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Salt Glands in the Tongue of the Estuarine Crocodile *Crocodylus porosus*

Abstract. *The apparent absence of salt glands in marine and estuarine Crocodilia has long been a puzzle. However, we have identified glands in the tongue of* Crocodylus porosus *which exude a concentrated secretion of sodium chloride. The glands are similar in ultrastructure to other reptilian salt glands and undoubtedly play a major role in electrolyte regulation.*

In the estuarine crocodile, *Crocodylus porosus*, a clear fluid exudes from numerous pores scattered over the upper surface of the tongue (*1*). The secretion is stimulated by injection of methacholine chloride, is hyperosmotic to the body fluids, and contains high concentrations of both sodium and chloride, pointing to the existence of a major and hitherto unknown salt gland in the Crocodilia.

Whether or not salt glands are present in Crocodilia has long been debated. Their presence was suggested more than 20 years ago (*2*) and doubted 10 years ago (*3*); recent reviews (*4, 5*) suggest that the marine or estuarine *C. porosus* and *Crocodylus acutus* probably lack significant extrarenal salt glands. However, the mechanism by which hatchling *C. porosus* are able to survive and grow in seawater (*6*) needs explanation and a resolution of the salt gland question is crucial to any understanding of their salt and water economy.

The search for salt glands has concentrated on the head because all known reptilian salt glands are cephalic. Because "crocodile tears" have been on record for at least 800 years (*7*) and because of the lack of any other visible secretions, there has been inevitable speculation that there may be orbital salt glands such as those in marine turtles (*2, 8*). However, the results of the only reported investigation (*3*) of orbital secretions were equivocal. Tears collected from both *C. porosus* and *C. acutus* showed some increase in sodium concentration after the animals were exposed to seawater, but the highest observed concentrations were considerably lower than any reported for salt gland secretions from other marine reptiles. That study has been used, with some reservations, both to accept (*8*) and to reject (*3–5*) the hypothesis that crocodiles possess functional orbital salt glands.

After our initial observation of salty secretions from the tongue, physiological and ultrastructural studies showed unequivocally that the lingual glands of *C. porosus* are salt glands comparable in structure and function to those of other marine reptiles.

Observations were made on 29 crocodiles (0.08 to 4.7 kg). Nine had been maintained in the laboratory in Sydney for 6 months or more. Twenty were captured freshly from salinities of 20 to 36 parts per thousand in the Liverpool-Tomkinson river system in northern Australia and were studied at Maningrida, Northern Territory. Of the nine laboratory animals, five had been kept in seawater for approximately 3 months, three with and two without fresh water to drink. The remainder had been kept in fresh water.

To observe and collect lingual gland secretions, crocodiles were washed thoroughly and restrained with the mouth propped open. The oral cavity was washed with distilled water and blotted dry. A watch for spontaneous secretory activity was maintained for 15 to 60 minutes. Secretion was seen in animals exposed to both fresh water and salt water, but the rates were too low to allow collection. Attempts, in some animals, to induce secretion by oral or intraperitoneal administration of 1*M* NaCl, at 10 mmole/kg, were unsuccessful. Accordingly, each animal was injected intraperitoneally with methacholine chloride (1 to 2 mg/kg), which initiates salt gland secretion in other marine reptiles (*2*). Secretion began within 3 to 15 minutes. In the 9 laboratory animals and 12 of the field animals, secretions were collected into a syringe as they welled up continuously from pores on the tongue. Tears were collected from four of the laboratory animals and plasma from eight of them. Osmotic pressure was measured with a Knauer Semimicro osmometer, Na and K were determined by flame photometry, and Cl was measured by coulometric titration. In eight field animals, Na secretory rates were measured by collecting secretions into filter papers placed on the tongue sequentially for measured time intervals. Papers were then soaked in 5 ml of distilled water to extract Na for analysis.

Lingual gland secretions from both laboratory and field animals were consistently three to five times more concentrated than the plasma and consisted principally of Na and Cl (Table 1). Among the laboratory animals, the secretion concentrations were somewhat higher in the two animals denied access to fresh water than in the remaining seven animals, but the difference was not statistically significant, and therefore the data were pooled (Table 1). In the field animals, there was no significant correlation between concentration and body weight or salinity at the capture site, and therefore these data were pooled also. Electrolyte concentrations were considerably higher in secretions from field animals compared to laboratory animals, almost certainly reflecting a greater degree of adaptation to salt water in the former group. However, some of the difference could be attributed to the smaller size of the field animals, which led to lower absolute secretory rates and the likelihood of more substantial evapo-

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Table 1. Electrolyte concentrations in plasma and lingual gland secretions of *C. porosus* and maximum observed secretory rates for Na. The means, the 95 percent confidence limits, and the number of samples (in parentheses) analyzed are shown. Field plasma data are taken from Grigg (*12*).

Condition	Weight (kg)	Osmotic pressure (mOsm/liter)	Na (m*M*)	Cl (m*M*)	K (m*M*)	Maximum Na secretory rate (μmole/100 g·hour)
Laboratory						
Secretions from lingual glands	0.8 to 4.7	1085 ± 153 (5)	509 ± 38 (9)	512 ± 50 (9)	11.2 ± 3.6 (5)	
Plasma	0.8 to 4.7	311 ± 12 (8)	145 ± 10 (8)	93 ± 18 (8)	3.4 ± 0.5 (8)	
Field						
Secretions from lingual glands	0.08 to 1.15		663 ± 34 (12)	632 ± 27 (10)	20.8 ± 1.6 (9)	49 ± 22 (8)
Plasma	0.89 to 46.44	304 ± 2 (99)	134 ± 2 (99)	118 ± 2 (99)	3.8 ± 0.2 (91)	

rative losses of water from the secretion during collection.

In the seven laboratory animals, Na, K, and Cl together accounted for 100 ± 2 percent ($\bar{x}$ ± 95 percent confidence limit) of the measured osmotic pressure and the index (Na + K)/Cl was not significantly different from 1.0. However, in the field animals, (Na + K) averaged 106 ± 6 percent (nine animals) of the Cl value suggesting the presence of some unidentified anion in the secretions. The reason for this apparent difference in composition is not yet clear. Tears collected from two seawater and two freshwater laboratory animals were all similar in composition and far less concentrated than lingual gland secretions: 155 to 275 m*M* Na; 147 to 190 m*M* Cl; 2.7 to 11.5 m*M* K.

Sodium secretory rates in field animals acclimatized to salt water ranged from 12 to 88 μmole/100 g·hour ($\bar{x}$ = 49) (Table 1). There was no significant correlation between secretory rate and body weight or capture site salinity. The figures represent the maximum rates sustained over a 10-minute sampling period under the conditions of the experiment and may not reflect rates under normal circumstances. However, the measurements do suggest that the salt glands are capable of secreting the 45 μmole of Na per 100 g·hour otherwise unaccounted for in recent measurements of ^{22}Na efflux in *C. porosus* hatchlings free-ranging in seawater (*9*).

The secretory organs are 30 to 40 discrete, compound, tubular glands in the mucous membrane of the posterior part of the tongue, each having a shallow duct leading to the surface (Fig. 1a). This broad, shallow duct leads down to a series of smaller ducts that branch repeatedly into the lobules of the gland. The larger branches are lined with columnar-cuboidal epithelium which passes into squamous epithelium in the smaller branches. Each lobule of the gland is densely packed with branching secretory tubules lined almost entirely by columnar epithelial cells. These cells show the extensive interdigitations of the lateral cell membranes and abundant mitochondria characteristic of principal cells from all other reptilian salt glands (Fig. 1b) (*4*). Blood vessels and unmyelinated nerve fibers are abundant in the interstitial tissue surrounding secretory tubules. The lingual glands resemble the sublingual salt glands of sea snakes in lacking the distinct peripheral tubules found in lizard and turtle salt glands (*4*). The ultrastructure of the glands supports the physiological evidence that they are specialized salt-secreting organs.

The discovery of lingual salt glands in *C. porosus* brings to five the number of distinct cephalic glands that have become specialized for salt excretion in marine and estuarine reptiles. The other recognized salt glands are the posterior sublingual glands of hydrophid and achrochordid snakes, the premaxillary gland of the homalopsid snake, *Cerberus rhyn-*

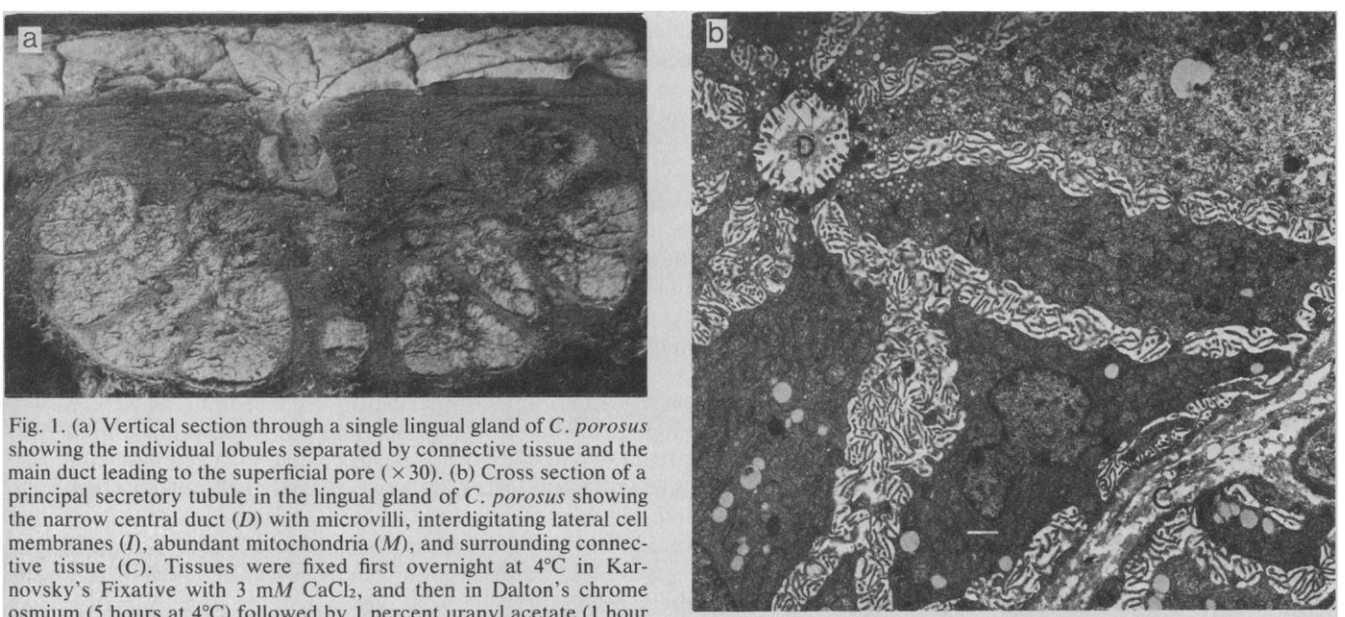

Fig. 1. (a) Vertical section through a single lingual gland of *C. porosus* showing the individual lobules separated by connective tissue and the main duct leading to the superficial pore (×30). (b) Cross section of a principal secretory tubule in the lingual gland of *C. porosus* showing the narrow central duct (*D*) with microvilli, interdigitating lateral cell membranes (*I*), abundant mitochondria (*M*), and surrounding connective tissue (*C*). Tissues were fixed first overnight at 4°C in Karnovsky's Fixative with 3 m*M* $CaCl_2$, and then in Dalton's chrome osmium (5 hours at 4°C) followed by 1 percent uranyl acetate (1 hour at 4°C); the preparation was dehydrated in acetone and infiltrated with Spurr's resin. The specimen is taken from a crocodile kept in salt water with fresh water available for drinking. [Scale bar, 1 μm]

chops, the nasal gland of iguanid and varanid lizards, and the lachrymal glands of emydid and chelonid turtles (*5*, *10*). Clearly, there have occurred a number of independent radiations of reptiles into marine and estuarine habitats and, not surprisingly, the salt glands that have evolved show a wide range of secretory capabilities. At one end of the spectrum are sea snakes such as *Aipysurus eydouxii* with glands capable of secreting Na at more than 200 μmole/100 g·hour while at the other extreme are snakes such as *Cerberus* whose premaxillary gland can secrete at perhaps 15 μmole/100 g·hour and some estuarine snakes that lack salt glands entirely (*11*). Our data suggest that, compared to those of other reptiles, the glands of *C. porosus* have a moderate secretory capacity, but their quantitative performance in the overall electrolyte budget remains unassessed. That functional salt glands are present is suggested by the survival and growth of hatchlings in seawater (*6*), and by plasma electrolyte homeostasis and low urinary sodium seen in animals from seawater (*12*). In view of both ultrastructure and physiological data, there is little doubt that the lingual glands are important salt glands in *C. porosus* and play an essential role in the maintenance of electrolyte homeostasis.

Since the initial identification of salt glands in *C. porosus*, we have seen similar lingual pores or secretions in *C. johnstoni, C. acutus,* and *Alligator mississippiensis* and also in *Caiman crocodilus* from which lingual glands were illustrated last century (*13*). The glands are probably present in all crocodilians and comparative investigations of their structure and function should provide valuable insights into patterns of evolution among crocodilians.

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Transbilayer Phospholipid Asymmetry in *Plasmodium knowlesi*–Infected Host Cell Membrane

Abstract. *The membranes from normal and* Plasmodium knowlesi*–infected rhesus monkey erythrocytes (90 to 95 percent infected with early ring stage) were analyzed for transbilayer distribution of phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserine (PS), by means of chemical and enzymatic probes. The external monolayer of the normal red cell membrane contained at least 68 to 72 percent of the total phosphatidylcholine and 15 to 20 percent of the total phosphatidylethanolamine. In the infected cell, the transmembrane phosphatidylcholine distribution appeared to be reversed, with only 20 to 30 percent of it being externally localized, whereas roughly equal amounts of phosphatidylethanolamine were present in the outer and inner surfaces. However, total phosphatidylserine in both the infected and normal red cells was exclusively internal. Unlike that in the normal intact cell, external phosphatidylethanolamine in the parasitized cell was readily accessible to phospholipase A_2. These results indicate that significant changes in molecular architecture of the host cell membrane are the result of parasitization.*

Knowledge of molecular changes induced by the malaria parasite in the structural framework of the host cell membrane is essential to an understanding of host-parasite interactions. Various biochemical, biophysical, immunochemical (*1–4*), and electron microscopic studies (*5*) have revealed that the malarial parasite produces distinct morphological changes in the host erythrocyte membrane at the time of entry and during development.

Recently, Schmidt-Ullrich and Wallach (*3*) demonstrated the presence of *Plasmodium knowlesi*–specific antigens in the membrane of parasitized red cells of the rhesus monkey. However, few attempts have been made to determine the molecular changes occurring in the structure of host erythrocyte membrane integral proteins, glycolipids, and phospholipids. Alterations in the lipids of the red cell membranes in *Plasmodium lophurae* infections have been suggested by Holz (*6*). We have studied transbilayer phospholipid asymmetry in plasma membranes of rhesus monkey red cells that were 90 to 95 percent infected with the early ring stage of *P. knowlesi*. We report here a dramatic change in lipid organization in the membranes of infected monkey erythrocytes.

Synchronous infections of *P. knowlesi* (W 1 strain) were maintained by serial passage of infected blood in rhesus monkeys, caged in a room illuminated with fluorescent lights from 7:00 a.m. to 7:00 p.m. The monkeys were bled when parasites were at the early ring stage and when parasitemia was 90 to 95 percent, as determined by Giemsa staining. Blood was drawn into heparinized glass tubes and washed thrice with phosphate-buffered saline (PBS) (*p*H 7.2). White blood cells and platelets from normal blood, and white blood cells, platelets, and schizonts from the infected blood were removed by means of a Ficoll-Hypaque gradient, as described by Wallach and Conley (*2*). The purified infected erythrocytes, when checked by Giemsa staining, were 90 to 95 percent infected with early rings contaminated with 2 to 3 percent trophozoites.

Ghosts from normal or parasitized erythrocytes were prepared by lysing the cells with saponin (*7*). Normal red cell ghosts were isolated by centrifuging the saponized hemolyzate at $2 \times 10^4 g$. The hemolyzate from parasitized cells was centrifuged at 370*g* and the pellet was discarded. The supernatant was further centrifuged at $10^4 g$ to remove additional parasites. Finally, the membranes of the parasitized cells were isolated by centrifuging the supernatant derived from the later step at $4 \times 10^4 g$. The membranes prepared in this way were completely free of parasites as determined by light microscopy.

Extraction of lipids from the membranes was carried out according to the procedure of Folch *et al.* (*8*). Separation of various phospholipids was done on silica gel 60F-254 (with 0.25-mm, 20 by 20 cm, Merck glass plates) as described

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