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Morphology of Lung of *Rana ridibunda* With Observations on Changes Occurring Under Different Conditions

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Abstract: The frogs used in this study were divided into three groups. The first group consisted of frogs collected from their natural habitat, and these animals were dissected immediately. The bodies of the frogs in the second group were totally submerged in water at +4 °C, and the third group of frogs was kept in a dry environment. The lung sections were examined under a light microscope after being stained. The lungs were found to have three folds: the primary, secondary and tertiary septa. Goblet cells were not only found among the ciliated cells but were also seen among pneumocytes. In particular, the goblet cells found among the pneumocytes were seen to form "mucous doors". The pneumocytes and goblet cells in the second group of animals were characterized by coarse granular areas in dark-stained nuclei, and the cytoplasm of the goblet and neuroepithelial endocrine cells also contained numerous scattered secretory granules. The pneumocyte nuclei in the third group of animals were lightly stained and possessed large nucleoli. The goblet and neuroepithelial endocrine cells of this group exhibited different stages of secretion and production. Therefore, this implies that the third group of frogs used their lungs during respiration, but a dry environment was not suitable for their survival. In contrast, the second group of frogs were in hibernation and did not use their lungs.

Key Words: lungs, respiratory epithelium, neuroepithelial endocrine cells, neuroepithelial bodies, frog, *Rana ridibunda*.

Farklı Yaşam Koşullarında *Rana ridibunda* Akciğer Morfolojisinde Meydana Gelen Değişiklikler

Özet: Bu çalışmada kullanılan kurbağalar üç grup altında toplandılar.Birinci gruptakiler, doğadan toplandıktan hemen sonra kesildiler. İkinci gruptaki kurbağalar, vücutlarının tamamı +4 °C su içinde tutuldular ve üçüncü gruptakiler, kuru ortam olarak susuz akvaryumda bırakıldılar. Akciğer kesitleri, uygun boyalarla boyandıktan sonra ışık mikroskobunda incelendiler. Akciğerler, birincil, ikincil ve üçüncül kıvrım adı verilen üç akciğer kıvrımına sahiptirler. Goblet hücreleri sadece silli hücreler arasında bulunmayıp, pnömositler arasında da gözlenmişlerdir. Özellikle pnömositlerin aralarında bulunanları, "*mukus kapıları*" oluşturmuşlardır. İkinci grubun pnömositleri ve goblet hücrelerinin nukleusları, kaba tanecikli ve koyu boyalıdır; ayrıca onların goblet hücreleri ve nöroepiteliyal endokrin hücrelerinin sitoplazmalarında çok fazla salgı taneciği gözlenmiştir.Üçüncü grubun pnömosit nukleusları açık renk boyalıdırlar ve büyük bir nukleolusa sahiptirler. Bu grubun goblet ve nöroepiteliyal endokrin hücreleri, farklı üretim ve salgılama sürecindedirler. Bu nedenle ikinci grup hayvanların, hibernasyonda oldukları ve solunum işlevinde akciğerlerini kullanmadıkları, üçüncü grup hayvanların ise, solunum için akciğerlerini kullandıkları, ama kurak şartların yaşamaları için uygun olmadığı söylenebilir.

Anahtar Sözcükler: Akciğer, solunum epiteli, nöroepiteliyal endokrin hücre, nöroepiteliyal cisimcik, kurbağa, *Rana ridibunda*.

Introduction

The respiratory systems of amphibians are interesting because the respiratory mechanism and the structural organization of their respiratory organs are different between species (1,2). Amphibians use one or a combination of different organs, such as gills, cutaneous body surface, buccopharynx and lungs, in respiration. All amphibians use the gills for respiration during the embryonic and larval stages. In aquatic salamanders, the gills function as a respiratory organ throughout their lives (1). Some aquatic urodelans, *Triturus alpestris* and *Triturus cristatus carnifex,* rely primarily on their gills and cutaneous surface and minimally on their lungs (3,4). Thus, the inner surface of the lungs is not folded. Since the lungs of many species of anuran amphibians play an important role in respiration, they possess lung septa, which increase the respiratory surface area (2). Since the diversity in the respiratory system and its function in amphibians are closely related to conditions in their habitats, environmental factors that change the conditions of their habitat may bring out some defects in the amphibian respiratory system. Various researchers have reported that changes in environmental conditions cause death in some amphibian species (5,6).

The purposes of this study were to examine the structural organization of the respiratory system of *Rana ridibunda*, to define the elements involved

during the respiratory function of the lungs, to observe the distribution and localization of these structural elements in the lungs, and to determine changes that may occur in the components of the lungs by experimentally exposing the frogs to different environmental conditions.

Materials and Methods

In this study, 19 adult male and female *Rana ridibunda* (Anura-Amphibia) specimens were used. The frogs, weighing from 10.1 to 68 g, were divided into three groups: five were dissected just after collection from their natural habitat, seven were totally submerged in water at +4 °C for 14 days, and the other seven individuals were kept in a waterless aquarium in dry conditions. The individuals of the third group were kept in the laboratory, where the temperature was 16-18 °C and the humidity varied between 49 and 58.5 %. The skins of the frogs were moistened with water twice a day. The duration of the experiment was reduced to 13 days because of the high death rate in the third group. After the spinal cord was destroyed, the lungs of the animals were fixed in Bouin's fluid for 24 hours. Lung sections of 5 μm thickness were examined under a light microscope after they were stained with Haematoxylin-Eosin (HE), Aldehyde Fuchsine (AF)-Light Green (LG) (7), and Periodic acid -Schiff (PAS) (8). In order to show the whole of the inner organization, the lung was divided into two pieces vertically. Both pieces were fixed and photographed.

The diameter of the pneumocyte bodies and their nuclei were measured with an ocular micrometer and it was determined whether there were any significant differences with regard to these dimensions between the groups of animals using Student's t-test and the Mann-Whitney U test (Table 1).

Results

First group

The frogs in this group were evaluated as control animals for the second and third groups of frogs.

It was determined that the three-fold (primary, secondary and tertiary septa) inner surface of the lung walls protruded deep into the lumen. They were all different in terms of both length and thickness (Figure 1). The primary septa with dilated apical ends were the longest and thickest (Figure 2). The apical ends of larger septa contained numerous bundles of smooth muscle cells and a wider vein. The secondary septum left the stem of the primary septum almost vertically, and the tertiary septum went into thinner branches from the secondary septum. The secondary septa were present across the areas between the central lumen and the lung walls, together with the tertiary septa (Figure 1). All these septa were interconnected. The lungs were divided by these septa into large and small air sacs, the alveoli.

The pulmonary arteries and veins going in and out of each hilum of the lungs were seen to run down along the lung walls and give branches to each septa. Finally, the capillary network was set in the wall of the air sacs by branching out of these vessels. On the septa, it was observed that the blood capillaries were located on opposite sides, forming a double capillary system.

The respiratory epithelium exhibited local variations in terms of the distribution of different cell types. The pseudostratified epithelium (PSE) which was found at the dilated apical end of the primary septa contained ciliated cells, goblet cells, basal cells and neuroepithelial endocrine cells (Figure 3). The single-layer respiratory epithelium had pneumocytes, goblet cells and neuroepithelial endocrine cells in the remaining parts.

Group	n	a/b AM±SD (min-max)	Groups-compared	T test	Mann-Whitney U test
1	435	0.73±0.11 (0.38-0.92)	-	-	-
2	325	0.61±0.12 (0.33-0.91)	1 and 2	P<0.05	-
3	325	0.83±0.13 (0.40-0.93)	1 and 3	-	p<0.05

Table 1. Arithmetic means (AM) and standard deviations (SD) of the proportions of pneumocytes and their nuclei (one unit is 0.042 mm); (a) diameters of nuclei, (b) diameters of pneumocytes, (n) numbers of cells.

The cubic or columnar pneumocytes had large round nuclei, occupying about 80-90 % of the cell volume and their nuclei exhibited small granular areas with large nucleoli. The cytoplasm of the cells was generally stained dark with the cytoplasmic dyes and was dense in appearance.

Goblet cells, which were found as individuals and in groups among the pneumocytes and ciliated cells, were numerous around the central lumen and were generally in the upper half of the lungs. In particular, goblet cells forming a group among the pneumocytes were observed in a fan shape because of their locational order. As it may be seen in Figure 4, they were closely located at opposite ends of the septa, and constituted a narrow area. Their cytoplasm occupied a large area in the apical portion of the cells. In some goblet cells, the apical cytoplasm appeared homogeneous and dark in colour when stained with eosin. In contrast, the cytoplasm of the other goblet cells was light in colour and honeycomb-shaped (Figure 5), with granular cytoplasmic structures that were stained pink with eosin and purple with AF and PAS in the honeycomb structure.

The nuclei of columnar ciliated cells showing granular areas had large nucleoli. Their cytoplasm was lightly stained, but the apical cytoplasm of the cell had a narrow band that was stained dark pink with eosin and was found under the cilia. Small granules stained pink with eosin were observed just under this band (Figure 6).

The conical basal cells were distinguished by a slightly stained cytoplasm (Figure 3).

In addition, in the sections stained with eosin, oval or round cells were observed whose cytoplasm could be described as clear and bright. These cells, which were present both individually and in groups, were usually located among the pneumocytes and goblet cells and around the blood capillaries (Figures 7 a, b). Their nuclei, with small granular areas and numerous invaginations, occupied about 60-65 % of the cell volume. Additionally, the respiratory epithelium in the sections stained with AF contained another type of cell, called aldehyde fuchsine positive, AF(+), cells whose secretory materials were stained purple with AF. These cells also appeared individually and in groups of 2-4 cells. AF (+) cells were present either in the basal region or near the basal region of PSE between the ciliated and goblet cells. In the remaining parts of the epithelium, these cells were seen among the goblet cells and pneumocytes near the blood capillaries. Most of these cells had small secretory granules in their cytoplasm.

The connective tissue possessed predominantly smooth muscle cells, fibrocytes, collagen and elastic fibers. The nuclei of the smooth muscle cells, with scarce granules, were oval in shape and were also folded.

Second group

In this group, the oval nuclei of the pneumocytes were stained dark, had coarse granular areas of karyoplasm and covered less space in the cell ($p < 0.05$) (Table 1). Their nucleoli could not be distinguished (Figure 8). The cytoplasm was stained lightly with cytoplasmic dyes. Erythrocytes in the blood capillaries were observed close to the pneumocytes and in other blood vessels on a large scale. They were dark in colour.

The nuclei of the goblet cells contained numerous large granular areas. Most of the goblet cells contained granular structures in large quantities in the honeycomb structure.

It was observed that the cytoplasm of the AF(+) cells was filled completely with large granular secretory material and this material was stained darker than that in the control animals (Figure 9).

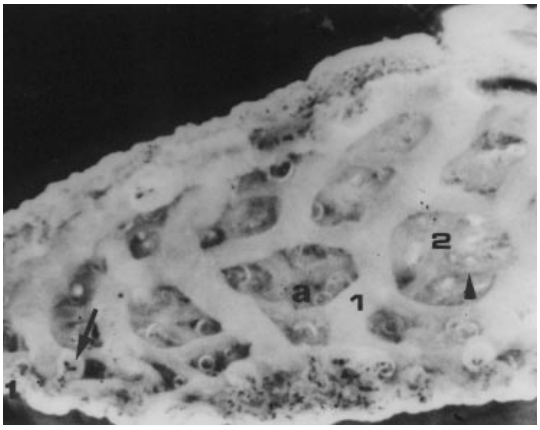
Third group

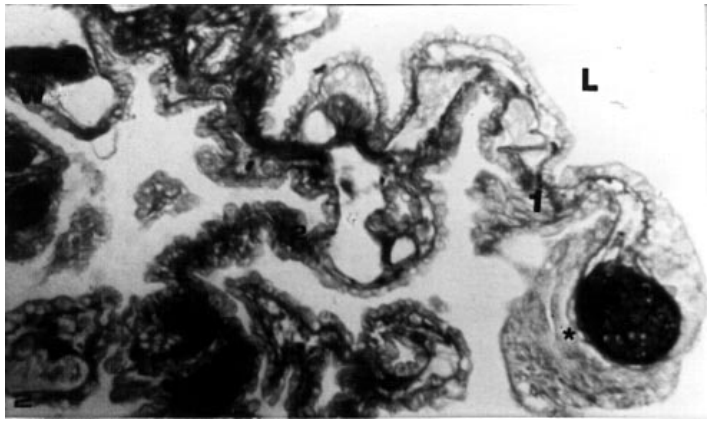
Three individuals died on the 5th, 10th, 12th days of this experiment, and the remaining experimental animals were killed on the 13th day. All the animals had clearly lost weight.

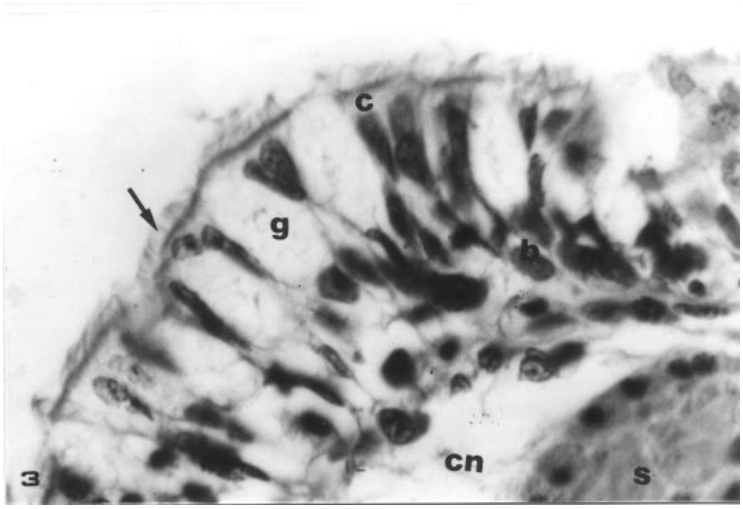
The nuclei of the cubic pneumocytes occupied a larger area in the cell ($p<0.05$) (Table 1). The granules of the nuclei with light coloured karyoplasm were small in number and their nucleoli were conspicuous (Figure 10). The cytoplasm, which was located in a narrow space around the nuclei, was stained slightly. It was noticeable that the blood capillaries closer to the pneumocytes had become wider.

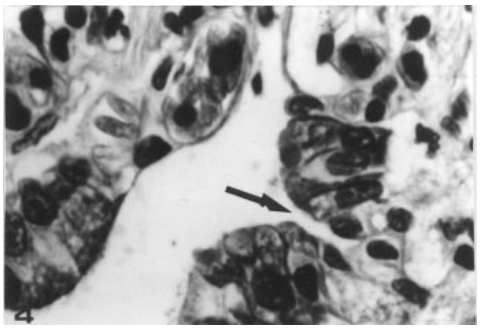
The goblet cells were short, stained homogeneously and were darker (Figure 12). Their nuclei were also darker and more granular than those in the control animals. The lungs of the individual which died on the fifth day of the experiment were fixed. It was oberved that the goblet cells, especially the ones containing granular structures, were excessive in quantity when compared to the goblet cells of the controls and the other animals of this group (Figures 7b, 11). The nuclei of the goblet cells, with small granular areas and a lightly stained karyoplasm, possessed large nucleoli.

Small granules in the ciliated cells of the control animals which were observed to be closer to the band









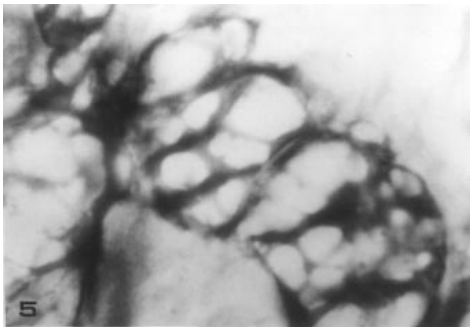
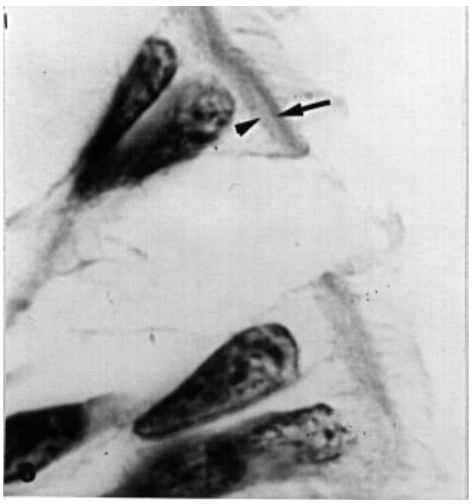


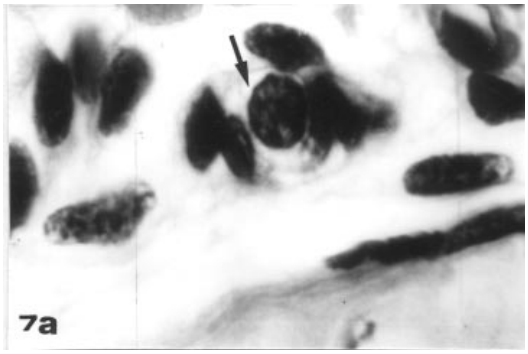
Figure 1. The inner organization of the lungs (1) primary septa, (2) secondary septa, tertiary septa (arrowhead), the dilated apical region (arrow), (a) lumen of the lung, x 8.
Figure 2. Primary and secondary septa of the first group: (1) primary and (2) secondary septa, (*) apical region of primary septa, (L) lumen, (w) the lung walls, AF-LG, X 112.
Figure 3. Apical region of primary septa: (b) basal cell, (c) ciliated cell, (cn) connective tissue, (g) goblet cell, (s) smooth muscle cell, cilia (arrow), HE, X 680.
Figure 4. A "narrow area" formed by the goblet cells (arrow), HE, X 240.
Figure 5. The honeycomb-shaped goblet cells in the first group AF, X 1700.

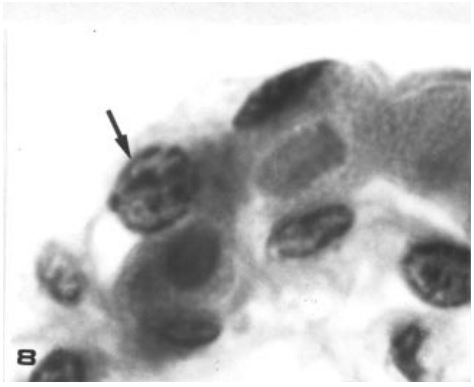
immediately under the cilia were not observed in the frogs of this group.

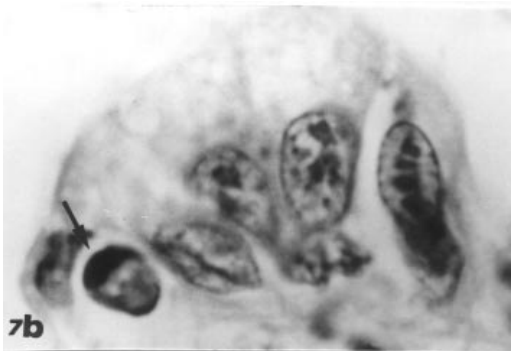
It was observed that the AF (+) cells contained large quantities of secretory granules in different sizes and were stained in varying densities.

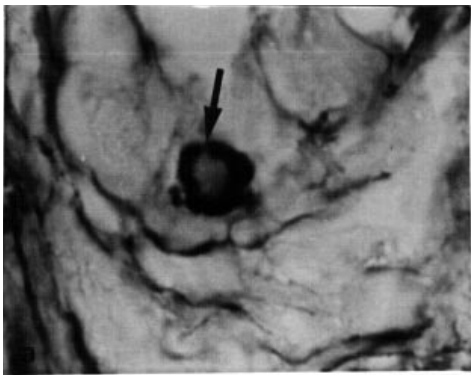
The most noticeable change observed in the connective tissue was the elongated elastic fibers (Figure 13) and the nuclei of the smooth muscle cells (Figures 14 a, b). In addititon, it was seen that the air sacs were wider.











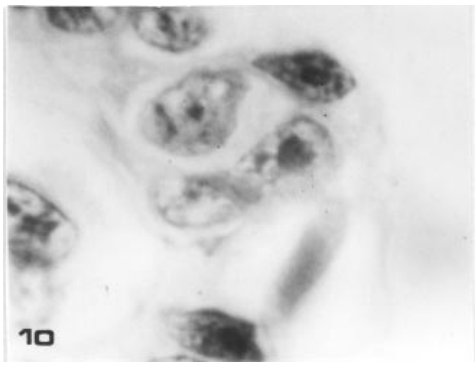
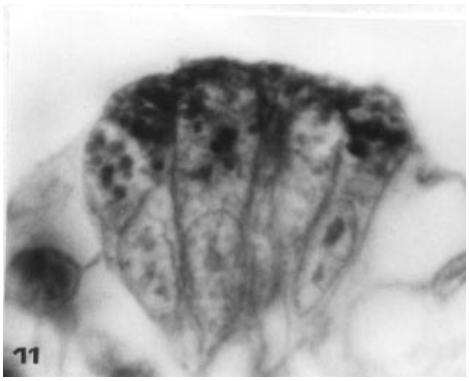
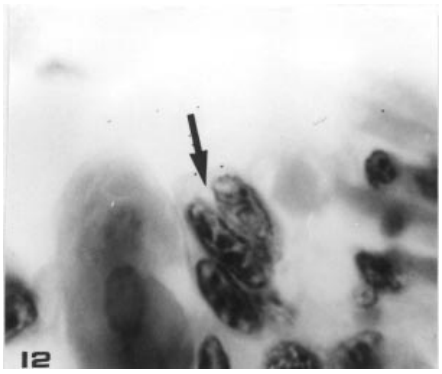


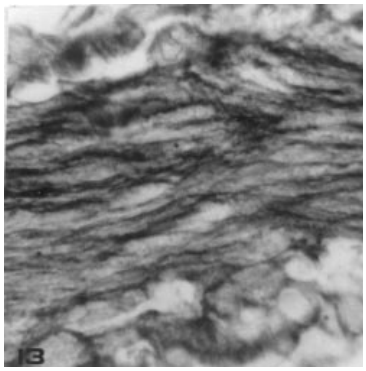
Figure 6. Ciliated cells in PSE: narrow band in apical cytoplasm of ciliated cells (arrow), and small granules under narrow band (arrowhead). HE. X 1700.
Figure 7. Arrow shows clear cell among pneumocytes (7a) and goblet cells (7b). HE. X 1700.
Figure 8. Arrow shows the inactive pneumocytes in the second group of animals. HE. X 1700.
Figure 9. Arrow shows inactive AF (+) cell in the second group of animals. AF-LG. X 1700.
Figure 10. The active pneumocytes in the third group of animals. HE. X 1700.

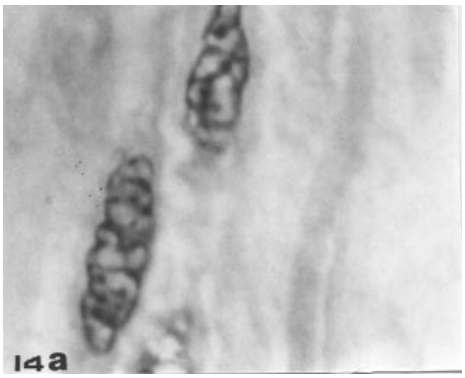
Discussion

In terms of their height, shape and appearance under the light microscope, the pneumocytes of *Rana ridibunda* are similar to the pneumocytes described in Urodela and Anura (3,4,9). In contrast to the lungs of reptiles, birds and mammals, characterized by the occurrence of two









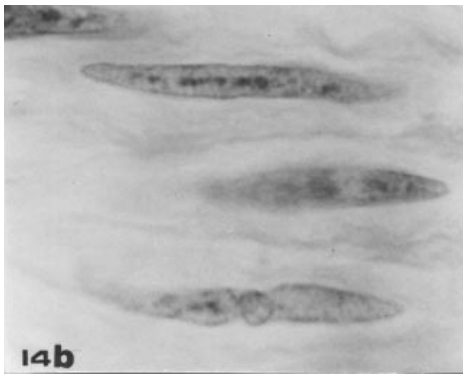


Figure 11. Goblet cells in the third group of animals and secretory granules in their cytoplasm. PAS. X 1700.
Figure 12. The short goblet cells in the third group of animals are indicated by the arrow. HE. X1700.
Figure 13. The extended elastic fibers in the third group of animals in the connective tissue of the septa. AF-LG. X1700.
Figure 14a. The nuclei of inactive smooth muscle cells in the first group of animals. HE. X 1700.
Figure 14b. The nuclei of extended smooth muscle cells in the third group of animals. HE. X1700.

pneumocyte types (I and II), amphibians have a single type of pneumocyte, combining the functions of type I and type II pneumocytes (10). Because the pneumocytes of *R. ridibunda* are all similar with regard to their appearance, it may be concluded that their type is unique. Relying on the assumption that some cells with large nuclei, light-coloured karyoplasm, and large nucleoli are active (11), it can be noted that all pneumocytes in the first group may not have been active at the same time. Moreover, most of those in the second group could have been inactive, and the pneumocytes in the third group were generally active (Table 1).

Goblet cells in amphibian lungs are usually described on the basis of their location among the ciliated cells (3,4,10). However, they were not only observed among the ciliated cells, but also found among the pneumocytes in *R. ridibunda.* It may also be noted that the goblet cells together with the ciliated cells of *R. ridibunda* may provide the function of cleaning inspired air. In addition, it can be suggested that the goblet cells, which were observed among the pneumocytes, formed door-like structures, a "mucous door" (the term introduced here) to clean the semi-cleaned inspired air. Rhodin and Dalhamn (1956) (12) defined darkly stained homogeneous apical cytoplasm goblet cells in rat tracheal epithelium as "the ones that are in the beginning of the secretory production phase", and goblet cells which are lightly stained and have a honeycomb structure with a wide apical region as "mature goblet cells." In addition, they suggested that the granular structures in the apical cytoplasm of goblet cells participate as a precursor of secretory material in mucin production. In accordance with this, it should be noted that goblet cells were found to be inactive by taking the nuclear morphology of the second group of the frogs into account. In addition, these cells were filled with granular structures since they did not form mature mucous material. The individual in the third group which died on the fifth day possessed a lot of active goblet cells (taking into consideration the characteristics of the nuclei) containing granular structures. Observation of these numerous active goblet

cells may suggest that this individual greatly needed mucous. In the rest of the animals in the third group, the observation of short goblet cells with dark-coloured apical cytoplasm leads us to think that these cells released their secretory material into the lumen from the fifth day to the end of the experiment.

Electron microscopy studies showed that the basal corpuscles of the cilia existed just under the ciliated cell surfaces (13). It may also be noted that in the ciliated cells of *R. ridibunda*, the darkly stained narrow band under the cilia was the structure formed by those basal corpuscles. In the ciliated cells that are present in the trachea of *Xenopus laevis*, some granules similar to mucous granules in the goblet cells have been observed (13). Since these granules were not observed in the third group, the granules can be considered mucous granules secreted from the cells, as described above.

The localization, distribution, number and secretory contents of the lung neuroendocrine cells have been defined in different animals using various methods (10,14,15). The endocrine cells can be recognised by their clear and bright cytoplasm in sections stained with HE (16). The endocrine cells of the gastrointestinal tract are of similar origin to the endocrine cells of the lung (16) as some of them are stained with Gomori stains (AF etc.). Therefore, it can be concluded that AF (+) cells and the cells with a clear cytoplasm were the endocrine cells of the lungs of *Rana ridibunda*. These endocrine cells in *R. ridibunda* resemble the lung endocrine cells of other vertebrates (9,17,18) with regard to their location and some morphological characteristics. They are found either individually (Neuroepithelial endocrine cells, NEE cells) or in a group (Neuroepithelial bodies, NEBs) just like in all other vertebrates (19). The numbers, distribution and secretory content of NEE cells and NEBs differ between the different species. It may be noted that NEE cells and NEBs, observed in different quantities in *R. ridibunda*, with regard to their general location, distribution and also the number of cells that NEBs contain, are similar to those in other amphibians (17,18,20). NEE cells that are generally observed in excess quantities among the pneumocytes and the blood capillaries are situated mostly at the basal side of the epithelium, just like in *Bombina orientalis* (21). NEBs with 2-3 cells are rarely found in *R. ridibunda*. They can occur all over the epithelium and are also situated near the basal region of the epithelium. According to the AF method, the AF(+) cells of the frogs in the first group had secretory granules with different sizes, densities and numbers, i.e., every one of these cells was in different functional phases. On the other hand, the observation of a large number of secretory dark stained granules filling all the cytoplasm in the second group of frogs leads us to think that these cells were inactive. Joosse (1964) described inactive neurosecretory cells with exactly the same characteristics (22). It may be noted that AF(+) cells in the third group of frogs were in different stages of secretion and synthesis processes of secretory materials.

The dark-coloured erythrocytes in the second group may have contained more hemoglobin to make use of the small amount of air left in the lungs. These findings are in accordance with those of Foxon (1). It may be reasoned that the expansion of the capillaries in the third group was to make use of more oxygen, providing more erythrocytes per unit of time. Elastic fibers together with smooth muscle cells may have the function of discharging the air in the lungs and inhaling the clean air. The lengthening of the elastic fibers and the smooth muscle cells in the third group may be explained by the flow of much more air into the air sacs, dilating their diameters.

In many anurans, the lungs predominate in the uptake of oxygen, and the skin in removal of carbon dioxide (1). Amphibians hibernate at +4 °C (1). In this period, they ensure the decreased supply of oxygen through the skin. For the second group, the observation of decreased activity in all cells and other structures of the lungs may indicate that those animals were inactive animals, that is to say, these frogs did not use their lungs and were in hibernation. Increased activity was observed in the cells and in other structures of the lungs in the third group. As buccopharyngeal respiration may be related with smell (1) and with the loss of the wet condition of the skin, it could have a limited role in the respiration. This may indicate that these animals used their lungs more than their skin during respiration. In addition, the death of some of the experimental animals within the third group shows that in spite of the increased activity in the structural elements of their lungs, the lungs of the experimental animals were not sufficient in terms of respiratory function and the dry conditions were possibly the main reason for death.

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