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## Effect of 24 hours light on circadian rhythms of secretory enzymes and morphology of rat von Ebner's glands

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### Abstract

Von Ebner's glands of the rat are minor salivary serous glands in the posterior portion of the tongue. They secrete two digestive enzymes, lingual lipase and amylase. In this investigation, circadian rhythm in feeding was established under a normal 12 h light/12 h dark cycle, with the rats eating primarily during the dark period. At lights on, the size of the acinar cells and the area of the inclusive secretory granules, and the amount of digestive enzyme activity (lingual lipase and amylase) remaining in the gland was significantly less than in the mid-afternoon, after very little daylight food consumption. However, after 7 days of continuous light the circadian rhythm was altered: the food consumption during the normal night-time hours (5 p.m. to 8 a.m.) went from 88% of total 24 h food consumption to 45%, and during normal daylight hours (8 a.m. to 5 p.m.) from 12% to 55%. These changes were correlated with histometric findings of a near reversal of the areas of acinar cells and secretory granules of a.m. and p.m. samples under continuous light. Lingual lipase activity in the glands went from 35% under 12 h light to 61% under continuous light in the a.m. and from 65% to 39% in the p.m. Amylase activity also showed nearly a reversal in activity remaining in the gland, from 36% at 12 h light to 58% at 24 h light in the a.m. and 64% to 41% for the p.m. samples. These results indicate that the von Ebner's glands of the rat have a circadian rhythm of secretion and storage of secretory proteins that is subject to light entrainment similar to that seen in other exocrine glands such as the parotid and pancreas. Published by Elsevier Science Ltd.

*Keywords:* Rat; Circadian rhythm; von Ebner's glands; Histometrics; Morphology; Acinar cells; Secretory granules; Lingual lipase; Amylase

### 1. Introduction

Circadian rhythm is the daily, 24 h, repetition of various functions of living organisms that relate to the light–dark cycle produced by the rotation of the earth. This rhythm is endogenous, but can be entrained to a different cycle by the environment, e.g., changes in the light cycle (Johnson, 1992). The biological clock or cir-

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adian rhythm has been studied in eukaryotic microorganisms, plants, insects, and mammals (Aschoff, 1960; Rusak and Zucker, 1979; Edmunds, 1988). The biochemical mechanism of the biological clock is of fundamental importance in the regulation of many cellular systems (Morse et al., 1990). Although some progress has been made in understanding the mechanism of the biological clock, it is not yet completely understood.

Circadian rhythm is exhibited by the secretory pathway in exocrine glands in the rat. Sreebny and Johnson (1969) were the first to show that there is a diurnal variation in the secretion and storage of secretory proteins in the parotid gland of the rat. Rats are nocturnal animals that feed mostly during the night and rest during the daylight hours. The exocrine glands discharge the contents of secretory granules during feeding and there is a substantial decrease in the glandular stores of secretory proteins by the end of the night. The partial depletion of these proteins triggers an increase in the rate of synthesis. During the day, when the secretory protein content has been restored, the rate of synthesis decreases (Sreebny et al., 1971). Morphological studies of acinar cells of rat parotid glands revealed time-dependent changes in the structures of acinar cells (Albegger et al., 1975, 1977). The maximum volume of the cells and the secretory granules occurs in the evening at the start of the eating cycle and the minimum volume is seen in the morning.

In the rat pancreas, secretory granules and other organelles involved in the secretory process are decreased in volume during the dark or feeding period and the volume of secretory granules is restored during the first hours of the light period (Müller and Gerber, 1985). Exocrine pancreatic secretion in rats also has a circadian rhythm, with secretory rates increasing in the dark period and decreasing during the light period (Maouyo et al., 1993).

von Ebner's glands, also known as lingual serous glands, are exocrine glands in the oral cavity. They are minor salivary glands embedded in the muscle tissue of the tongue beneath the vallate papillae and foliate furrows (Hand, 1970; Hand et al., 1999). These glands secrete two digestive enzymes, lingual lipase and lingual amylase. Lingual lipase digests triacylglycerols at the acid pH of the stomach, producing diacyl- and monoacylglycerols and fatty acids. This preduodenal fat digestion is especially important when there is pancreatic insufficiency, as in the premature and neonatal animal, or in disease states such as cystic fibrosis (Field and Scow, 1983; Field and Hand, 1987; Field et al., 1989). von Ebner's glands differ from other minor salivary glands in that they are not muco- or mucoserous glands but serous glands in most species (Hand et al., 1999; see Nalavade and Varute, 1972 for exceptions). In addition, regulation of protein secretion from these

glands is similar to the pancreas and different from the parotid gland. Major protein secretion in von Ebner's glands (Field and Hand, 1987) and the pancreas (Peikin et al., 1979) is stimulated by cholinergic agonists, whereas in the parotid gland proteins are secreted after  $\beta$ -adrenergic stimulation (Johnson and Sreebny, 1973; Garrett and Thulin, 1975). To our knowledge there have been no reports on circadian rhythm in von Ebner's glands.

The purpose of the current work is to investigate the effects of the 12 h light/12 h dark cycle and constant light on the morphology of, and the secretion and storage of two digestive enzymes in, von Ebner's gland.

## 2. Methods and materials

### 2.1. Animals, light entrainment

For 1 week before the start of the experiment, male Sprague–Dawley rats, 7 weeks old,  $284 \pm 5$  g, were housed one animal per cage under controlled lighting conditions of either 12 h light/dark (lights on at 8 a.m. and off at 8 p.m.) or continuous light. The rats used in the light/dark cycle were from the breeding colony at the DVA Medical Center and were progeny of rats from Charles River Laboratories, Raleigh, NC, certified free of sialodacryodentitis and rat corona viruses. For the experiments with continuous light, virus-free rats were obtained from Harlan Sprague–Dawley Inc., Indianapolis, IN. For each experiment, three to six rats were anaesthetized by intraperitoneal injection with sodium nembutal (50 mg/kg), killed by exsanguination at 8 a.m. or 3 p.m., and their tongues removed. The total number of rats on which enzyme assays were performed was 17 and 18 from the continuous and light/dark experiments, respectively. The entire von Ebner's gland was dissected (0.05–0.10 g wet wt) from the tongue and each gland was frozen on dry ice. For morphological examination, portions of von Ebner's glands were separately dissected from near the circumvallate papilla and foliate furrows of 12 and 14 rats from the continuous and light/dark lighting groups, respectively, and placed in fixative. The excision of von Ebner's gland from the tongue was done with the aid of a dissecting microscope at  $\times 10$  magnification.

### 2.2. Food measurement

The rats were allowed access to a commercial pelleted diet and water ad libitum. Food was weighed at 8 a.m. and 5 p.m. on the day before killing, and again at 8 a.m. on the day of death. Care was taken to include pieces of food that had fallen into the cages. The food consumed during the period from 8 a.m. to 5 p.m. was considered to be the p.m. sample. The food

Table 1  
Effect of light entrainment (12-h light/12-h dark vs 24-h light) on the amount of food consumed

Light entrainment	Food consumed (g/rat/h)	
	a.m. (5 p.m.–8 a.m.)	p.m. (8 a.m.–5 p.m.)
12 h light/12 h dark (LD)	2.06 ± 0.06 <sup>e,a,c</sup>	0.47 ± 0.09 <sup>e,a,d</sup>
24 h light (LL)	0.95 ± 0.07 <sup>f,b,c</sup>	1.98 ± 0.12 <sup>f,b,d</sup>

<sup>a,b,c,d</sup> Data with the same letters indicate statistically significant differences,  $p = 0.0001$ , Student  $t$  test on the LD data, a.m. vs p.m.; the LL data, a.m. vs p.m.; a.m. data LD vs LL; p.m. data LD vs LL.

<sup>e</sup> Average food consumed by 23 rats from 5 p.m. to 8 a.m. for a.m. (total g = 711.90/15 h) and from 8 a.m. to 5 p.m. for p.m. (total g = 96.30/9 h). The data are expressed as g/rat/h ± SEM.

<sup>f</sup> Average food consumed by 21 rats from 5 p.m. to 8 a.m. for a.m. (total g = 301.20/15 h) and 22 rats from 8 a.m. to 5 p.m. for p.m. (total g = 392.58/9 h).

consumed from 5 p.m. to 8 a.m. was considered to be the a.m. sample. The data are expressed as grams of food eaten per hour per rat. The total weight (g) of food consumed and the total weight (g)/rat in each time period and light cycle are also presented. The percentage of the a.m. or p.m. samples of the total food eaten in 24 h/rat was calculated.

### 2.3. Morphology and histometrics

The samples were fixed in 4.0% glutaraldehyde in 0.1 M NaH<sub>2</sub>PO<sub>4</sub>, 0.15 M sucrose, 0.001 M CaCl<sub>2</sub>, and 2.5% dimethylsulphoxide, pH 7.4, then postfixed in osmium and embedded in EMbed 812, as previously described (Yu and Redman, 1990). Semithin sections (approx. 0.1 µm) were cut, mounted on glass slides and stained with methylene blue and Azur II (Richardson et al., 1960).

Histometric analysis was as previously described (Yu and Redman, 1990). In brief, one slide from each of two blocks from each gland area (vallate and foliate) of each animal was selected for analysis based on technical quality. Technical considerations in the selection of slides included the presence of a sufficient number of acini in the section, clarity of staining, and the absence of artefactual scattering of the secretory granules due to proximity to razor cuts. These selected slides were randomized and coded by a laboratory technician in order to ensure that all measurements were made blind. Areas of entire acinar cells and portions of these cells occupied by secretory granules were measured and tallied using Jandel's software SigmaScan by tracing the camera lucida images on a digitizing pad. The digitizing pad was calibrated to µm<sup>2</sup>. Ten acinar cells were measured on each slide. Only cells that both bordered a lumen and contained a nucleus were used. The average of the 20 cells (two slides per animal per gland area) so measured was the value used for comparisons.

### 2.4. Enzyme assays

Each frozen gland was homogenized in 3 ml glucose-free Tyrode's solution with a Polytron (Brinkmann Instruments, Westbury, NY) for 30 s. The generator was washed with 1 ml glucose-free Tyrode's solution. The homogenate was centrifuged at 850 g for 15 min at 4°C. The supernatant was divided into two tubes so that the sample was thawed only once before performing the lingual lipase and amylase assays. The samples were stored in a -70°C freezer.

### 2.5. Lingual lipase assay

The sample was thawed and diluted 1:50 with saline. Activity was determined by the method of Field and Scow (1983). A unit of lingual lipase activity is defined as a micromole of fatty acid produced per minute at 37°C.

### 2.6. Amylase assay

The sample was thawed and diluted 1:5 with pH 5.9 NaH<sub>2</sub>PO<sub>4</sub>/NaCl buffer used in the assay. Activity was determined by the method of Bernfeld (1955). A unit of amylase activity is defined as a milligram equivalent of maltose formed in 3 min at 30°C.

### 2.7. Chemicals

The radioisotopes used in the lingual lipase assay, tri [9,10-<sup>3</sup>H]oleoylglycerol and [9,10-<sup>3</sup>H]oleic acid, were from Dupont NEN Research Products, MA. All other chemicals were reagent grade.

### 2.8. Statistics

The Student  $t$ -test was performed using StatView for the data shown in Table 1 and in Fig. 2. Comparisons were made between a.m. and p.m. for each light

Table 2

Comparisons under differing light entrainment and time of death of the areas of cells and their secretory granules in rat von Ebner's gland vallate papillae and foliate furrows combined

Light entrainment	a.m. <sup>g</sup>	p.m. <sup>g</sup>
	<i>Area of cells (μm<sup>2</sup>)<sup>h</sup></i>	
12 h light/12 h dark	108.4 ± 2.8 <sup>a,c</sup>	122.5 ± 4.3 <sup>a</sup>
24 h light	136.3 ± 4.1 <sup>b,c</sup>	117.3 ± 6.9 <sup>b</sup>
	<i>Area of secretory granules (μm<sup>2</sup>)<sup>h</sup></i>	
12 h light/12 h dark	61.6 ± 4.9 <sup>d,f</sup>	83.8 ± 4.5 <sup>d</sup>
24 h light	98.8 ± 4.4 <sup>e,f</sup>	64.7 ± 9.3 <sup>e</sup>

<sup>a,b,c,d,e,f</sup> Data with the same letters indicate statistically significant differences,  $p < 0.05$ , Student–Newman–Keuls. There are no statistically significant differences between the areas of the cells or their secretory granules under 12-h cycled or 24-h light at the p.m. death.

<sup>g</sup> Rats were killed at 8 a.m. for a.m. samples and at 3 p.m. for p.m. samples.

<sup>h</sup> Average area (μm<sup>2</sup>) of 10 cells on each of four slides from seven rats subjected to 12 h cycled light and from six rats subjected to continuous light. Data are expressed as μm<sup>2</sup> ± SEM.

entrainment (light/dark or continuous light), and light/dark vs continuous light for a.m. or p.m. data. The data shown in Table 2 were evaluated by the Student–Newman–Keuls method using SigmaStat. Differences were considered statistically significant when  $p < 0.05$ .

### 3. Results

#### 3.1. Food measurement

As shown in Table 1, there were significant differences between the a.m. and p.m. rates of feeding under both lighting conditions and also between light/dark or continuous light for a.m. and p.m. measurements. The rats housed under the light/dark conditions consumed 711.9 g or 31.0 ± 0.9 g/rat in the 15 h from 5 p.m. to 8 a.m., or 88% of the food consumed in 24 h. However, after 7 days of continuous lighting, 299.9 g or 14.3 ± 1.0 g/rat was consumed during these hours or 45% of the food. Under light/dark conditions, 96.3 g or 4.2 ± 0.8 g/rat (12%) was consumed in the 9 h from 8 a.m. to 5 p.m. compared to 392.6 g or 17.8 ± 1.1 g/rat (55%) under continuous lighting conditions. The total amount of food consumed/rat in 24 h was similar under either condition, 35.1 ± 1.3 g for light/dark compared to 31.8 ± 1.1 g for continuous light,  $p = 0.063$ .

#### 3.2. Histometric measurement

The results of the histometric analysis of the areas of the cells and the secretory granules are shown in

Table 2. As there were no significant differences between the vallate papillae and foliate furrows, the data were combined and comparisons were made on the average of area estimates of both gland areas for 40 cells for each rat. The differences in both the areas of the cells and of the secretory granules between the a.m. and p.m. samples were significant under both lighting conditions. The differences in the areas of cells and secretory granules for the a.m. samples between light/dark and continuous light also were significant. However, for the p.m. samples, there was no significant difference between the two lighting conditions. These data reflect the changes in the feeding pattern brought about by the light changes. For the light/dark samples, the p.m. areas were greater than the a.m. areas for both cells and secretory granules. In the continuous-light samples, this order was reversed. The areas of the p.m. samples of light/dark and continuous light were now close to each other.

#### 3.3. Morphology

Photomicrographs of sections of lingual serous glands are seen in Fig. 1. As there were no significant differences between the vallate papillae and foliate furrows for the areas of the acinar cells and their secretory granules, the origin of the tissues was not considered when selecting the illustrations. In Fig. 1 we see that circadian rhythm is maintained when the cycle is light/dark. After a night of eating in the dark, the cells are small and depleted of secretory granules (Fig. 1a). During daylight non-eating hours, the area of secretory granules is increased, and the cells are enlarged (Fig. 1). The reverse is seen under continuous lighting conditions. The a.m. sections (Fig. 1c) have larger cells with more secretory granules than in the p.m. sections (Fig. 1d).

#### 3.4. Enzyme assays

Activities of lingual lipase and amylase remaining in the tissue at the time of death are shown in Fig. 2. The a.m. and p.m. data were averaged and these averages added together to get a value for the total enzyme activity in the gland at two specific times of the 24-h day. The percentage of each a.m. or p.m. datum of the total values were calculated. For lingual lipase, under light/dark the percentage of enzyme activity remaining in the glands at the a.m. sampling, 34.7 ± 2.6, was significantly different from the p.m. sample, 65.3 ± 4.2,  $p = 0.0001$ . The continuous-light samples also showed this difference, 60.9 ± 5.8%, a.m., 39.1 ± 3.3%, p.m.,  $p = 0.006$ . For amylase, under light/dark conditions the enzyme activity remaining was 36.2 ± 2.2% in the a.m. and 63.8 ± 5.8% in the p.m. samples,  $p = 0.0004$ . Under continuous lighting conditions, amylase activity

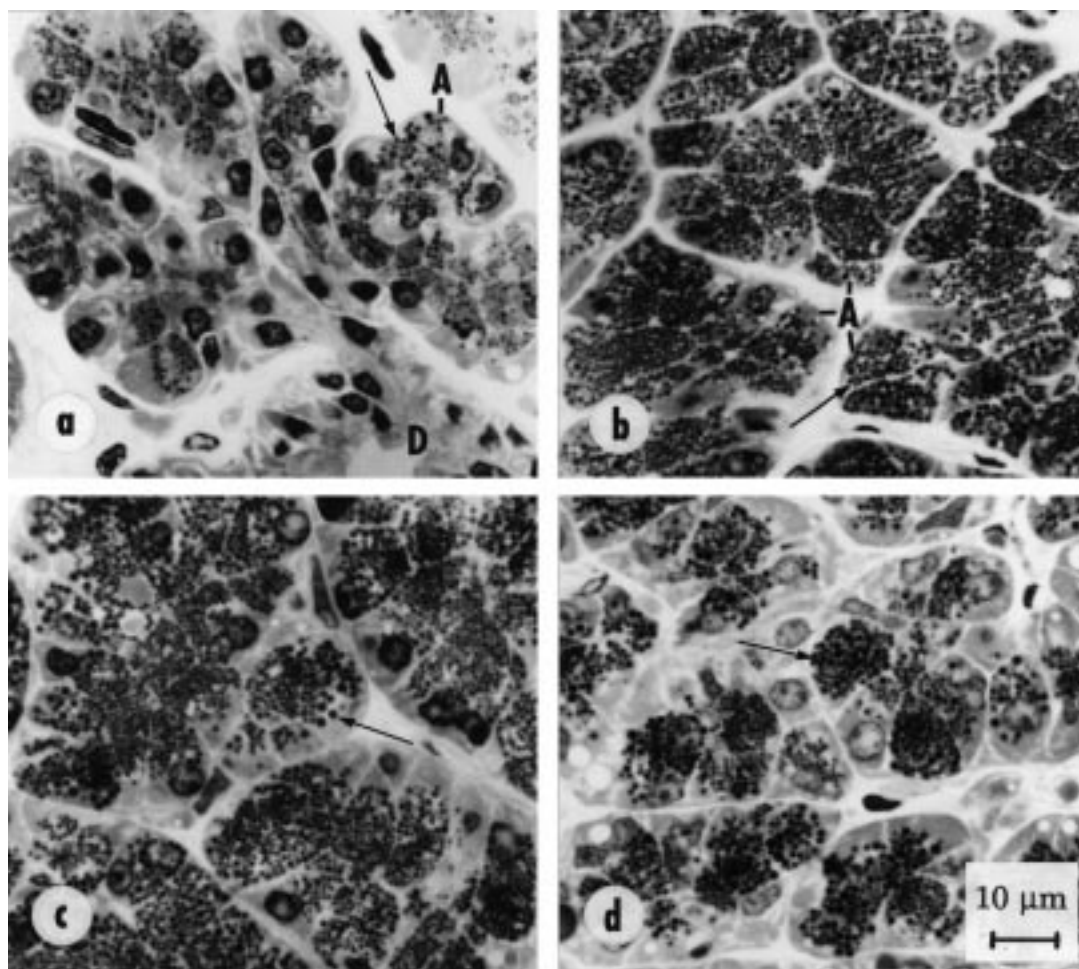


Fig. 1. Photomicrographs of epoxy resin sections of lingual serous glands of rats housed with lights on from 8:00 a.m. to 8:00 p.m. daily (a, b) or with lights on continuously for seven days before the termination of the experiment (c, d). Glands in (a) and (c) were associated with foliate furrows, while those in (b) and (d) were associated with vallate papillae. Rats were killed starting at 8:00 a.m. (a, c) or 3:00 p.m. (b, d). With the lights in a 12 h on/12 h off cycle, the acini (A) are smaller and contain fewer secretory granules (arrows) at 8:00 a.m. than at 3:00 p.m. The large duct (D) in (a) has a dilated lumen. After seven days of continuous light, the pattern is reversed. Methylene blue–azure II;  $\times 850$ .

remaining was  $58.3 \pm 4.5\%$  in the a.m. and  $41.7 \pm 3.3\%$  in the p.m. samples,  $p = 0.01$ . The a.m. samples under light/dark were significantly different from continuous light for lingual lipase,  $p = 0.0007$  and amylase,  $p = 0.0004$ . The p.m. samples under the different light conditions were significantly different,  $p = 0.0002$  and  $0.0062$  for lingual lipase and amylase, respectively. These results revealed that, under light/dark conditions, in the a.m. (after the dark period), enzyme activity in the gland has been depleted, however the p.m. enzyme activity has been replenished. Whereas, with continuous lighting, the reverse is seen, with more enzyme activity remaining in the a.m. than in the p.m.

#### 4. Discussion

Although von Ebner's glands differ from other minor glands in most species in that they are pure serous glands (Hand, 1970; Hand et al., 1999) and they differ from the parotid, a major exocrine salivary gland, in the regulation of protein secretion (Field and Hand, 1987), the results shown here indicate that they do resemble other exocrine glands with regard to light entrainment and circadian rhythm. These experiments show that changes in circadian (24-h periodicity) rhythm are entrained by light in rat von Ebner's gland. Continuous lighting affects the time of food consumption, and the discharge of the secretory granules con-

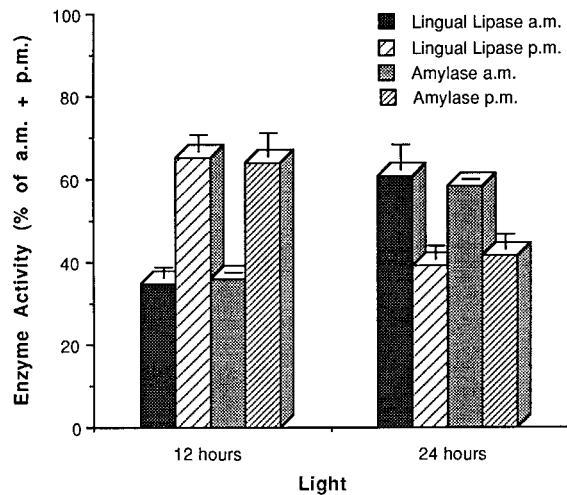


Fig. 2. Lingual lipase and amylase activities remaining in von Ebner's glands after 7 days of 12 h light/12 h dark or continuous lighting as % of a.m. + p.m. activities combined. The von Ebner's glands from rats that were killed in the a.m. under 12 h light/12 h dark conditions were depleted of enzyme activity, whereas those killed in the p.m. were replenished with enzyme. Almost the reverse was seen under continuous lighting. The enzyme activities expressed as units/gland are: lingual lipase 12 h light, a.m.  $24.2 \pm 1.8$  ( $n = 9$ ) vs p.m.  $45.4 \pm 2.9$  ( $n = 9$ ),  $p = 0.0001$ ; lingual lipase 24 h light, a.m.  $122.6 \pm 11.7$  ( $n = 8$ ) vs p.m.  $78.7 \pm 6.6$  ( $n = 8$ ),  $p = 0.006$ ; amylase 12 h light, a.m.  $162.5 \pm 9.8$  ( $n = 9$ ) vs p.m.  $286.0 \pm 25.9$  ( $n = 9$ ),  $p = 0.0004$ ; amylase 24 h light, a.m.  $309.3 \pm 23.9$  ( $n = 8$ ) vs p.m.  $221.7 \pm 18.0$  ( $n = 8$ ),  $p = 0.01$ .

taining enzyme activity of two digestive enzymes, lingual lipase and amylase. These events were observed by assays of enzyme activity remaining in the gland at 8 a.m. or 3 p.m. and changes in the morphology of von Ebner's gland at these times, under exposure to either light/dark or continuous-light conditions.

Under normal light conditions, rats eat in the dark and discharge their exocrine-gland secretory granules containing digestive enzymes during the eating period. The experiments described here confirm that this occurs in von Ebner's glands. These events have been seen in other exocrine glands such as the parotid (Sreebny and Johnson, 1969; Sreebny et al., 1971; Albegger et al., 1975, 1977) and the pancreas (Müller and Gerber, 1985; Maouyo et al., 1993). When the light was on 12 h and off 12 h, the rats consumed most of their food during the dark hours (Table 1). The acinar cells were smaller and the secretory granules occupied less space in the cells when the rats were killed at 8 a.m. than when they were killed at 3 p.m. At 3 p.m., the secretory granules have been restored due to protein synthesis and storage so that they will be ready to be released when needed during the dark feeding time.

However, when the light was on constantly the usual circadian rhythm was not maintained. There was more daytime eating under the conditions of continuous light than light/dark. The total amount of food consumed/rat in 24 h was similar under either condition (cf. Rosenwasser et al., 1981). Changes in morphology of the acinar cells and secretory granules and the enzyme activity of lingual lipase and amylase remaining in the glands were almost reversed, with large cells and more activity at 8 a.m. and less at 3 p.m. than was observed under light/dark conditions. These results correlate with the amount of food consumed from 5 p.m. to 8 a.m. or from 8 a.m. to 5 p.m.

The observed reversal in the rhythm of feeding and enzyme activity remaining in the gland and morphological changes after 7 days of constant light suggests that a 1.5 h/day shift in circadian rhythm occurred. However, we cannot determine from our experiments exactly what phase changes in circadian rhythm actually occurred in the 7 days of continuous illumination. Siegel (1961) observed that with a normal light/dark cycle and ad libitum food and water intake, the maximum feeding of rats was during the dark hours. Under continuous light, this pattern was gone in 6 days, with more feeding during the usual daylight hours and less during the normal nighttime hours. They also observed a return to the original feeding pattern by day 6 when the lighting conditions were returned to light/dark. Mice raised in continuous light have a displaced circadian rhythm with periods between 25.0 and 25.6 h (Aschoff, 1960). In addition, Rosenwasser et al. (1979), when measuring food consumption in rats under continuous illumination, found no difference in the daily total amount of food consumed whether in light/dark or continuous light, but in continuous light the rhythm was attenuated from 24 h in light/dark to 24.25–24.7 h. These findings are consistent with the observations described here.

We have shown that light entrainment affects the eating cycle of rats. However, we do not know whether light entrainment it affects enzyme synthesis and storage in von Ebner's gland per se or whether the morphological and enzyme changes we observed were a consequence of the changes in the eating cycle that was entrained by light. In a review article, Johnson (1992) has enumerated many experiments that indicate that metabolic response to eating is not a response to circadian changes, but rather a response to feeding changes that are entrained by light. However, there has been some indication that some digestive enzyme secretion is independent of food consumption. In the rat exocrine pancreas, a minor cycle of secretion was found that was independent of food intake (Maouyo et al., 1993). Further experiments are needed to determine whether significant changes in morphology and

enzyme secretion in von Ebner's glands might occur under light entrainment when food is withheld.

Recently, circadian rhythms have been studied from a genetic viewpoint to ascertain more about molecular control and entrainment (Dunlap, 1998). Genes have been cloned that make protein components of the biochemical clock. Mutations in these genes have been shown to alter or even abolish circadian rhythms. Among these are *per*, *tim* and *doubletime* in *Drosophila melanogaster* (Young, 1998), *frq* in *Neurospora crassa*, (Aronson et al., 1994) and *Clock* in mice (Vitaterna et al., 1994). These studies will further clarify the genetic and biochemical factors that are involved in the pathway of light-entrained circadian rhythm.

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