Histological Changes in the Skin of *Rana pipiens* Produced by Either KCl Loading or Fasting (43866)

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Abstract. **The present study was performed to determine if either potassium loading or fasting results in histological changes in the skin. *Rana pipiens* were loaded with KCl and skin biopsies obtained (Group I). These biopsies were compared with biopsies from NaCl loaded frogs (Group II). In blind studies of microscopic sections, 13 of 17 biopsies of a mixture of I and II were correctly diagnosed by one observer and similarly, 14 of 17 of Group I and II were correctly diagnosed by a second observer ($P = 0.0245$ and 0.0063, respectively). The characteristics used to distinguish skins from KCl treated frogs versus controls treatment included: (i) an abundance of large euchromatin cells on or near the surface; (ii) changes in the basal cell layer with elongation and rotation of the nuclei; (iii) lighter cells in the spinosal layers; and (iv) sometimes the skin became thicker. The water-soluble nondialyzable material of the frog skin was extracted, and we found that it increased by 4.4 times following KCl loading ($P < 0.05$). However, the protein fraction was not increased by loading the frog for 3 days with $NaHCO_3$. We conclude that potassium loading results in characteristic histological changes in the skin and that this is probably related to the ability of the skin to excrete potassium. In addition, a comparison of biopsies of skin from fed frogs with samples from frogs fasted for 40 to 49 days showed a change in the thickness of the skin. Skins of fed frogs averaged 57.0 ± 1.4 μ thick compared with fasted, 39.9 ± 2.7 μ ($P < 0.001$).**

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Over the last several decades, water and electrolyte transport of amphibian tissue has been widely studied. In addition, the frog skin and toad urinary bladder are accepted models of the mammalian nephron. Both the frog skin and the toad bladder are readily obtainable and easily accessible, thereby, making excellent models for electrolyte transport studies.

Huf (1) first reported on sodium absorption by frog skin. There followed extensive studies on sodium absorption and on water absorption and loss by frog skin.

The literature through 1965 (2–4) on the histology of frog skin, and of the skin of *Rana pipiens* in particular, emphasized increased knowledge of the fine structure of the skin. Although the turnover of the epithelium by loss of superficial cells, and replacement by the proliferation of the basal layer of cells was known, no mention is made in papers through 1965 of any change in structure related to the change in function. This omission is obviously due to the fact that changes in function other than changes in the rates of absorption of sodium salts and of water were not described at this time.

In 1977, Shoemaker and Nagy (5) indicated that the skin had no excretory function other than the loss of water. In 1980, Frazier and Vanatta (6) reported ammonia excretion by frog skin, and, since then, excretion of H^+, K^+, HCO_3^-, Na^+, and sulfate (7–10) has been described. In each case, the excretion increased in response to injecting the frog with solutions containing the indicated ion.

In 1987, Page and Frazier (11) reported changes in

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the histology of frog skin in response to a metabolic acidosis, which also causes increased H^+, and NH_4^+ excretion. Vanatta and Frazier (12) reported distinguishing changes in the histology of frog skin in response to the production of a metabolic alkalosis, a condition which causes frog skin to excrete bicarbonate.

This is a report of a third and fourth change in the histology of the skin in response to two different stresses. These stresses are injecting the frog with KCl and prolonged fasting. In addition, we report a change in the protein content of the skin associated with the KCl injection as an additional finding.

Materials and Methods

KCl or NaCl Loading. *Rana pipiens* of either sex, weighing 20–45 g were used. They were divided into two groups. All injections were made into the dorsal lymph sac. Group I contained 10 frogs. Each frog was injected with 60 m*M* KCl, 0.05 ml/g twice daily for 5 days. Group II was made up of seven frogs. Each frog was injected with 120 m*M* NaCl, 0.10 ml/g twice daily for 5 days. The dose of KCl was selected by trial and error. Increasing the dose by another 25% caused death of a significant number of frogs. The dose of approximately isotonic NaCl was well tolerated and was considered to represent a marked increase in NaCl excretion. The dose of NaCl has been found to produce Na excretion by the frog (12). None of the animals were observed molting during any of the loading experiments.

At the end of the treatment period, all frogs were pithed, and skin was removed from the thigh. Each skin sample was fixed in glutaraldehyde, cross-sectioned at 1 μ*M* thickness with a glass knife using a Porter-Blum ultramicrotome and stained as previously reported (11). The stain is a modification of the Paragon multiple stain according to the method of Martin *et al.* (13).

Fasting or Fed Experiment. A total of 21 frogs, all received in the same shipment, were divided into two groups. For Group A, 10 frogs were force-fed daily for 40–49 days. The diet was as described previously (12). For Group B, 11 frogs were fasted for 40–49 days. They were handled and sham fed daily.

All frogs were kept in containers in which they could choose between a surface above water level, or varying degrees of submersion in deionized water. On the 40th day, five frogs from each group were pithed, and, on the 49th day, the remainder in each group were pithed. After pithing, abdominal skin was dissected free. The skins were fixed, sectioned, and stained as previously reported (11).

The term, "KCl skins," will be used to indicate sections of skins prepared from frogs that were treated with KCl as described for Group I above, similarly, "NaCl skins," will be used for Group II, "fed skins," for Group A, and "fasted skins" for Group B.

Evaluation of Histological Changes. The questions of whether or not histological changes were distinguishing features of a given treatment of the frog was determined by having an observer identify a set of slides representing a mixture of skins from two different treatments. The identity of each slide was masked, and the slides were randomly numbered. The observer then looked at each slide and made a diagnosis as to which treatment he thought the slide represented. Only after diagnosing all the slides in the set was the accuracy of the observers diagnosis checked against the records of the actual treatment. A *P* value for the data was then determined using the binomial theorem.

Experiments on Extraction of Frog Skin. Three groups of frogs were used in these experiments. Group C contained five control frogs; Group D (six frogs) was injected with KCl on the same dose and schedules as Group I above; and Group E (five frogs) was injected with 0.1 ml/g body wt of 120 m*M* $NaHCO_3$, two times daily for 3 days.

The animals were pithed, and the abdominal, leg, and thigh skin was removed with care to dissect all muscle free from the skin. The pooled skins were homogenized and extracted with 2.5 ml of a mixture of chloroform, methanol, and concentrated HCL (20:40:1), centrifuged, and the residue was washed with 2 ml chloroform:H_2O (1:1) and then further washed with 3 ml of 0.1 *N* NaOH, all at 4°C (14). The aqueous phase of each wash was pooled and then extensively dialyzed against distilled water at 4°C. Following dialysis, the samples were lyophilized and weighed.

Measurement of Thickness of Epithelium. The average thickness of the epithelial cell layer of the fasted and fed skins was determined on cross-sections of skin using a calibrated grid in an optical microscope. Three 125 μ*M* lengths of skin were randomly selected from the skin of each animal, and the area of the epithelial layer was determined by counting the squares covering them, estimating at the margins to quarter squares. This total area was then calculated to thickness by dividing by the length. The identity of the treatment of the frog that produced each skin was unknown to the person who made the determination. Following measurement of the thickness of each specimen, the slides were decoded, and the average for each group (fasted or fed) was determined algebraically and compared using the Student's *t* test.

In a separate set of observations to determine cell size, a 125-μm section of each slide was counted for cell number, and the thickness determined as described. Again, the group to which the slide belonged was hidden from the observer until all observations

were completed. Cell number divided by thickness was then an index of the cell size. All means given are ±SEM.

Results

The abilities of observer A (J.C.V.) and observer B (L.W.F.) to correctly identify skins as NaCl of KCl based on histological features are given in Table I. In each case, the statistical significance was determined by the binomial theorem. Two of the three KCl slides missed by the two observers were from the same frogs.

Histology. *Skin of fed and NaCl frogs.* The epidermis of fed frogs and NaCl loaded frogs were essentially the same as the skin of *Rana pipiens* described by Moore (2). The skin consists of three to six layers of stratified squamous epithelium. The basilar layer is a single layer of cells. The majority of nuclei are spheroid and stain darker than nuclei of other cells of the epithelium. The cytoplasm is sparse. Occasionally, chromatic granules are visible in the nuclei. Presumed keratohyalin granules are occasionally present in the cytoplasm; there is more variation in this trait from one frog to another than within the section of skin of a given frog (Fig. 1).

The spinous layer is not remarkable. Occasionally, keratohylaline granules make it a granulosal layer. There is more cytoplasm present with each cell. Often the more superficial cells are lighter staining than the deeper cells. Both the nuclei and the cytoplasm may have darker staining granules. A few mitochondria-rich flask or euchromatin cells are present.

The most superficial one or two layers of cells are usually thin and cornified (Fig. 1). The nuclei are often distinguishable, but are flat. The nuclear cytoplasm may or may not have chromatic granules.

Skin of KCl loaded animals. The changes that enabled us to distinguish whether or not the skin was from a frog which had been loaded with KCl are described as follows:

1. Large euchromatin cells (EC). The most striking histological feature in these studies was the frequent appearance of large cells with a euchromatin nucleus and cytoplasm which takes up very little stain. These were found both on the surface of the skin and in the granulosal layer. The nuclei contained dark-staining granules usually near the nuclear membrane (Fig. 2, arrow). When these EC occur in the granulosal layer, they are large and oval in shape. There is usually just one of these cells surrounded by normal-staining epithelial cells (Fig. 2). When EC are on the surface of the epidermis, they are more flattened and may occur in a line of 2–5 cells contiguous to one another on the surface of the epithelium (Fig. 3). These cells are not a large percentage of the total number of cells. They were not always found in the skin of KCl-loaded frogs. It is reasonable to assume the cells migrate from the granulosal layer to the surface, but we have no direct evidence for this.
2. The cells comprising the basal layer of the skin of KCl loaded frogs often show a distinct pattern. The nuclei tend to elongate, with a line drawn through the long axis of the nuclei pointing toward the skin surface, and often nearly perpendicular to the surface (Fig. 4, arrow). The nuclei of the basal layer of fed and NaCl loaded skins tend to be more nearly rounded and are denser staining (Fig. 2 and 5). The nuclei of these cells in KCl skins tend to be lighter and to have some granularity.
3. There is a tendency of the cells of the granulosal layer to become lighter near the surface and the nuclei are more likely to show granules in the KCl loaded skin (Fig. 3 and 4).
4. The skin of the KCl loaded frog tends to be a little thicker than that of the control skin. There was a 40% increase in the average thickness of the KCl loaded skins.

It is to be emphasized that what enabled the observers to identify whether a skin was from a KCl-loaded animal or from a control animal was the frequency with which the characteristic changes were observed, not simply whether they were present or absent in the skin. It was necessary to carefully evaluate three or more sections (each 1.5 to 3.3 mm in length) before making a diagnosis. In some of the control skins, we saw occasional EC cells, and also occasional changes in the basilar layer. In some of the KCl-loaded skins, there was an occasional microscopic

Table I. Evaluation of Correctness of Histological Diagnosis

	Treatment	Correct	Incorrect	Total	Correct *P* value[a]
Observer A	NaCl	6	1	7	13 of 17
	KCl	7	3	10	$P = 0.0245$
Observer B	NaCl	7	0	7	14 of 17
	KCl	7	3	10	$P = 0.00636$

[a] *P* value determine by binomial theorem.

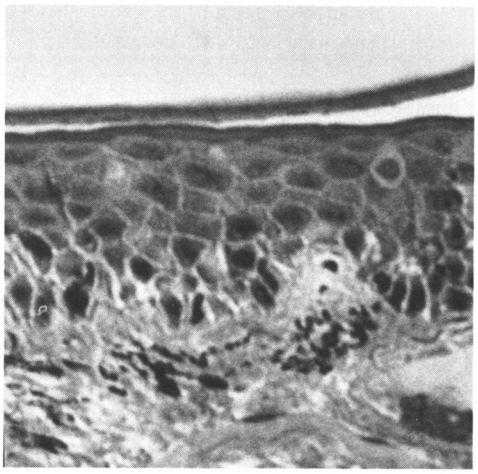

Figure 1. Photomicrograph of skin from fed frog. The pattern is similar to Figure 6. The outer layer of cornified cells has split off. Both the cytoplasm and nuclei of the cells stain darker than those of the fasting frog (Fig. 6). ×825.

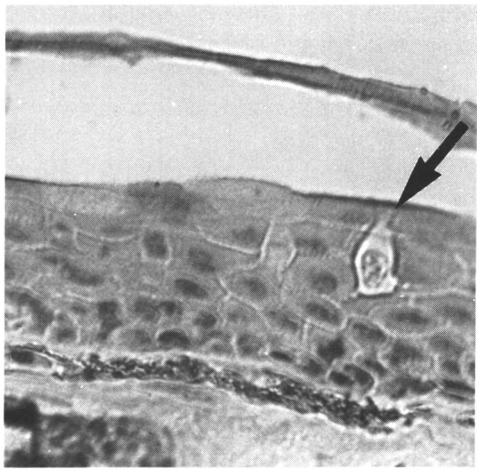

Figure 2. Photomicrograph of skin of NaCl-loaded frog. The euchromatin cell layer on the surface (arrow) is prominent. The basilar layer does not have as striking a change as other sections. ×1150.

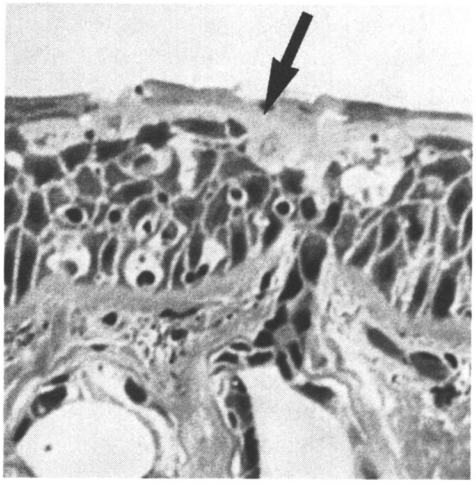

Figure 3. Photomicrograph of skin from KCl-loaded frog. The epithelium is obviously thicker than that of the skin in Figure 1 and 6. The long continuous layer of euchromatin cells (arrow) is often present but is not seen throughout all sections. The cells of the basal layer have their nuclei predominantly elongated with the long axis of the nuclei either pointing upward at an angle or perpendicular to the surface of the skin. ×1150.

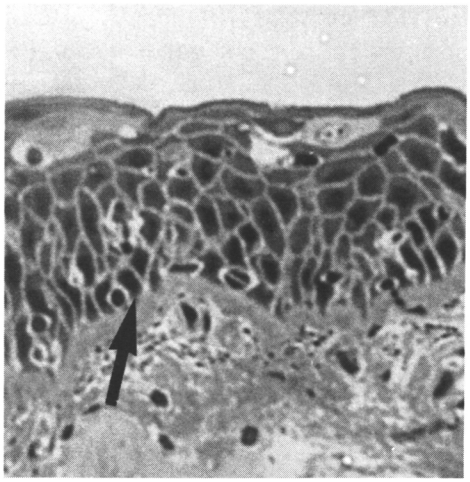

Figure 4. Photomicrograph of skin from KCl-loaded frog. A few euchromatin cells are present. The predominant finding is the change in the orientation of the cells of the basilar layer to a more vertical position (arrow). ×1150.

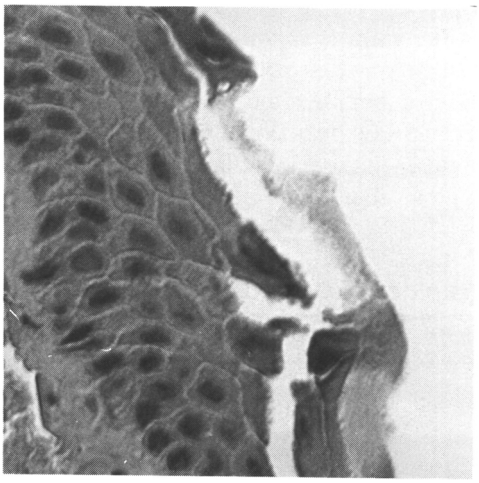

Figure 5. Photomicrograph of skin of NaCl-loaded frog. ×1150.

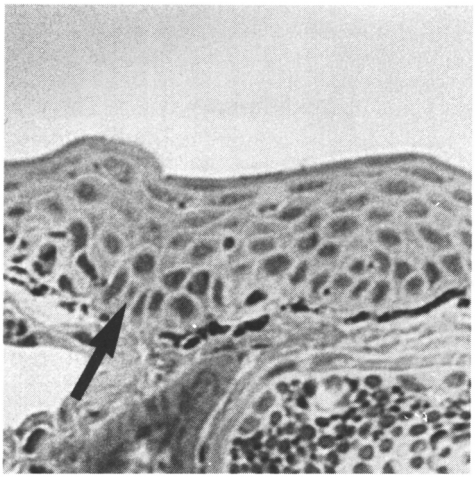

Figure 6. Photomicrograph of fasting frog skin. Cells in the basilar layer (arrow) in this epithelium all have little cytoplasm. The nuclei have their long axes predominately parallel with the skin surface. ×825.

field that had very few of the described changes. However, when scanned as indicated above, the described changes in the KCl skins enabled the observer to make the diagnoses with the accuracy reported.

Skin of fasted frogs. The epidermis of the fasting frogs compared with that of the fed frogs in general had lighter staining characteristics (Fig. 6). Both the cytoplasm and the nuclear region of the cells stained lighter.

The KCl skins were further evaluated to see if the observers could differentiate them from skins obtained from $NaHCO_3$-loaded frogs. The histological changes on $NaHCO_3$-loaded frogs that were previously reported by Vanatta and Frazier (12) are indistinguishable from those produced by KCl loading. We previously noted that there were no marked differences between the skin of sham injected frogs and frogs injected with NaCl.

The differences in histology of the fed skins compared with the fasted skins were evaluated by appearance. A total of 21, consisting of 11 fasted and 10 fed, were diagnosed with the identity unknown to the observer. He correctly identified all of the fed skins and 7 of the 11 fasted skins, for a total of 17 correct out of 21 ($P = 0.0036$).

The observer realized that the primary differentiating feature was a judgment of the thickness of the epithelial layer. This then led to a measurement of the average thickness of the epithelial layer of each skin as described under methods. The fed group averaged 57.0 ± 1.4 μM in thickness (mean ± SEM). The fasted group averaged 39.9 ± 2.7 μM $P < 0.001$. No other distinctive differences were noted.

On the evaluation of the cell size, the average cell count for the 125-μ sections were fed 67.8 ± 2.7, and fasted 52.8 ± 3.1 ($P < 0.002$). The average thickness were fed, 53.8 ± 1.58, and fasted, 41.4 ± 2.68 μ, ($P < 0.001$). There was virtually no difference in the average area occupied by each cell, the changed epithelial thickness was interpreted as being due entirely to the change in the number of cells and not to an increase in cell volume.

The aqueous soluble material extracted from the skin was dialyzed, and the nondialyzable material freeze dried and weighed. The result are given in Table II. In three determinations on the skins of KCl loaded frogs the weight of material per gram of skin averaged over four times the average of two determinations on the skin of control frogs. (2.68 ± 0.47 for KCl compared with 0.60 ± 0.45 for control, $P < 0.05$).

The residue of the first extract of the $NaHCO_3$-loaded skin was reextracted to evaluate the efficiency of the extraction procedure. A small amount of protein was recovered in the second extraction, but it was less than 5% of the protein obtained in the original extraction.

Table II. Nondialyzed Water-Soluble Material Extracted from Frog Skin

Treatment of frogs	Dry weight of extract (mg/g wet wt of skin)	*P* value
Control ($n = 2$)[a]	0.607 ± 0.453	
KCl ($n = 3$)[a]	2.68 ± 0.465	<0.05[b]
$NaHCO_3$ ($n = 2$)[a]	0.704 ± 0.391	>0.80[b]

[a] N = number of pooled skin specimens extracted.
[b] Determined from Student's *t* test (unpaired), compared with control frogs.

Discussion

It is noted that the changes that occur in response to KCl-loading involve all the layers of the epithelium, with some of the more prominent changes in the basilar cells. This suggest that there is a coordination of the functions of the different cells. If this is true, then studies of the epithelium should include not only the characteristics of individual cells, as often done in cell cultures, but also studies need to be performed on the epithelium as a tissue.

In a classic monograph by Porter and Bonneville (15), the authors note that dense chromatic material in the nuclei (heterochromatin) is thought to be DNA in an inactive state, while the lighter euchromatin nuclear material is DNA that is actively transcribing information in the form of RNA. This concept is likewise noted by Lentz (16) in a similar monograph.

It thus seems clear that the accepted interpretation is that the euchromatin cells that we noted are encoding information as RNA, and it is reasonable to assume that this RNA is a step in enabling the cell to change its function so that it will excrete more K^+ ion. The similar histological response is noted in the epithelium of frogs stressed by a metabolic alkalosis (12).

Presumably, these nuclear changes produce functional changes in the epithelial cells and are in some way linked to the changes in function that have been previously reported. The denseness of the nuclei of the NaCl-loaded group and of the fed frogs indicates that the nuclei are heterochromatic and contain DNA in an inactive state (17, 18). Consultation with fellow faculty members specializing in cell biology have not given rise to any alternate theory explaining these histological changes.

The findings are consistent with the concept that the euchromatin cells of the KCl-loaded frog skin are involved in the production of one or more proteins used in the excretion of K^+ by the skin. The surprising finding is that, although the $NaHCO_3$-loaded skins had similar histological changes, the skin did not have a significant increase in the water-soluble protein content compared with the control. The possibility that there might be a change in the concentration of spe-

cific proteins still exists and should be explored in the future.

There are two possible explanations for the observed difference in thickness between the fasted and fed skins. It may merely represent a decrease in tissue volume associated with the weight loss known to occur with prolonged fasting. On the other hand, there may be a quiescent phase of the excretory systems of the skin, since one might reasonably predict that the skin would need to excrete less K^+, H^+, and NH_4^+ during the fasted state.

We have used a principle in these studies that is often used successfully in determining mechanisms by which animals deal with maintaining electrolyte balance. The principle is to give the animal a load of the electrolyte involved that is near the maximal that the animal can tolerate. This was done as early as 1948 by Berliner and Kennedy (19), who used this method to establish that the K^+ excretion by the kidney involved more than simple glomerular filtration, but required that the tubule excrete K^+ as well. It should be noted that Pitts and coworkers used a similar method in studying acid-base balance (20–21). They injected HCl iv to produce an acidosis in dogs, and injected $NaHCO_3$ to produce an alkalosis.

Analogous to the work of Berliner, we first used the load described to show that frog skin does increase its excretion of potassium in response to such loads (7). The further studies reported are an attempt to add to the understanding of the mechanisms by which the skin changes its function in order to increase this K^+ excretion.

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1. Huf E. Uber Aktiven Wasser-und Salztransport Durch Die Froschauf. Arch Fed Ges Physiol **237**:143–166, 1936.
2. Moore JA. Physiology of Amphibian. New York: Academic Press, p261, 1964.
3. Voûte CL. An electron microscopic study of the skin of the frog *Rana pipiens*. J Ultrastruct Res **9**:497–510, 1963.
4. Voûte CL, Ussing HH. The morphological aspects of active sodium transport. J Cell Biol **361**:625–638, 1968.
5. Shoemaker VH, Nagy KA. Osmoregulation in amphibians and reptiles. Ann Rev Physiol **39**:449–471, 1977.
6. Frazier LW, Vanatta JC. Evidence that the frog skin excretes ammonia. Comp Biochem Physiol **66A**:525–527, 1980.
7. Frazier LW, Vanatta JC. Excretion of K^+ by the frog skin with rate varying with K^+ load. Comp Biochem Physiol **69A**:157–160, 1981.
8. Vanatta JC, Frazier LW. The epithelium of *Rana pipiens* excretes H^+ and NH_4^+ in acidosis and HCO_3^- in alkalosis. Comp Biochem Physiol **68A**:511–513, 1981.
9. Vanatta JC, Frazier LW. Increased Na^+ excretion by the skin of *Rana pipiens* after NaCl loading. Comp Biochem Physiol **79A**:115–118, 1984.
10. Vanatta JC, Frazier LW. The role of the skin of *Rana pipiens* in sulfate excretion. Comp Biochem Physiol **88A**:281–282, 1987.
11. Page RD, Frazier LW. Morphological changes in the skin of *Rana pipiens* in response to metabolic acidosis. Proc Soc Exp Biol Med **184**:416–422, 1987.
12. Vanatta JC, Frazier LW. Histological changes in the skin of *Rana pipiens* produced by metabolic alkalosis. Tissue and Cell **21**:219–226, 1989.
13. Martin JH, Lynn JA, Nickey WM. A rapid polychrome stain for epoxy-embedded tissue. Amer J Clin Pathol **46**:250–251, 1966.
14. Frazier LW. Stimulation of membrane phospholipid metabolism by agents that increase acidification in toad urinary bladder. Proc Soc Exp Biol Med **196**:208–213, 1991.
15. Porter KR, Bonneville MA. Fine Structure of Cells and Tissues (4th ed). Philadelphia: Lea & Febiger, p3, 1973.
16. Lentz TL. Cell Fine Structure (2nd ed). Philadelphia: WB Saunders, p6, 1971.
17. Voüte CL, Hanni S, Ammann E. Aldosterone induced morphological changes in amphibian epithelia *in vivo*. J Steroid Biochem **3**:161–165, 1972.
18. Whitear M. Flask cells and epidermal dynamics of frog skin. J Zool (London) **175**:107–149, 1975.
19. Berliner RW, Kennedy TJ. Renal tubular secretion of potassium in the dog. Proc Soc Exp Biol Med **67**:542–545, 1948.
20. Swan RC, Pitts RF. Neutralization of infused acid by nephrectomized dogs. J Clin Invest **34**:205–212, 1955.
21. Swan RC, Axelrod DR, Seip M, Pitts RF. Distribution of sodium bicarbonate infused into nephrectomized dogs. J Clin Invest **34**:1795–1801, 1955.