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Morphological Changes in the Skin of *Rana pipiens* in Response to Metabolic Acidosis[1] (42495)

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Abstract. The skin of *Rana pipiens* excretes H^+ and this excretion is increased by metabolic acidosis. The mitochondria-rich (MR) cells of the skin have been found to mediate this H^+ transport. The purpose of this study was to determine if there is a change in the MR cells of the skin during metabolic acidosis and if the isolated split epithelia of frog skin maintains its capacity to excrete H^+. Metabolic acidosis was induced by injecting 120 m*M* NH_4Cl (0.025 ml/g body wt) into the dorsal lymph sac three times a day for 2 days. The frogs were sacrificed and collagenase-split skins from the abdomen of normal and metabolic acidotic frogs were mounted between 2-ml chambers. H^+ fluxes into both the mucosal and serosal media were measured and reported in units of (nmol) $(cm^2)^{-1}$ $(min)^{-1}$. An increase in H^+ flux was seen on both the mucosal and serosal sides of the acidotic split skins. The isolated epithelia were fixed, postosmicated, and dehydrated in the chamber. They were then embedded in Spurr's resin and 1-μm sections were cut and stained with Paragon multiple stain. Coded slides were used to count various cell types. Sections were randomly selected and approximately 40,000 cells were counted. Four basic cell types were noted and confirmed by TEM photomicrographs; basal (B) cells, granular (G) cells, keratinized cells, and MR cells. The ratio of G + B cells:MR cells in the normal skins was 1.0:0.021. The ratio in acidotic skins was 1.0:0.34. The average percentage of cell population of MR cells in the normal skins was 2.08 + 0.18 and in acidotic skins 3.20 + 0.36 ($P < 0.005$). We conclude that the split skin maintains the capacity to acidify the mucosal fluid. Additionally, during metabolic acidosis there is an increased number of MR cells in the skin and this increase may be an adaptive mechanism of the skin to excrete excess H^+ during acidosis.

It has been shown previously by Vanatta and Frazier (1) that the skin of *Rana pipiens* has the capacity to excrete hydrogen ion. Further studies by Frazier (2) have demonstrated the ability of frog skin to increase H^+ excretion in response to chronic NH_4Cl- induced metabolic acidosis. In the isolated frog skin, four basic cell types have been identified by Farquhar and Palade (3): cuboidal to columnar basal (B) cells forming the *stratum germinativum,* polyhedral granular (G) cells forming the *s. granulosum* and *s. spinosum,* keratinized squamous cells forming the *s. corneum,* and the flask-shaped mitochondria-rich (MR) cells located throughout the *s. granulosum* and *s. spinosum* and extending apically into the *s. corneum.* Differentiation of each cell type in frog skin and amphibian urinary bladder has also been determined by their individual dynamic physiological functions by Rosen and Friedley (4), Scott *et al.* (5), Schwartz *et al.* (6), Voute *et al.* (7), and Whitear (8). The histochemical analysis of Rosen and Friedley (4) indicated that carbonic anhydrase activity is localized in the MR cell of frog skin and the MR cell may be the mediator of H^+ transport. It has also been shown by Frazier (9) that in toad urinary bladder, a tissue similar in function to frog skin, a chronic metabolic acidosis will increase the number of MR cells and this increases the capacity of the bladder to excrete H^+.

The purpose of the present study was to determine if the isolated epithelium of the split frog skin maintained the capacity to excrete H^+. Additionally, we wanted to confirm that there was an increase in H^+ excretion during chronic metabolic acidosis and to learn if this increase was associated with changes in the number of MR cells. Our results indicate that the split skin does acidify and that there is an increased number of MR cells in response to chronic metabolic acidosis that accompanies

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the increased H^+ excretion seen during acidosis.

Materials and Methods. *Inducing acidosis and isolation of epithelia.* The frogs used in this study were *R. pipiens* of southern origin and were supplied by Carolina Biological Company (Burlington, NC). The frogs were kept without food from day of receipt until use in a holding tank containing tap water. Chronic metabolis acidosis was induced by injecting 120 m*M* NH_4Cl (0.025 ml/g body wt) into the dorsal lymph sac three times a day for 2 days. A dose similar to this over a 48-hr period has been shown to induce a marked acidosis in the toad *Bufo marinus,* as confirmed by plasma HCO_3^- concentration (10). The toad is similar to the frog *R. pipiens* in terms of its' body pH and buffering systems. Normal and acidotic frogs were sacrificed by double pithing and abdominal skin was removed. The skin was collagenase split and isolated similar to the method of Fisher *et al.* (11). The skin was stretched flat and the tela subcutanea was gently scraped from the serosal surface with a scalpel. The outside surface of the skin was glued as a flat sheet to the rim of a Lucite ring (o.d., 3 cm; i.d., 2.6 cm) with a tissue adhesive (Zipbond, Tescom Corp., Minneapolis, MN). The mucosal or outside surface was bathed in frogs Ringer's solution consisting of (in m*M*); NaCl, 114.5; KCl, 3.0; $CaCl_2$, 0.9; sodium phosphate (dibasic), 1.5. The final pH was 7.20. The basolateral surface was bathed in Ringer's solution with collagenase (0.4 mg/ml) in a petri dish. The skins were allowed to incubate in a water bath at 29°C for 1.5 to 2 hr. The subcutaneous tissue was then carefully split away leaving a thin, isolated sheet of epithelium on the Lucite ring.

Determination of H^+ excretion. The split skins were mounted as sheets between two modified Ussing chambers made of Lucite. Each chamber was 2 ml in volume, and the exposed area of each skin was 1.98 cm^2. The phosphate-buffered Ringer's solution was added to both the mucosal and serosal chambers. The mounted skins were allowed to equilibrate for 15 min. The chambers were then drained and fresh Ringer's solution was added to both chambers. The flux was allowed to proceed for 120 min at room temperature. Throughout the experiment air was bubbled into the serosal bathing fluid. At the end of the flux period the mucosal and serosal solutions were removed and the pH of both the original and final bathing solutions was determined using a Radiometer Model PHM64 digital pH meter. The H^+ excretion was calculated from the change in pH and the concentration of buffer in the mucosal solution as described previously (10). H^+ excretion was then normalized for area of skin exposed and time and reported in units of nmole $(cm^2)^{-1}$ $(min)^{-1}$.

Histology. After the flux period and pH determinations, the skins were histologically prepared for the TEM and cell counts similar to the methods described by Carson *et al.* (12). The fixing and dehydration of the tissue were performed in the chambers to prevent curling of the skin. The skins were fixed overnight by introducing 2% glutaraldehyde in a 0.1 *M* cacodylate buffer to the chambers. The tissues were washed for 15 min in 0.1 *M* cacodylate buffer and postosmicated for 90 min in 1% osmium tetroxide, with 0.1 *M* cacodylate buffer containing 5 m*M* $CaCl_2$. After washing, the skins were dehydrated in a graded series of ethanol: 30, 30, 50, 70, 90, 95, and 100 at 10 min each. The skins were removed from the chamber and placed in 100% absolute ethanol for 15 min. The tissue was then placed in Spurr's resin (13) for two series of 15 min. The skins were removed from the O-ring and embedded as sheets in Spurr's resin using a flat embedding mold and incubated overnight in an oven at 60°C. The embedded sheets were randomly cut into rectangles (2–4 mm) with a razor blade after heating the hardened plastic on a hot plate. The cut sheets were then glued with either Zipbond or epoxy to modified Beam capsules that have a broad, flat surface instead of the cone shape. They were glued to the studs with an orientation that would allow longitudinal sections of the skin to be made. Sections (1 μm) were randomly cut with a glass knife using a Porter-Blum ultramicrotome. Sections were placed on glass slides and stained with Paragon multiple stain according to Martin *et al.* (14). Coded slides were used to count the various cell types. Sections were randomly selected and approximately 40,000 cells were counted from both the normal and the acidotic frogs on an A. O. light microscope

(×400). Photographs were made on a Zeiss light microscope. Ultrathin sections cut with a diamond knife were stained with uranyl acetate and lead citrate and examined in a JEOL JEM100CX-II transmission electron microscope (TEM) for confirmation of cell types.

Hydrogen permeability. An additional series of frog skins were examined for comparison of H^+ permeability. Normal whole skins, isolated split skins, and the complementary subcutaneous tissue were mounted on the chambers. The mucosal side contained the buffered Ringer's solution adjusted to a pH of 4.0 and the serosal side contained the buffered Ringer's solution adjusted to a pH of 7.5. This produced almost a 3000-fold H^+ concentration gradient from which the H^+ could permeate from mucosa to serosa. The flux period was for 60 min and the final pH of each solution was recorded. A permeability coefficient, K_{trans}, was obtained by calculating the rate of appearance of H^+ on the serosal side in relation to the concentration gradient on the mucosal side using a modification of the equation given by Maffly *et al.* (15), and was expressed in cm $(sec)^{-1}$.

$$K_{trans} = \frac{\text{change in } [H^+] \text{ on serosal side}}{\text{initial } [H^+] \text{ on mucosal side} \times \text{area of skin} \times \text{time}}.$$

All the statistics to determine significance for the decoded cell counts and the permeability studies were performed using Student's *t* test. Duncan's multiple-range test along with Student's *t* test were used to test levels of significance in the H^+ excretion data. Probability of significance levels was set at <0.05.

Results. The isolated epithelia of normal frog skin and 48-hr NH_4Cl-induced metabolic acidotic frog skin were histologically prepared for identification of cell types and cell counts as described above.

Coded Paragon multiple-stain slides were used to count the different cell types in the frog skin. Figure 1 shows a typical section in black and white of isolated frog epithelium from which counts were made. It is apparent that there are at least four cell types present: (i) the cuboidal to columnar basal (B) cell, (ii) the polyhedral granular (G) cell, (iii) the keratinized squamous (Sq) cell, and (iv) the clear, flask-shaped, mitochondria-rich (MR) cell.

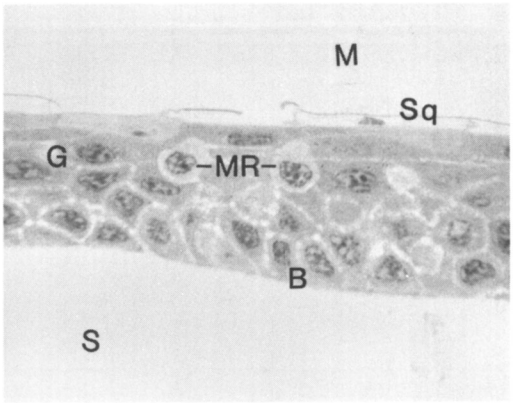

FIG. 1. Light micrograph of isolated split epithelium of the frog skin in metabolic acidosis. The four cell types are identified as cuboidal basal (B) cell lining the serosal (S) side, polydedral granular (G) cells, keratinized squamous (Sq) cells lining the mucosal (M) side, and the clear, flask-shaped mitochondria-rich (MR) cells (×499).

Figures 2 and 3 are transmission electron micrographs confirming each of the cell types; Fig. 3 shows a typical MR cell located in the *stratum granulosum* and extending apically beneath the stratified corneum.

Changes in the cell population in frog split skin in response to chronic metabolic acidosis are shown in Table I. The G + B:MR cell ratio and percentage of cell types found in the isolated split skin of both the normal and acidotic frogs demonstrate a significant increase in the number of MR cells in the frog skin in response to a 48-hr metabolic acidosis ($P < 0.005$).

The excretion of hydrogen ions into both the mucosal and serosal solution of two groups of frogs, isolated split skins and intact (whole) skins, are shown in Table II. Both groups had normal frogs and frogs in 48-hr metabolic acidosis. H^+ excretion into the mucosal fluid was increased significantly in the acidotic intact skins ($P < 0.005$). However, this was not found to be true in the split skins ($P > 0.10$). Further observation in Table II demonstrated that there was a significant difference in the H^+ flux among (i) normal split epithelia and intact skins on the serosal bathing fluid ($P < 0.001$), (ii) acidotic split epithelia and intact skins on both mucosal and serosal bathing fluids ($P < 0.025$ and $P < 0.001$, respectively), and (iii) normal split epithelia and split subcutaneous

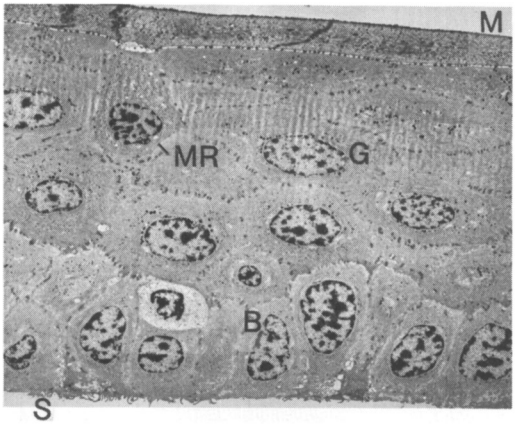

FIG. 2. Electron micrograph confirming the cell types of the isolated split epithelium of frog skin. Legend same as Fig. 1 (×826).

tissue on both mucosal and serosal bathing fluids ($P < 0.001$, for each). The probabilities in Table II were calculated using Duncan's multiple-range test, excluding the difference between the split epithelia and the split subcutaneous tissue. Student's t test showed the same probability levels as Duncan's Multiple Range Test.

H^+ permeability studies were performed to test the inference of the above data that passive backflux of H^+ is occurring from mucosa to serosa in the isolated split skin. The permeability changes of normal intact (whole) skin, normal split epithelia, and the subcutaneous counterpart are listed in Table III. The H^+ permeability coefficient, K_{trans}, indicates that both collagenase split epithelia and the subcutaneous counterpart had over a twofold increase in H^+ permeability from mucosa to serosa ($P < 0.005$ and $P < 0.001$, respectively).

Discussion. The identification of cell types and arrangement of the split epithelia of *R. pipiens* through TEM, and light microscope observations (Figs. 1–3) confirm the reports of Farquhar and Palade (3). The epidermis is five to seven cell layers thick with intermittent glandular cells. Four basic cell types are identified: (i) the cuboidal basal (B) cell, (ii) the polyhedral granular (G) cell, (iii) the keratinized squamous (Sq) cell, and (iv) the clear mitochondria-rich (MR) cell. The MR cells are flask shaped and the body is located in the *s. spinosum* and *s. granulosom* bound by a few desmosomes to the surrounding cells. The MR cell has a light cytoplasmic matrix due to the absence of secretory granules and cytoplasmic filaments and granules. They are packed with mitochondria with most of these organelles in the main process of the cell. The main process of the MR cell is found, by other SEM and TEM studies in our laboratory, to extend just beneath the *s. corneum.* This is in agreement with the reports of Brown *et al.* (16).

The results shown in Table I demonstrate that in response to chronic metabolic acidosis there is an increase number of MR cells in the cell population of the acidotic frog skin ($P < 0.005$). This is similar to work of Frazier (9) which demonstrated an increase in the number of MR cells in the acidotic toad urinary bladder by SEM cell counts. SEM cell counts could not be done in frog skin due to the presence of the corneum giving a uniform surface appearance.

Frazier (9) found a G:MR of 1.0:0.12 in normal toads, excluding B-cell count due to SEM mucosal surface counts. Choi (17) similarly found a G:MR of 1.0:0.12, excluding B cells because they were undifferentiated cells. Our results indicate a G + B:MR of 1.0:0.021 in normal frog skin, which indicates a significantly lower MR cell population in the frog skin, in spite of the fact that basal cells were also counted. It is interesting to note that the frog skin secretes H^+ at a rate which is only about 25% of that found in toad bladder (18). Regardless, cell counts on Paragon slides

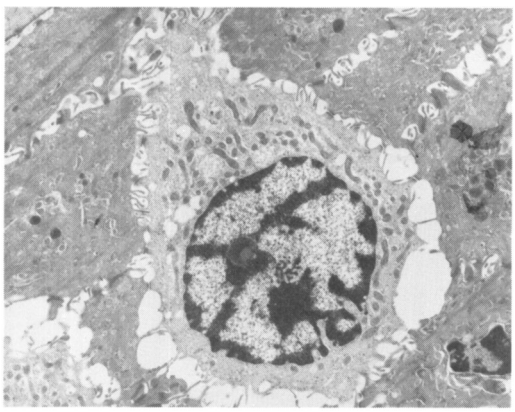

FIG. 3. Electron micrograph of typical MR cell. The cell is flask shaped, and packed with mitochondria (×3422).

TABLE I. CHANGES IN CELL POPULATION IN FROG SPLIT SKIN IN RESPONSE TO A METABOLIC ACIDOSIS

Skin	Ratio of Cell Types	
	Granular + Basal	Mitochondria-Rich
Normal[a] ($N = 16$)	1.0 (97.92 ± 0.18%)	0.021 (2.08 ± 0.18%)
48-hr metabolic acidosis[b] ($N = 18$)	1.0 (96.70 ± 0.36%)	0.034 (3.30 ± 0.36%) $P < 0.005$[c]

[a] Represents a total of 18,178 cells counted from Paragon slides.
[b] Represents a total of 21,123 cells counted from Paragon slides.
[c] Determined by Student's t test on the percentage of cells in each group.

showed a significant proliferation of the MR cell population in acidotic frogs. A report of Al-Awqati and Schwartz (19) demonstrated an increase in MR cells in the collecting tubule of acidotic rabbits. However, they state there are two different MR cells: a H^+ secreting cell and a HCO_3^- secreting cell and instead of an actual proliferation in the MR cells there is a reversal in the polarity of the HCO_3^- secreting cells. Our data were unable to support this hypothesis. It is assumed there is only the acid-secreting MR cell in the frog skin until data can be obtained supporting the plasticity of MR cell polarity. Therefore, it can be concluded there are two possible origins of the newly proliferated MR cells. They could arise from (i) differentiation of the basal cell, or (ii) division of an existing MR cell.

Since there is an increase in the MR cell population of frog skin in response to metabolic acidosis, it would be expected that H^+ excretion would also increase to remove the acid challenge. However, Table II shows there is not a significant increase in H^+ excretion of the isolated split skin after 48-hr metabolic acidosis ($P > 0.10$). On the contrary, the acidotic normal intact skin demonstrates a significant increase in H^+ excretion ($P < 0.005$).

Further observations in Table II demonstrates the comparison of H^+ flux between split epithelia and whole skins in both normal and acidotic states. A significant difference of H^+ flux is found between normal split epithelia and intact skins on the serosal side ($P < 0.001$), the acidotic split epithelia, and intact skins on both the mucosal and serosal sides ($P < 0.025$ and $P < 0.001$, respectively), and normal split epithelia and split subcutaneous tissue on both mucosal and serosal bathing fluids ($P < 0.001$, for each). These discrepancies give rise to several suggestions. The subcutaneous tissue has no capacity to excrete H^+ and is, therefore, not a contributing factor in the acidification of the mucosal fluid as seen in the intact skins.

TABLE II. COMPARISON OF H^+ EXCRETION BY COLLAGENASE-SPLIT AND WHOLE SKINS OF FROGS BOTH NORMAL AND IN METABOLIC ACIDOSIS

Group and state of skins (N)	H^+ excretion (nmole/cm^2 × min) cell→mucosal bathing fluid		H^+ excretion (nmole/cm^2 × min) cell→serosal bathing fluid	
Normal				
Split epithelia (11)	0.48 ± 0.13[a]		0.67 ± 0.09[a]	
Intact (whole) (10)	0.55 ± 0.09	N.S.[b]	−0.23 ± 0.07	<0.001[b]
Split subcutaneous tissue (8)	−0.02 ± 0.06	<0.001[b]	0.007 ± 0.070	<0.001[b]
48-hr metabolic acidosis				
Split epithelia (11)	0.71 ± 0.08		−0.70 ± 0.10	N.S.[b]
Intact (whole) (10)	1.04 ± 0.12	<0.025[b]	0.06 ± 0.05	<0.001[b]

[a] Mean ± SEM
[b] P is the probability that the value is different from the split epithelia value. N.S., nonsignificant.

TABLE III. H^+ PERMEABILITY CHANGES IN FROG SKIN AS A RESULT OF COLLAGENASE-SPLIT SKIN TREATMENT

Skin	Mean K_{trans} m→s ± SEM ($\times 10^{-7}$ cm/sec)	P value[a]
Intact (whole)[b] ($N = 8$)	1110 ± 290	
Split epithelia[b] ($N = 7$)	2290 ± 130	<0.005
Split subcutaneous tissue[b] ($N = 8$)	2370 ± 170	<0.001

[a] P is the probability that the value is different from the intact skin.

[b] All frogs were in a normal acid-base state.

Furthermore, the large difference between the acidification rates of the serosal bathing fluids of the split epithelia and the intact skins strongly implies that a significant amount of passive backflux is occurring in the split epithelia. This offers a probable explanation for the significally lower H^+ excretion rates found on the mucosal fluid of the acidotic split epithelium, the decreased mucosal H^+ excretion rates in the normal split epithelia, and the increased serosal acidification rates in both normal and acidotic split epithelia.

The implication that passive backflux into the serosal media is occurring can be reasonably inferred since the enzymatic treatment of collagenase could cause the split epithelia to lose part of their integrity. Therefore, H^+ permeability studies were performed to test this hypothesis. In Table IV the H^+ permeability coefficient, K_{trans}, demonstrates that both isolated split epithelia and the subcutaneous counterpart show over a twofold increase in H^+ permeability from mucosa to serosa ($P < 0.005$ and $P < 0.001$, respectively). This increase in permeability could be caused by one or more of the following treatments to the whole skin: (i) the removal of the serosal tela subcutanea by scalpel, (ii) the removal of the subcutaneous tissue being bound directly to the basal cells of the epithelium thus encouraging additional backflux, or (iii) the collagenase is acting on the split epithelium at the cellular level to produce a loss of tissue integrity. The permeability of H^+ is of the same magnitude as that of water, and may suggest the H^+ can pass through the epithelium, via cell membranes and/or intercellular spaces with the same ubiquity and ease as water (14, 20, 21). Lindinger and McDonald (22) made the interesting observation that acid loading in *R. catesbiana* resulted in an increase in skin permeability with an increase of water and ion flux from mucosa to serosa.

It has been shown by Benos (23) that the normal sodium transport in frog skin may be altered by mucosal acidification. In our studies on permeability we were interested in comparing the intact and split skin. The intact skin was acidified to the same pH as the split skin. Therefore, we assume that if the acidification of the mucosal fluid altered sodium metabolism it would have done so in both the intact and split skin preparations.

A report by Burch and Halushka (24) suggests that changes in mucosal pH could result in increases in prostaglandin synthesis. Since it is well known that prostaglandins can alter epithelial membrane permeability the differences we observed in split and intact skin could be due to a difference in these two preparations to synthesize prostaglandins. Future experiments will have to be done to confirm this possibility.

Since our studies showed an increase in H^+ permeability in the normal isolated split skin, this infers that H^+ permeability may be further enhanced in the split skin during acidosis. In summary, this permeability study suggests that in response to chronic metabolic acidosis the isolated split skin is significantly acidifying the mucosal solution but a significant amount of H^+ is backfluxing passively into the serosal solution and, consequently, absolute values are not seen. A corollary to our findings in the permeability studies is that something in the split-skin preparation has changed the resistance of the tissue, at least for H^+. Further electrical studies are planned to characterize the changes in resistance and electrolyte movement in the split-skin preparation.

As mentioned above, Lindinger and McDonald (22) have shown that the bullfrog adapts to a metabolic acidosis challenge by an immediate buffering by extracellular fluids, renal excretion of NH_4^+ with little urine pH change, and an increase in cutaneous permeability resulting in a net uptake of water. In the frog, an acid-base disturbance is com-

pensated for by blood buffers and both renal and cutaneous proton secreting mechanisms. Our data support the concept that frog skin increases its ability to excrete H^+ in response to chronic metabolic acidosis by adaptive cellular changes in the skin. This adaptation is a result of an increase in the number of H^+ secreting MR cells in the frog skin. In an earlier study (9) we have also demonstrated that the toad urinary bladder increases H^+ excretion in response to an acidosis and this is accompanied by an increase in the number of MR cells in the bladder. Since amphibian transport epithelia are considered as functional analogs to the mammalian distal nephron, our data may infer that a similar adaptive mechanism occurs with the intercalated (MR) cells of the mammalian distal nephron in response to chronic metabolic acidosis. However, further research must be done on the mammalian nephron before this assumption can be validated.

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