Journal Title: **Cell & Tissue Research**

Volume: 236 **Issue:** 1
Month/Year: 1984
Pages: 121-128

Article Author: Richard L. Leino, J. Howard McCormick

Article Title: Morphological and morphometrical changes in chloride cells of the gills of Pimephales promelas after chronic exposure to acid water

ISSN:

English only!

Notes/Alternate Delivery:

☐ Paged to SZ ☐ Paged to HS ☐ Emailed Loc

Cell Tissue Res (1984) 236:121–128

Cell and Tissue Research

Morphological and morphometrical changes in chloride cells of the gills of *Pimephales promelas* after chronic exposure to acid water

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Summary. Fathead minnows, *Pimephales promelas*, were exposed for 129 days to Lake Superior water acidified with sulfuric acid by means of a flow-through toxicant injection system. The effects of chronic acid stress (pH 6.5, 6.0, 5.5, 5.0) on gill histology were examined. Most of the histological effects were seen at pH 5.5 and 5.0 and were confined primarily to changes in numbers, distribution, and morphology of chloride cells. At low pH levels there tend to be more chloride cells in the gill epithelium and an increased percentage of these cells in the secondary lamellae. In contrast to normal chloride cells, chloride cells from fish exposed to low pH frequently had apical pits while some had bulbous apical evaginations. The occurrence of structural changes in chloride cells during exposure to acid water suggests that chloride cells may be involved in acclimation to acid stress.

Key words: Chloride cells – Acid stress – Gill – Electron microscopy – Fathead minnow

The effects of acid water on fish are not thoroughly understood and may be complicated by the presence of toxic cations such as lead and aluminum, or other pollutants such as chlorinated hydrocarbons resulting from or accompanying acid precipitation (Schofield 1977; Henrikson and Wright 1978; Cronan and Schofield 1979; Fromm 1980). Laboratory experiments with water relatively free from toxic substances show that dramatic increases in hydrogen ion concentration can produce rapid histopathological (Daye and Garside 1976) and physiological (e.g., Leivestad and Muniz 1976; Ultsch 1978; Neville 1979; Packer and Dunson 1970, 1972; McWilliams and Potts 1978; Packer 1979; Fromm 1980; Ultsch et al. 1981; McDonald and Wood 1981) changes in fish gills reducing their ability to perform their respiratory or hydromineral and acid-base regulating functions. However, in some species and in some individuals of a given species acid stress may not be fatal except at very low pH levels (Hultburg and Stenson 1970; Beamish 1976; Schofield 1976; Fromm 1980), and studies are lacking on the effects of chronic rather than acute acid stress on the histology and histopathology of fish gills. Since gills are important organs of ionic and acid-base regulation they presumably respond in some manner to help fish acclimate to chronic acid stress. The purpose of the present study is to examine some changes which may reflect such an acclimation response involving chloride cells of the gills of fathead minnows, *Pimephales promelas*, exposed for several months to acidified water.

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Acknowledgements. We are indebted to M. Lindemann and J. Anderson for their technical assistance. The study was supported by a grant from the University of Minnesota Graduate School

Materials and methods

Exposure

The present research was done in conjunction with a study involving the effects of pH on reproduction in the fathead minnow at the U.S.E.P.A. Environmental Research Laboratory, Duluth, MN. Fathead minnows were exposed to one of the following pH levels: 5.0, 5.5, 6.0, 6.5 or 7.5 (Lake Superior water). Exposures began on 8 July 1981 with juvenile minnows, 35 days after hatching, and continued until spawning was generally completed on 13 November 1981. At that time, six pairs of fish from each pH regimen in two series of replicate exposures (A and B) were anesthesized with MS-222 and placed in fixative for histological processing.

Exposures were performed with six pairs of fish per tank: these 40 l glass aquaria received a continuous flow (250 ml/min) of untreated Lake Superior water adjusted to the experimental pH with reagent grade sulfuric acid. Correct acid concentrations were maintained by a multichannel toxicant injection system for flow-through bioassays as described by Defoe (1975). The pH and temperature for each system were constantly monitored and recorded. Water hardness, alkalinity, free CO_2, and dissolved O_2 were measured or calculated weekly. The fish experienced a 16 L, 8 D photoperiod, and were fed frozen brine shrimp. General characteristics of the water used in the exposures are listed in Table 1. Other chemical properties of the Lake Superior water included: Cl^-, 0.03 mM/l; Na^+, 0.05 mM/l; K^+, 0.01 mM/l; Ca^{2+}, 0.34 mM/l; Mg^{2+}, 0.13 mM/l. A more thorough analysis of Lake Superior water used by the U.S.E.P.A. Laboratory was presented by Biesinger and Christensen (1972).

Table 1. Water quality characteristics during exposure of fathead minnows to H_2SO_4 – acidified Lake Superior water[a]

Characteristic	Treatments				
	Unaltered lake water	pH 6.5	pH 6.0	pH 5.5	pH 5.0
pH (last 30 days)	7.4 ± <0.1[b] (7.1–7.7)	6.6 ± <0.1 (6.4–7.1)	6.1 ± <0.1 (6.0–6.2)	5.5 ± <0.1 (5.4–6.0)	5.0 ± <0.1 (4.5–5.3)
pH (entire exposure period)	7.5 ± <0.1[b] (7.0–7.9)	6.5 ± <0.1 (6.0–7.1)	6.0 ± <0.1 (5.6–6.4)	5.5 ± <0.1 (5.2–6.0)	5.0 ± <0.1 (4.5–5.6)
Free CO_2[c] ($mgCO_2/l$)	3.2 ± 0.4[c] (1.5–6.1)	14.5 ± 1.6 (3.0–24.8)	23.8 ± 1.9 (12.8–39.0)	34.8 ± 3.9 (21.0–78.4)	41.4 ± 5.8 (13.9–88.0)
Acidity (mg/l as $CaCO_3$)	4.6 ± 0.6[d] (3.6–5.9)	12.0 ± 1.4 (9.6–14.3)	16.7 ± 1.8 (14.1–19.6)	21.9 ± 2.1 (19.1–25.0)	21.5 ± 2.6 (17.7–25.5)
Alkalinity (mg/l as $CaCO_3$)	42.6 ± 0.2[c] (41.2–44.3)	22.4 ± 1.8 (14.8–37.4)	11.1 ± 0.8 (7.4–14.8)	5.5 ± 0.5 (3.7–9.8)	2.1 ± 0.3 (1.0–4.4)
Hardness (mg/l as $CaCO_3$)	45.3 ± 0.3[c] (43.6–47.5)	45.2 ± 0.2 (44.2–46.6)	45.3 ± 0.4 (44.1–48.5)	45.3 ± 0.2 (43.6–46.6)	45.0 ± 0.3 (41.7–47.5)
Temperature (° C)	24.3 ± 0.1[b] (23.3–26.4)	24.3 ± 0.1 (23.3–26.4)	24.3 ± 0.1 (23.3–26.5)	24.3 ± 0.1 (22.2–26.5)	24.3 ± 0.1 (23.3–26.5)
D.O. (mg/l)	5.8 ± 0.3[c] (3.6–7.3)	5.9 ± 0.4 (3.5–7.4)	6.0 ± 0.3 (3.6–7.3)	6.0 ± 0.2 (4.5–7.7)	6.0 ± 0.3 (4.5–7.7)

[a] Water quality values are for that period after pH was gradually adjusted to test level, except where otherwise indicated
[b] Mean, range, and 95% C.L. of daily reading from both A and B replicates
[c] Mean, range, and 95% C.L. of weekly analyses from both A and B replicates
[d] Mean, range, and 95% C.L. of weekly analyses for A and B replicates (15 October–12 November)
[e] Free CO_2 calculated from pH and alkalinity analyses

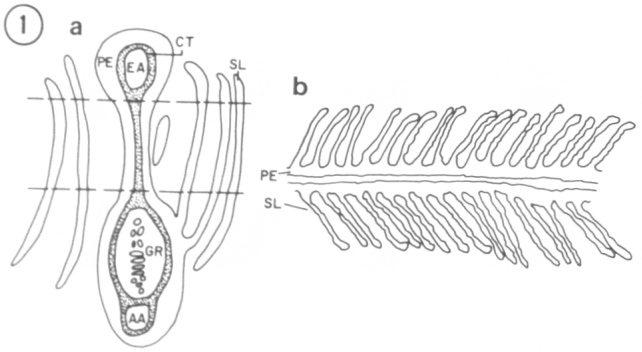


Fig. 1a. Diagram of cross-section of gill filament; for cell counts, longitudinal sections from region between two lines were used. ×100. **b** computer assisted tracing of longitudinal section of gill filament. ×60. *PE* primary lamellar epithelium, *SL* secondary lamella, *EA* efferent artery, *AA* afferent artery, *CT* connective tissue core, *GR* cartilagenous gill ray

Preparation and examination of tissues for light microscopy

The fish were fixed in cold 2% formaldehyde – 2.5% glutaraldehyde in 0.08 M phosphate buffer, pH 7.2, for at least 48 h. The first gill from each gill chamber was then excised and embedded in Dupont JB-4 methacrylate (Bennett et al. 1976). The gills were oriented with their medial hemibranches placed downward in embedding molds. Cell counts were made from 2 µm-thick longitudinal sections of comparable areas on each gill, i.e., from the portion of the filament lying between the efferent artery and cartilagenous gill ray (Fig. 1a, b). This region has a primary lamellar epithelium of relatively uniform thickness and cell composition, with many chloride cells. A section of this region also includes the central part of the secondary lamellae. Sections were stained in buffered eosin (Bennett et al. 1976) followed by Harris' hematoxylin. The slides were coded and randomized by a colleague and examined under oil immersion. Chloride cells bordering on the external epithelial surfaces were counted on a minimum of two filaments. If less than 100 cells were present, additional entire filaments were examined until the cell total exceeded 100. Data were analyzed by means of the Statistical Package for the Social Sciences (Nie et al. 1975) in conjunction with a CDC Cyber 171 computer. The Mann-Whitney U test and correlation analyses were used to evaluate differences in chloride cell numbers and morphology versus pH.

Preparation of tissues for electron microscopy

Gills were fixed as described for light microscopy and post-fixed for 2 h in cold 1% OsO_4 in 0.1 M phosphate buffer. The second gill from each side was embedded in Epon-Araldite after dehydration in graded ethanols and passage through proplylene oxide. Some gill tissue was treated with colloidal thorium for detection of polyanionic mucosubstances (Revel 1964) before being dehydrated and embedded.

Results

Three changes involving chloride cells were found to be correlated with decreasing pH: 1) increases in numbers of chloride cells; 2) increases in the percentage of these cells

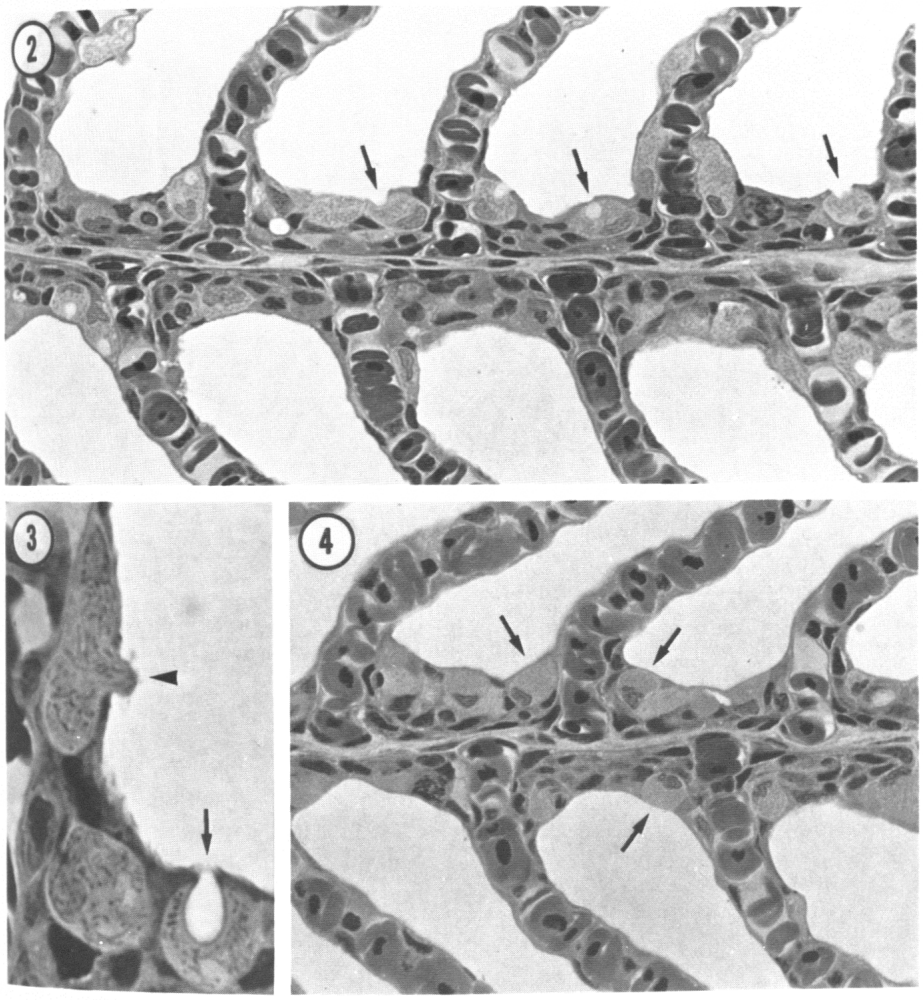


Fig. 2. Light micrograph of gill, pH 5.0. Note apical pits (*arrows*) in many chloride cells. ×480

Fig. 3. Light micrograph of gill, pH 5.0, showing chloride cells with apical pit (*arrow*) and apical evagination (*arrowhead*). ×1560

Fig. 4. Light micrograph of gill, non-acidified water (pH 7.5). Chloride cells (*arrows*) have flat or slightly convex apical surfaces. ×480

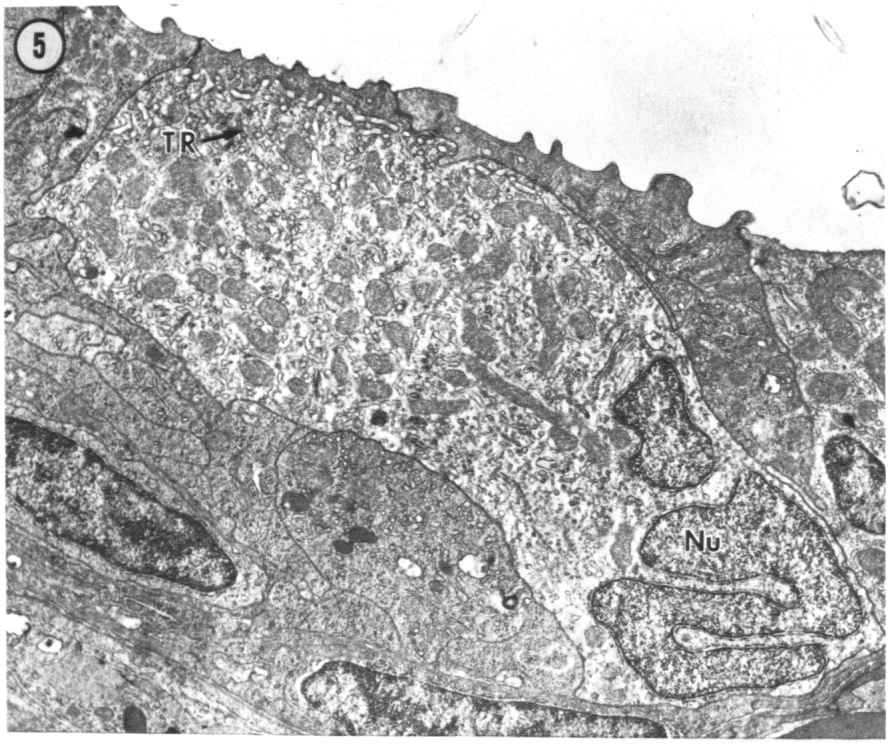
in the epithelium of secondary lamellae, and 3) increases in the percentage of chloride cells with apical pits.

The fine structure of a typical chloride cell of *P. promelas* kept in untreated Lake Superior water is similar to that of chloride cells of other species adapted to fresh water (Philpott and Copeland 1963; Threadgold and Houston 1964; Morgan and Tovell 1973; Laurent and Dunel 1980). The cytoplasm contains numerous long, sometimes branched mitochondria and an extensive tubular reticulum continuous with the lateral and basal plasmalemma (Fig. 5). Near the apex of these cells the tubular reticulum is modified into a flattened network of channels with some associated vesicles (Fig. 5); this network has been called the tubulovesicular system (Sardet et al. 1979; Philpott 1980; Bradley 1981). Superficial to the tubulovesicular system lies an electron-dense band of cytoplasm rich in microfilaments. This band is thin (0.1 μm) in some cells but prominent (0.4 μm) in others. The plasma membrane lateral to the zone of microfilaments forms 0.2 to 0.6 μm deep tight junctions (zonulae occludentes) with adjacent pavement epithelial cells or, occasionally, with other chloride cells. Other commonly seen cytoplasmic constituents include a sparse rough endoplasmic reticulum, free polyribosomes, several small Golgi elements, multivesicular bodies, and secondary lysosomes. A nucleus, usually indented and somewhat flattened, lies in the basal part of the cell.

The apical surface of the chloride cells of *P. promelas* adapted to untreated lake water is typically flat (Figs. 4, 5) or slightly convex; it usually has a few short microvilli as resolved by scanning electron microscopy (Leino et al. 1983). Apical pits are rare and, if observed, shallow.

In contrast to the typical appearance of chloride cells from fish in lake water, *P. promelas* exposed to acidified water, particularly at pH 5.5 and 5.0, have variable numbers of chloride cells which develop apical pits. These pits vary from distinct, but shallow concavities to deep, often teardrop-shaped crypts (Figs. 2, 3, 6). The plasma membrane of the pits is lined by a surface coat which stains strongly with colloidal thorium (Fig. 8). Frequently, the lumen of the deeper pits contains a colloidal thorium-positive material (Fig. 9). Some acid-exposed chloride cells have evaginations rather than pits (Figs. 3, 7; Leino et al. 1983). These evaginations contain mitochondria and tubular reticulum.

Chloride cells from acid-adapted *P. promelas* form prominent tight junctions (0.4 μm mean width) (Figs. 6, 7) usually with pavement cells, and although preliminary evidence suggests that there are more "companion", "accessory", or "immature" chloride cells in acid-adapted fish, these cells do not interdigitate with mature chloride cells, i.e., there are no apical pit-companion cell complexes as described in saltwater-adapted euryhaline teleosts (Sardet et al. 1979; Dunel-Erb and Laurent 1980; Laurent and Dunel 1980). The cytoplasmic contents of the acid-exposed and unexposed chloride cells are qualitatively similar except that occasionally groups of tubules of the tubular reticulum are arranged in parallel arrays in acid-stressed cells (Fig. 10).



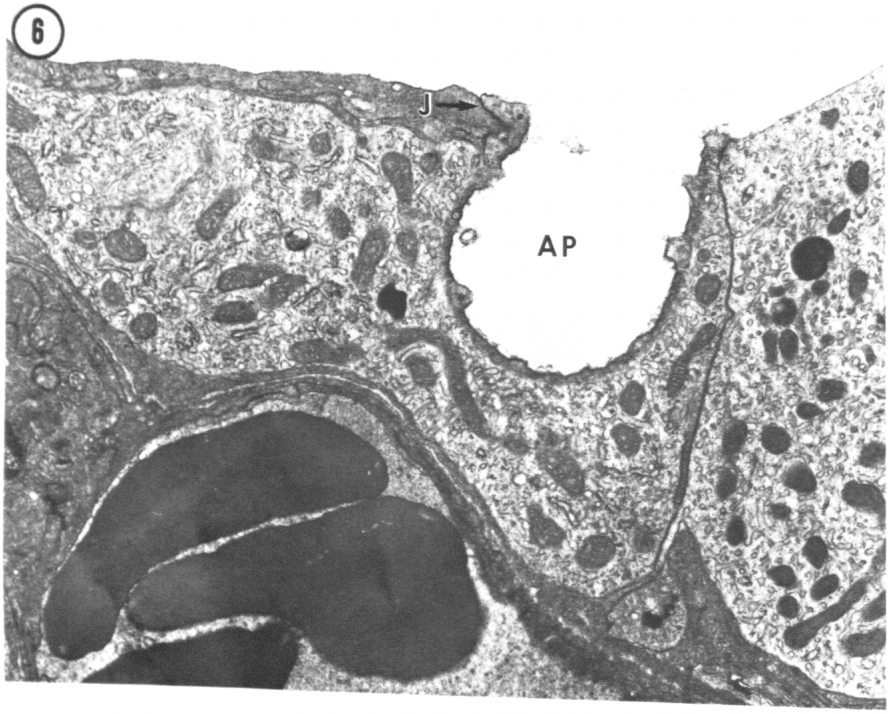


Fig. 5. Electron micrograph of chloride cell, non-acidified water. Note flat apical surface. *TR* tubular reticulum, *Nu* nucleus. ×9770

Fig. 6. Electron micrograph of chloride cell, pH 5.0, showing apical pit (*AP*). *J* tight junction. ×11450

As previously mentioned, apical pits are rare in chloride cells of control fish. They appear in greater numbers as the pH is lowered until at pH 5.0 pits occur in a mean of 22.4% (range 4.1–39.9%) of the cells (Table 2; we examined relatively thin i.e., 2 μm sections of cells; so the true percentage of cells with pits is probably higher). The graph (Fig. 11) shows a sharp and significant ($P<0.006$) rise in the mean numbers of chloride cells with pits at pH 5.5.

One female in the pH 5.0 group, killed on day 123 because she was dying, had 53.8% chloride cells with apical pits, well above the highest percentage in the surviving fish. The percentage of pitted chloride cells (14.4%) in her healthy-appearing mate, killed at the same time, was close to the mean of the pH 5.0 survivors which formed the sample group (see Fig. 11).

There was a significant correlation between increasing acid concentration and 1) the percentage of chloride cells with pits ($P\leqq0.001$), 2) the numbers of chloride cells per mm of gill ($P\leqq0.03$), and 3) the percentage of chloride cells located entirely in the epithelium of the secondary lamellae ($P\leqq0.05$) (Table 3). Similarily, comparison of the highest (pH 7.5) and lowest (pH 5.0) pH groups revealed the following differences (Mann-Whitney U test): minnows kept at pH 5.0 had a greater percentage of chloride cells with pits ($P<0.001$), a greater number of chloride cells per mm of gill ($P<0.006$), and a larger percentage of chloride

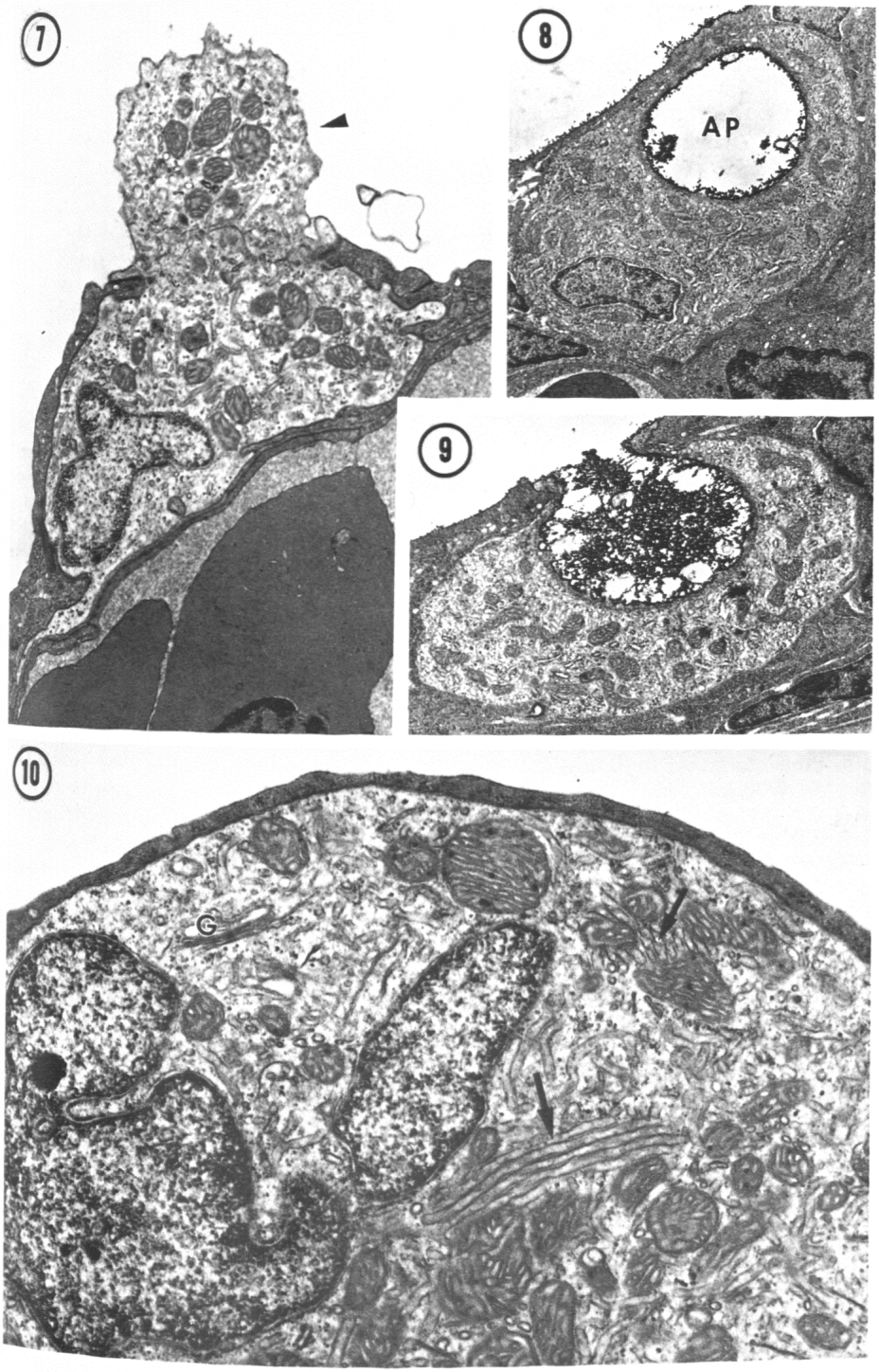


Fig. 7. Electron micrograph of chloride cell in secondary lamella, pH 5.0, showing apical evagination (*arrowhead*). ×11620

Fig. 8. Chloride cell in gill treated with colloidal thorium, pH 5.0, showing apical pit (*AP*) lined with colloidal thorium-positive surface coat. ×7000

Fig. 9. Apical pit of chloride cell, pH 5.0, filled with colloidal thorium-positive material. ×7000

Fig. 10. Chloride cell, pH 5.0, with parallel arrays of tubular reticulum (*arrows*). *G* Golgi apparatus. ×20720

cells in the secondary lamellae ($P<0.02$). There were no significant differences in the measurements between males and females at the same pH.

Discussion

When fish are exposed to acid water they tend to lose Na^+, Cl^- and other ions and to become acidotic (Packer and Dunson 1970, 1972; Leviestad and Munz 1976; McWilliams and Potts 1978; Neville 1979; Packer 1979; McDonald et al. 1980; McDonald and Wood 1981; Booth et al. 1982; Nieminen et al. 1982; Lee et al. 1983). In fresh water, chloride cells are thought by some investigators to be involved in both NaCl uptake and acid-base balance; indeed these two processes are interrelated (Evans 1975; Bornancin et al. 1980; Randall et al. 1982). Therefore, it is not surprising that the numbers, distribution, and morphology of chloride cells change in response to acid stress.

Previously, the change in morphology of chloride cells from an "unpitted" to a "pitted" condition has been described only when euryhaline teleosts have been transferred from fresh water (no pits) to salt water (pits develop). It is not known why chloride cells of saltwater-adapted fish have apical pits (Hossler et al. 1979). One suggestion is that pits increase surface area for the exchange of ions (Philpott 1968) or other substances. Another suggestion is that they

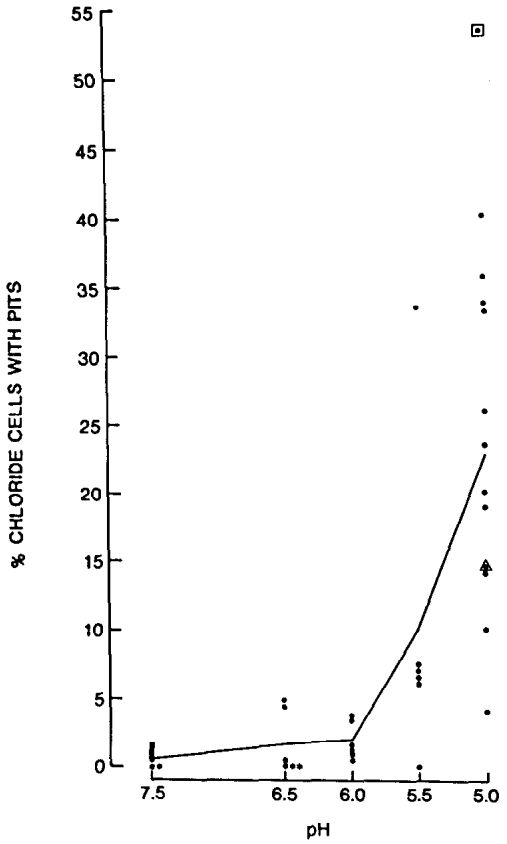


Fig. 11. Changes in percentage of chloride cells with apical pits as pH declines. Line connects mean percentage at each pH. *6 observations, **3 observations, ⊡ value for fish that became moribund on day 123 of experiment; ▵ value for its mate, removed at the same time

provide a microenvironment different from the more exposed epithelial surfaces. For example, apical pits often contain a polyanionic mucosubstance which could function in selective binding and concentration of cations, in filtration, in ion exchanges or in other ways (Philpott 1968).

The development of apical pits and apical evaginations in chloride cells of acid-stressed *P. promelas* could be an adaptation to increase surface area for taking in Na^+ and Cl^-. Between pH 5.6 and 4.5, this species exhibits a sharp decline in serum osmolarity (C.E. Firling, personal communication) suggesting a loss of Na^+ and Cl^-. To survive at low pH it must compensate for these ion losses. Perhaps the increased surface area of pits and apical evaginations provides for more ion exchange sites, e.g., involving Na^+/H^+, NH_4^+ or Cl^-/HCO_3^- exchanges; the polyanionic mucosubstance of the surface coat and in the pits of chloride cells could serve to attract cations such as Na^+. Bierther (1970) has reported a high content of both Na^+ and Cl^- in the mucosubstance of apical pits of freshwater-adapted sticklebacks (*Gasterosteus aculeatus*), a species whose chloride cells often have pits in ordinary fresh water.

Alterations of the apical morphology of acid-stressed chloride cells could, of course, be involved in some mechanism other than to increase NaCl uptake or H^+ egress. For example, they could be associated with uptake of calcium ions which acid-stressed fish tend to lose or with egress of sulfate ions which could conceivably be taken in by sulfuric acid-exposed fish (Ahuja 1970). Little is known about

Table 2. Morphologial and morphometrical differences involving chloride cells after exposure to various pH levels

Exposure pH	No. of fish examined	No. of chloride cells examined	% of chloride cells with pits	No. of chloride cells per mm of gill lamella	% of chloride cells in secondary lamella[a]
7.5	12	2286	0.6±0.6	19.1±5.1	2.4±4.5
6.5	6	1540	1.6±2.4	14.9±3.8	2.2±2.0
6.0	6	977	1.8±1.4*	17.8±7.5	2.3±3.5
5.5	6	1377	9.9±11.7**	26.0±11.1	5.3±5.0
5.0	10	2966	22.4±11.5**	29.6±10.6**	7.1±6.7*

Results are presented as the mean ± standard deviation
Significance of difference from control pH: * $P<0.03$, ** $P<0.006$, Mann-Whitney U one-tailed test

[a] In *P. promelas*, as in many freshwater teleosts, most chloride cells in the primary lamellae lie in close contact with the vascular compartment of the secondary lamellae near where it emerges from the primary lamellae; therefore the function of most chloride cells in the primary as well as secondary lamellae appears to be associated with the arterial rather than the venous circulation of the gill

Table 3. Morphological and morphometrical changes involving chloride cells: correlation with pH

Increase in (log 10)	pH 7.5–5.0		pH 6.5–5.0	
	correlation coefficient	sig.	correlation coefficient	sig.
% of chloride cells with pits[a]	$r=0.75$	$P\leqq0.001$	$r=0.77$	$P\leqq0.001$
No. of chloride cells/mm of gill lamella	$r=0.36$	$P\leqq0.03$	$r=0.61$	$P\leqq0.001$
% of chloride cells in secondary lamella	$r=0.37$	$P\leqq0.009$	$r=0.33$	$P\leqq0.05$

[a] 0% values arbitrarily set at 0.2% to obtain a logarithmic plot

branchial involvement in exchanges of divalent ions (Heisler 1982). Payen et al. (1981) demonstrated Ca^{2+} uptake through the primary lamellar epithelium of the gills of trout adapted to fresh water and suggested that chloride cells are responsible for this uptake.

In contrast to saltwater-exposed chloride cells with apical pits, pitted acid-exposed chloride cells retain extensive tight junctions and lack interdigitations with "companion" or "accessory" cells. This suggests that pits have some function common to acid and hyperosmotic stress such as amplifying surface area and supports the view that "leaky" tight junctions and interactions of chloride and companion cells are adaptations concerned with efflux of Na^+ or Cl^- in salt water.

The present experiments did not rule out the possibility that chloride cells may also be sensitive to changes in CO_2 concentrations. Calculated free CO_2 concentrations due to the addition of acid to the Lake Superior water used in

our experiments were not negligible (Table 1). They were, however, well under the levels of free CO_2 calculated by Mount (1973) who exposed *P. promelas* to acidified hard water. Mount (1973) reported a reduction in egg production at pH 5.9, 105 mg CO_2/l and a cessation of spawning at pH 5.2, 135 mg CO_2/l. No change in these processes was observed in the present study even at the lowest pH: 5.0, 41 mg CO_2/l (McCormick, unpublished). These differences in the effects on reproduction may be at least partly due to the differences in CO_2 concentrations between the hard and soft water used in the respective studies. Whether CO_2 can induce structural changes in chloride cells is not known. Increased ambient CO_2 concentrations can affect acid-base and salt balancing mechanisms (for example, Na^+/H^+, NH_4^+ or Cl^-/HCO_3^- exchanges) in cyprinids (Istin and Maren 1971; Romaneko and Krisalnyy 1977) as well as in other families. However, moderate levels of ambient CO_2 are not necessarily deleterious to acid-stressed fish (Neville 1979). Indeed, increased serum [HCO_3^-] in fish which acclimate to a combination of elevated ambient [CO_2] (Eddy et al. 1977) and chronic acid stress could provide chloride cells with counter ions for Cl^- intake (Cl^-/HCO_3^- exchange). Therefore, CO_2 concentrations could be related to functional and, presumably, morphological changes in chloride cells. However, changes in the structure of chloride cells are also seen with acid stress and low ambient [CO_2]. For example, apical pits occur in chloride cells of *P. promelas* from well-aerated, acidified Lake Superior water and of pearl dace, *Semotilus margarita*, from experimentally acidified Canadian lakes with very soft water and low CO_2 content (Leino, unpublished preliminary observations: Apical pits were seen in chloride cells of dace from Lake 223, acidified to about pH 5.0, and Lake 114, with periodic addition of acid to simulate acid rain, but not in dace from Lake 302, non-acidified – Experimental Lakes Area, northwestern Ontario, Canada; see Schindler and Turner 1982).

The present study revealed that in the laboratory *P. promelas* exposed for 129 days to Lake Superior water acidified to various pH levels down to 5.0 remained healthy in appearance. They had near normal gill structure except for changes in chloride cell numbers, distribution, and morphology. This suggests that chloride cells may be involved in acclimation to acid stress either by promoting salt or acid-base balances or by some other mechanism. The findings also suggest that simple microscopic examination of gill morphology may be useful to detect sublethal acid stress and to predict the imminence of more adverse effects on populations of fish exposed to increasing environmental acidification.

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Accepted September 13, 1983