of Sheep Fed on Concentrates

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ABSTRACT

The objective of this study was to explore the time course of morphological alterations of rumen papillae after changing the diet from hay (*ad libitum*) to a mixed hay/concentrate diet. A total of 24 sheep were subjected to different periods of mixed hay/concentrate feeding ranging from 0 weeks (control; hay *ad libitum*) to 12 weeks (1-1.5 kg hay plus 780 g concentrate per day in two equal portions). Macro- and mesoscopic examinations, as well as scanning electron microscopical techniques were employed to study the rumen papillae of the different groups. Scanning electron microscopy (S.E.M) examination revealed that time and change of diet has greatly influenced ruminal papillae. This influence was expressed by the pronounced change of the papillae from small, tongue shaped when the animals were fed on hay to large, heavily cornified, finger_ and foliate_ shaped when fed on concentrates for 4-6 weeks. Morphometric analysis indicated that the increase in the length and number of papillae was also correlated to the duration of feeding concentrates for 4-6 weeks. The total surface of papillae increased in the 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. It is concluded that the most of the adaptation events were significantly established in 4-6 weeks; changes in 12 weeks were similar, but less developed

Keywords: Rumen papillae, Morphology, Sheep

The rumen is covered by stratified squamous epithelium that consists of leaf like papillae, which greatly increase the absorptive surface area (Steven and Marshall, 1970). These papillae vary in prominence according to age, diet and location (Scott and Gardner, 1973; Habel, 1975; Dyce *et al.*, 2002). A diet rich in concentrates is generally associated with high concentration of short chain fatty acids (SCFA) and, consequently, this will lead to increase in the transport of SCFA across the ruminal wall (Dirksen *et al.*, 1984; Gäbel *et al.*, 1987, 1991). This will eventually stimulate

some aspects of rumen morphologic and metabolic development, leading to greater development of rumen papillae than observed with diet based on forage (Weigand *et al.*, 1975; McGavin and Morrill, 1976; Goodlad, 1981; Lane and Jesse, 1997; Zitnan *et al.*, 1999; Swan and Groenewald, 2000; Dyce *et al.*, 2002). Absorption rates are likely to be further enhanced by morphological adaptation which takes approximately six weeks to reach peak levels (Etschmann *et al.*, 2009). The morphological and functional adaptations of the ruminal epithelium due to change in diet, are rapid (4-6 days or 2 to 3 weeks), and are particularly evident in atrium. These adaptations are not permanent but are actually reversible (Wardrop, 1961b; Palmquist and Ronning, 1963; McGavin and Morrill, 1976, Gäbel *et al.*, 1987). Little has been done quantitatively to estimate the effect of duration of feeding on the morphology of the rumen mucosa of sheep. Therefore, the objectives of this study are to characterize the effect of feeding concentrates on the morphology of the rumen papillae of sheep and to determine the exact time at which these changes occur after changing of the diet from hay ad libitum to hay plus concentrates.

MATERIALS AND METHODS

Experimental animals

total of 24 German dairy sheep of different sex were used in this study. Animals were 9-10 months old at the time of the experiment, and their weights ranged between 33.5-50 kg. The animals were divided into 8 groups of three animals in each.

Feedings

Prior to the experiment the sheep were fed only hay ad libitum for at least 8 weeks, in order to adapt them to a low-energy food. Thereafter, the sheep were either solely fed hay (1-1.5 kg hay/day) (one control-group) or received, in addition to that, 780 g concentrate (seven experimental-groups).

The seven experimental groups were assigned according to duration of feeding concentrates to 2, 4 days, 1, 2, 4, 6 and 12 weeks. Introduction of concentrate diet was preceded by an adaptation period of 4 days in which the concentrate feed was increased gradually. The concentrate diet was supplied in equal portions at 07.00 am and 02.30 pm. All animals had free access to tap water and salt block.

Composition of Diet

The nutrient content of both concentrate and hay rations were shown in tables 1 and 2 respectively.

Table 1: Composition of the concentrate diet

Nutrients	%	Nutrients	%
Dry matter	89.1	Organic ADF	13.28
Crude ash	6.42	Organic NDF	25.46
Crude protein	18.03	ADL	3.99
Crude fiber	9.65	Vitamin A	7200 Iu
Calcium	0.65	Vitamin D3	1800 Iu
Phosphorus	0.59	Selenium	0.5 mg
Magnesium	0.27	Copper	10 mg
Potassium	1.35	DCAB	+299 meq/kg DM
Sodium	0.42	Net energy lactation (NEL)	6.7 MJ/kg
Chloride	0.46	Metabolic energy (ME)	10.41 MJ/kg
Sulfur	0.21		

Table 2: Composition of hay diet

Nutrients	%	Nutrients	%
Dry matter	93.5	ADL	4
Crude ash	4.9	Non fibers carbohydrates (NFC)	22.5
Crude protein	8.8	Metabolizable energy (ME)	9.3 MJ/Kg
Crude fiber	29.3	Net energy lactation (NEL)	5.5 MJ/Kg
Potassium	1.44	Usable crude protein (nXP)	120.3 g/Kg
Sodium	0.032	Degradable crude protein (UDP)	17.6 g/Kg
Organic ADF	34	Ruminal nitrogen balance (RNB)	-5.2 g/Kg
Organic NDF	56.5		

Sampling and processing of ruminal papillae

Following the feeding trial and at the end of each experimental period, samples were obtained from identical site of the rumen (left wall of the recessus ruminis ventral sac; adjacent to the left longitudinal groove) (Gäbel *et al,* 1987). The ruminal mucosa was cleaned by immersion in a transportation buffer solution (see table 3). The mucosa was then stripped from the muscle layers and was sliced into sections and immersed in 4 % paraformaldehyde and Karnovsky's fixative solutions for both gross and electron microscopy.

Table 3: Composition of the transport buffer

Compound	Concentration (mmol / L)
Sodium	145.2
Potassium	5
Calcium	1
Magnesium	1
Bicarbonate	25
Chloride	120
Dihydrogenphosphate	0.4
Hydrogenphosphate	2.4
Glucose	5
pH value	7.4
Osmolarity	300 mosmol/L

For gross anatomy, sections (3 x 3 cm) of ruminal epithelium were fixed in 4% formalin and examined under a stereomicroscope to study the gross morphology of the ruminal papillae, including the colour, shape and clumping of the papillae. For scanning electron microscopy, small pieces of tissue (0.5 x 0.5 cm), were fixed in modified Karnovsky's fixative solution (Romeis, 1989), and postfixed with 1% Osmiumtetroxide (OsO_4) buffered with cacodylate (pH 7.2). After washing several times in cacodylate buffer (0.1 M), specimens were dehydrated through a graded series of ethanol alcohol, and dried using hexamethyldisilazane solution (HMD) (Co. Roth, Karlsruhe, Germany) and in a critical-point dryer (overnight). The specimens were mounted onto aluminum stubs with Leit-C glue (Co. Plano, Marburg, Germany) and sputter-coated with gold (30-40nm) for 2 min.

Scanning was carried out with scanning electron microscope (DSM 950, Co. Zeiss, Oberkochen, Germany) with an accelerating voltage of 10 kV. Samples were examined at magnification of 30 to 7500X. Photographs of the scanning electron microscope were taken in digital form directly to a personal computer.

For quantitative morphology of the ruminal papillae including the length, width and density, five papillae were selected randomly and were measured in each animal, so that a total of 120 papillae were measured (15 papillae per group). The morphometric procedure was carried out with a stereomicroscope and standard measuring slide at 10x magnification and the following morphometric parameters were measured:

1. Length of papilla (distance between the base and the tip of the papilla).
2. Width of papilla (at the middle of the papilla).
3. Density of papillae (number of papillae / cm^2 mucosa).
4. Total surface of papillae per cm^2 mucosa: was determined as length x width x 2, multiplied by the number of papillae / cm^2 mucosa (density of papillae).

Statistical analysis

The morphometrical data were prepared with the Excel program version XP (Co. Microsoft, Redmond / USA) and were finally statistically analyzed using SPSS program, version12.0 (Co. SPSS Software GmbH, München, Germany). Type and duration of feeding were considered as main effects. The statistical analyses were performed on the mean values per animal. The results were expressed as the mean average ± standard error. Values were analyzed by Analysis of Variances (ANOVA) procedure (statistical software STATGRAPHICS version 12, Statistical Graphics Cooperation / USA). We used a mixed model with animal as random factor and group as a fixed factor. In case where ANOVA was significant, Post-Hoc-tests (Scheffe) were carried out in order to study the significant differences among the distinct groups. Results were regarded to be significantly different at $P < 0.05$. The significant differences of the parameters of the rumen papillae were illustrated in a separate table. $^{A-h}$ Means in the same column and kind of sampling sharing the same superscript letters differ significantly.

RESULTS AND DISCUSSION

Colour of the ruminal papillae

The present study revealed that changes in colour of the papillae were diet-dependent. Papillae from hay-fed sheep or sheep fed concentrate up to 2 weeks were light brown in colour. However, papillae with dark brown colour were observed in 6 and 12 weeks concentrate-fed groups. Interestingly, 4 weeks concentrate-fed group showed papillae with both light and dark brown colours.

Shape and Size of the ruminal papillae

The present study showed a remarkable effect of the diet and the duration of the concentrate feeding on both shape and size of the papillae as demonstrated by stereomicroscope and scanning electron microscopy. The variations in shape of papillae were clear among the different groups (Fig. 1 and 2). A complete sequence of transitional forms ranging from small, flattened tongue-shaped papillae present in the rumen of hay-fed group to tongue-rounded finger-like papillae present in 2, 4 days concentrate-fed sheep were observed. Al though in 1 week concentrate-fed sheep, the papillae resembled those of 2 and 4 days concentrate-fed groups, being rounded finger-like papillae. Yet, they were thicker. The rumen of two weeks concentrate-fed sheep has displayed small, thick and irregular-shaped papillae. A large, heavily keratinized, finger-, foliate- -shaped papillae were present in the rumen of 4 and 6 or 12 weeks concentrate-fed sheep, respectively.

Scanning electron microscopy revealed that the surface of papillae was not smooth; it possessed one shallow longitudinal groove in hay-fed sheep. Changing the diet and duration of feeding concentrates have altered or modified this groove in one way or another. In 2 and 4 days, the single longitudinal groove was doubled and became deeper. In 2 weeks, the two major longitudinal grooves were much deeper.

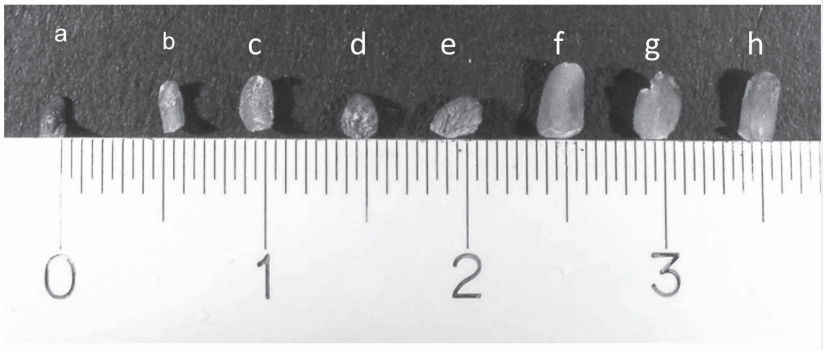

Fig. 1: Photograph illustrating changes in shape and size of sheep-ruminal papillae in relation to the type and the duration of feeding. a. Hay-fed group b. 2 days concentrate-fed group c. 4 days concentrate-fed group d.1 week concentrate-fed group e. 2 weeks concentrate-fed group f. 4 weeks concentrate-fed group g. 6 weeks concentrate-fed group h. 12 weeks concentrate-fed group.

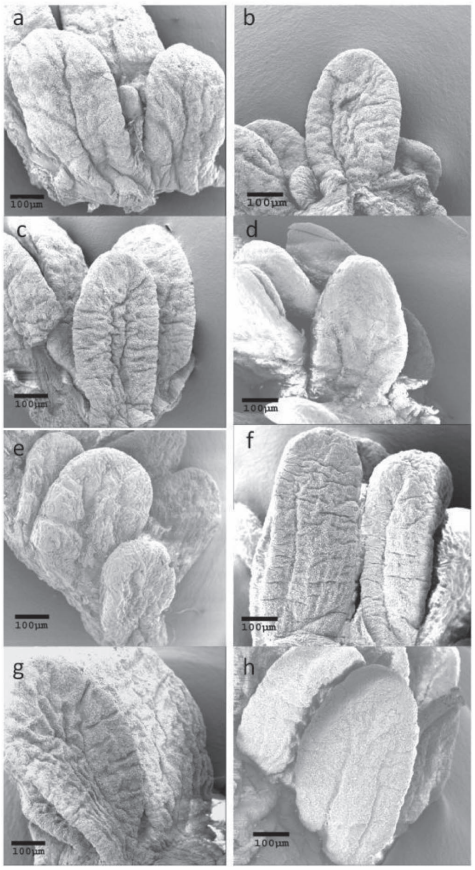

Fig. 2: S.E.M photograph shows the ruminal papillae isolated from hay and concentrate fed-sheep a. Hay-fed group b. 2 days concentrate-fed group c. 4 days concentrate-fed group d.1 week concentrate-fed group e. 2 weeks concentrate-fed group f. 4 weeks concentrate-fed group g. 6 weeks concentrate-fed group h. 12 weeks concentrate-fed group. (Scale 100µm)

In 4 weeks, the two major longitudinal grooves changed once again to one narrow groove but many short and deep transverse grooves have appeared. Finally, in 6-12 weeks, the grooves displayed a reticular form (Fig.2). 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 of the superficial horn cells (microvilli-like processes) which varied in their arrangement and degree of development in the different experimental sheep groups. Cellular protrusions were well developed and had different shape and arrangement in the form of foliate structures or more tortuous flap-like projections in sheep fed concentrate for 4-12 weeks compared to the less developed nipple-like projections which characterized the hay-fed sheep and sheep fed concentrate up to 2 weeks (Fig.3).

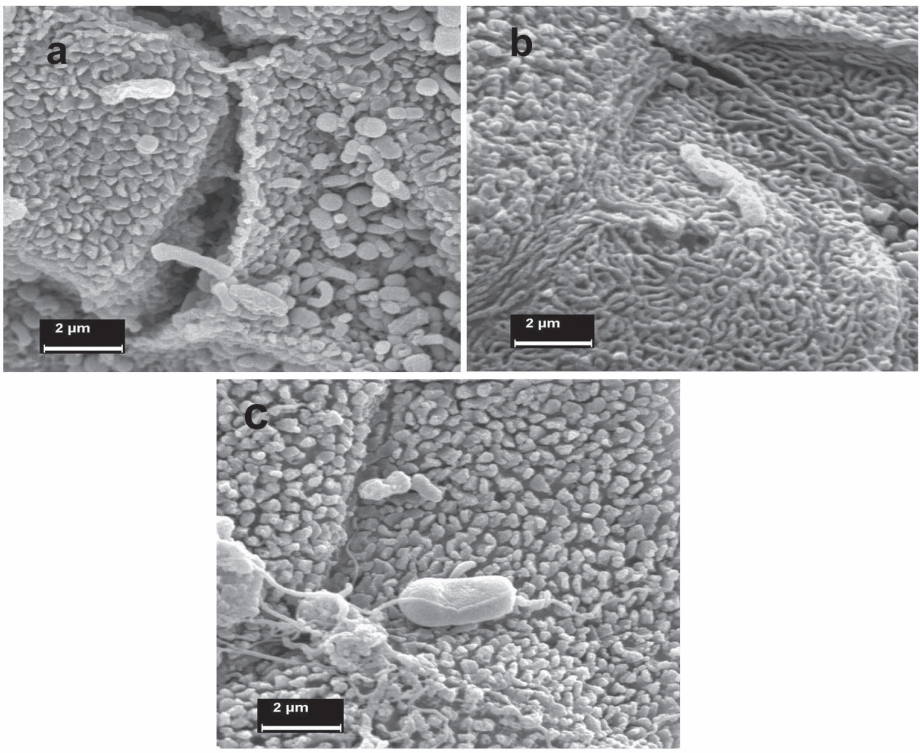

Fig. 3: S.E.M photographs of sheep ruminal papillae illustrating the changes in shape and size of cytoplasmic protrusions of horn cells in relation to the type and the duration of feeding (Scale 2µm) a. Hay-fed group b. 4 weeks concentrate-fed group c. 6&12 weeks concentrate-fed group.

Quantitative morphological analysis

Using quantitative morphological analysis, a remarkable effect of the diet and the duration of the concentrate feeding on the development of the ruminal papillae was observed (Fig.4 and tables 4 and 5). The adaptation of the rumen to concentrate feeding occurred rapidly within two days, where the length, density and total surface of papillae increased significantly ($P < 0.05$) compared to hay-fed sheep.

Table 4: Effects of the duration of concentrate feeding on the length, width and number of ruminal papillae (Mean ± SD)

Experimental groups of animals	Papillae		
	Length (mm)	Width (mm)	Number/ (cm^2) mucosa
CF0W	2.21 ± 0.40	1.77±0.2	38.67 ± 2.89
CF2D	3.1± 0.5	1.83 ± 0.18	64.33 ± 3.06
CF4D	3.23 ± 0.33	2.13 ± 0.18	51 ± 2
CF1W	2.50 ± 0.6	1.96 ± 0.43	63.33 ± 4.04
CF2W	2.61 ± 0.42	2.37 ± 0.32	50.33 ± 2.52
CF4W	4.67 ± 0.36	2.75 ± 0.42	48.33 ± 3.51
CF6W	3.89 ± 0.38	2.6 ± 0.21	49.33 ± 2.08
CF12W	3.63 ± 0.29	2.55 ± 0.37	51.33±1.53

Table 5: Significant differences of the length, width and number of ruminal papillae (Mean ± SD)

Experimental groups of animals	Significant letter	Significant -Length of papillae	Significant - Width of papillae	Significant - Number/ (cm^2) mucosa
CF0W	a	-	-	-
CF2D	b	a d e	-	a c e f g h
CF4D	c	a d e	a	a
CF1W	d	-	-	a c e f g h
CF2W	e	a	a b d	A
CF4W	f	a b c d e g h	a b c d e	-
CF6W	g	a b c d e	a b c d	A
CF12W	h	a b c d e	a b c d	A

[a-h] Means in the same column and kind of sampling sharing the same superscript letters differ significant

Length and width of the papillae

Among concentrate-fed groups, the mean length and width of the papillae of 4 weeks concentrate-fed group were strikingly elevated (4.67 mm, 2.75 mm), compared to that of hay-fed group (2.21 mm, 1.77 mm). The mean of the total surface of papillae increased significantly in 2 days concentrate-fed sheep (308.21 mm^2 / cm^2 mucosa) to 2 folds of that of hay-fed sheep (734.86 mm^2/cm^2 mucosa). It reached the maximum value in 4 weeks concentrate-fed group (1248.44 mm^2 / cm^2 mucosa), which was about 4 folds that of the hay-fed group (Fig.4).

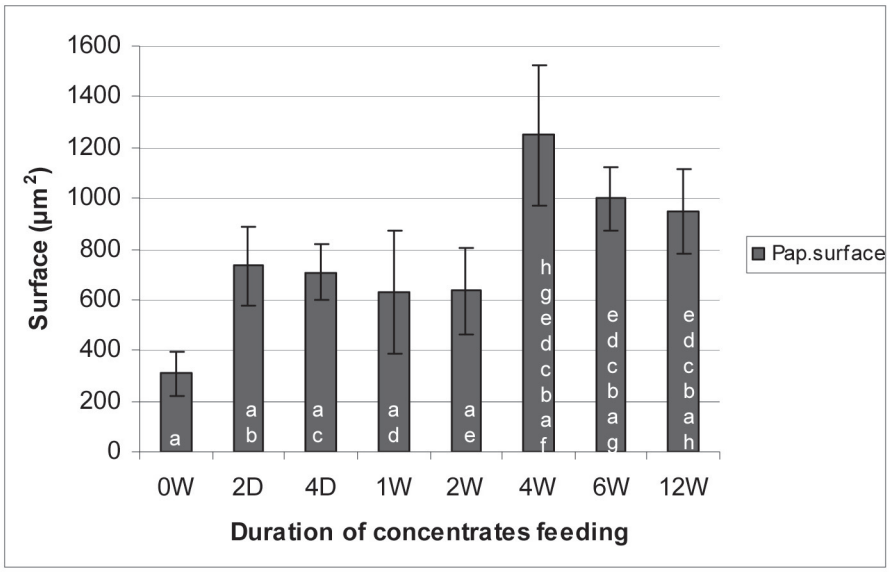

Fig. 4: Total surface of ruminal papillae from different sheep groups (fed hay only or hay plus concentrate feeding for different periods).
[a-h] Means in the same column and kind of sampling sharing the same superscript letters differ significantly.

Number of papillae

The number of papillae increased significantly ($P < 0.05$) in concentrate-fed groups compared to that of hay-fed ones (tables 4 and 5). In 4 weeks concentrate-fed group the number of papillae was 48 papillae / cm^2 mucosa, the difference was not significant eventhough the number was higher than that of hay-fed group (38 papillae / cm^2 mucosa). On the other hand, among concentrate-fed groups, the decrease in the number of papillae per cm^2 mucosa with increasing the duration of concentrate feeding was clearly observed.

This study showed for the first time that complete sequence of transitional forms of papillae are under the influence of the change of diet and duration of feeding from small, tongue shaped papillae (hay-fed group) to large, heavily cornified, finger and foliate shaped ones (4 to 12 weeks concentrate-fed groups).

Generally, the development and growth of ruminal papillae are modulated by diet due to mechanical stimuli provided by the roughage volume, chemical stimuli caused by short chain fatty acids, age of the animal and the time of weaning (Flatt *et al.*, 1958; Loe *et al.*, 1959; Harrison *et al.*, 1960; Stobo *et al.*, 1966; Banks, 1986; Anderson *et al.*, 1987; Franco *et al.*, 1992; Zitnan *et al.*, 1999; Swan and Groenewald, 2000). On the other hand, 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 a lesser extent, 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 increasing absorptive capacity. Scott and Gardner (1973) found that the papillae are largest and most dense in the ventral and cranial sacs of the rumen, region where the papillae are exposed to the highest concentrations of soluble nutrients. In this context, the sequential changes from tongue to finger or foliate 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).

The surface of the papillae of 4-12 weeks concentrate-fed groups showed deep and numerous grooves compared to the few shallow ones present on the surface of papillae of hay-fed group. These deep grooves could increase the surface of papillae and hold back the ingesta for a longer time, hence, increasing the absorptive capacity of the epithelium. At high magnification, the epithelial cells have distinctly granular appearance due to the presence of a covering of cytoplasmic protrusions. These were well developed and had different shape and arrangement (foliate structure or more tortuous flap-like projections) in sheep fed concentrate for 4-12 weeks compared to the less developed nipple-like projections, which characterized hay fed-sheep. Evidence was presented that these cellular protrusions represent attachment sites of desmosomes (Kligman, 1964; Scott and Gardner, 1973) and their development are affected by the nutritional regimes (McGavin and Morrill, 1976; Yamamoto *et al.*, 1994). Thus, these cellular protrusions might increase the total absorptive surface of the rumen epithelium.

In addition to changes in the shape, this study also revealed diet-dependent changes in colour of the papillae. Papillae from hay-fed sheep or sheep fed concentrate for 2 weeks exhibited light brown colour. However, dark brown coloured papillae were observed in 4, 6 and 12 weeks concentrate-fed groups. The dark brown colour of papillae appears to be a combination of keratinized tissue, (resulting from rapid growth and limited abrasion), high supply of iron, and an acid pH (Nockels *et al.*, 1966), or represents products of microbial activity (Sinclair and Kunkel, 1959). Contrary to this notion, Nockels et al. (1966) have stated that the colour of ruminal papillae remain light eventhough the sheep received high amounts of SCFA.

Morphometrical evaluation used in this study revealed a remarkable effect of the diet and the duration of the concentrate feeding on the development of the ruminal papillae. 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 that of hay-fed sheep. However, 4 weeks concentrate-fed group represented the maximum time of most significant structural differences among the different concentrate-fed groups. The mean length and width of the papillae of 4 weeks concentrate-fed group were more than 2 and 1.5 times the papillary length

and width, respectively, of that of the hay-fed group. Thus this enlargement of papillae leads to an increase in the total surface of papillae. The total surface of papillae increased significantly after 2 days of concentrate feeding (2 folds of that of hay-fed sheep) and reached the maximum value (4 folds) within 4 weeks of concentrate feeding. These observations are in accordance with those of Zitnan et al. (1999) who found that the nutritional regimes affect the morphometrical development of the mucosa. Goodlad (1981) stated that differences, which were attributed to the SCFA, can be only observed for a couple of days after onset of feeding and it was explained by a transient decrease in the cell cycle duration. 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 ruminal epithelium (Mentschel *et al.*, 2001).

The number of papillae per cm^2 mucosa increased significantly in concentrate-fed group (except in 4 weeks concentrate-fed group) compared to that of hay-fed ones. Among concentrate-fed groups, the number of papillae per cm^2 mucosa decreased with increasing the duration of concentrate feeding. 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 reduction in the number might be due to increase in thickness of individual papillae, thus, accommodating fewer papillae per unit area (Tiwari and Jamdar, 1970a).

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