Leo and buoy B2 around eddy Maria.) Figure 3c shows eddy Maria moving around to the north and eddy Leo moving in a manner suggesting that the line between the eddies was shortening. In Figure 3d the buoys achieve a synchronism of rotation around a large feature that would seem to preclude the possibility that the centers of the two eddies were still separated. The new eddy was named eddy Mario (see Fig. 2).

Our observations support the idea that two eddies with characteristic signature layers coalesced in a process lasting about 20 days. During the coalescence, both eddies seemed to move around a point on the contracting line joining their centers. Part of the signature layer of eddy Maria was lost as a result of the coalescence, and the lower boundary was uplifted about 230 m. The signature layer of eddy Leo stayed mainly intact as it was depressed by at least 100 m beneath the uplifted part of eddy Maria's signature layer. The temperature and salinity structure of the combined eddy was very similar to that of an eddy observed 1 year earlier (eddy J) and believed to be the result of coalescence. The buoy tracks were reminiscent of the two-dimensional coupling of vortices revealed by computer simulation (*8*).

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The Chloride Cell: Definitive Identification as the Salt-Secretory Cell in Teleosts

Abstract. *Chloride-secreting isolated opercular membranes from the seawater-adapted teleost* Sarotherodon mossambicus *contain the several cell types also seen in the branchial epithelium. The vibrating probe technique has been used to localize conductance and chloride current specifically to the so-called chloride cells, thereby establishing these cells definitively as the extrarenal salt-secretory cells.*

Seawater-adapted teleost fish live in a dehydrating environment but cannot produce a concentrated urine. Numerous ion transport studies have made it clear that the branchial epithelium, in addition to serving as a respiratory organ, functions as the site of extrarenal salt secretion in these fish (*1*). It is generally accepted (*2*) that the anatomical basis of branchial salt secretion is chloride cells, large, granular, acidophilic cells originally described by Keys and Willmer in seawater eel gills (*3*). The gill epithelium is composed of four principal cell types (*4*), however, and each of these cell types has, at one time or another, been implicated as the salt-secretory cell type (*3*, *5*). Despite numerous ultrastructural, enzymatic, and kinetic studies (*1*, *2*), there is still no direct experimental support for the idea that the chloride cell is the site of branchial salt secretion.

We have used the vibrating probe technique (*6*) to localize current and conductance pathways in the opercular membrane isolated from the seawater-adapted euryhaline teleost, the tilapia *Sarotherodon mossambicus* (formerly *Tilapia mossambica*). This epithelium possesses a rich population of chloride cells characterized by an ultrastructure typical of branchial chloride cells (*7*) and displays ion transport properties that respond to various hormonal and pharmacological agents in a manner analogous to the branchial responses (*7*, *8*). We provide here the first conclusive evidence that chloride cells are sites of active chloride secretion and high ionic permeability.

Opercular membranes were isolated from seawater-adapted tilapia as described in (*7*). The pigmented serosal connective tissue was gently removed with fine-tipped forceps, and the translucent epithelium was mounted horizontally, apical side up, in an Ussing chamber containing tilapia Ringer solution. The apical side was approached with the vibrating probe under visual control; we used bright-field optics at ×100 or ×320. The transepithelial voltage was measured with agar bridges, each within a few millimeters of either side of the tissue, and connected to calomel electrodes. The epithelium was voltage-clamped by standard techniques with series resistance compensation.

The vibrating probe apparatus developed by one of us (C.S.) is similar to that described by Jaffe and Nuccitelli (*6*), except that the tip was smaller (7 μm in diameter) and vibrated at higher frequencies (1.6 or 6 kHz) (*9*). The tip of a platinized gold electrode was vibrated along a line 10 to 15 μm long, and its signal was analyzed with computational circuitry to measure a weighted average of the voltage gradient along the line of vibration. We calibrated this measured voltage gradient and then converted it into current density by dividing by the medium bulk resistivity. It was assumed that the bulk resistivity closely approximated the medium resistivity near the tip because the acoustic streaming at the tip vigorously mixed the bulk solution with the solution at the tip (*6*, *9*). With the assumption that chloride cells represent point sources of current density at the apical surface, we calculated the current per chloride cell by multiplying the current density above a chloride cell by the area of a hemisphere centered at the chloride cell with a radius equal to the height of the probe above the surface. We checked the probe signal corresponding to zero current density periodically throughout the experiments by moving the probe into an area of the bath where there was no current. The signal drift was usually negligible during an experiment and corresponded to no more than 5 percent of the current density measured over a chloride cell.

The short-circuit current (I_{sc}) across the tilapia opercular membrane represents chloride-carried negative charge flow into the apical solution (*7*). To investigate the distribution of current sources on the epithelium, the vibrating probe was positioned so that the closest excursion of its vibration was 5 to 10 μm above the apical surface with the line of vibration at a 60° angle with the surface. Under short-circuit conditions, the vibrating probe output revealed peak negative current densities over 90 to 95 percent of the chloride cells examined in eight different opercular membranes. Current density peaks were never observed over nonchloride cells. Figure 1 is a typical transect in which the vibrating probe was moved at a constant height (20 μm) above the tissue between two chloride cells. Negative current density is greatest when the tip of the probe is

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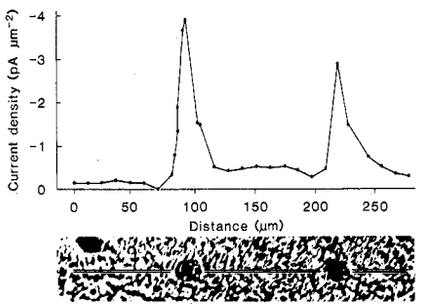

Fig. 1. Demonstration that negative current flow through the isolated opercular membrane into the apical solution is associated only with chloride cells. The opercular membrane was mounted horizontally in an Ussing style chamber containing tilapia Ringer solution (160 m*M* Na^+, 151 m*M* Cl^-, 15 m*M* HCO_3^-, 3 m*M* K^+, 2 m*M* Ca^{2+}, 1 m*M* Mg^{2+}, 1 m*M* SO_4^{2-}, 1 m*M* $H_2PO_4^-$, 5 m*M* tris; *p*H adjusted to 7.9 when gassed with 100 percent O_2 at 25°C). Chloride cells were identified visually during experiments at ×100 or ×320 on the basis of their large size (approximately 20 μm in diameter), as no other cell type in the opercular membrane is this large (*7*), and by staining with the fluorescent mitochondrial probe 2-(dimethylaminostyryl)-1-ethylpyridiniumiodine (DASPEI) (*11*) at the end of each experiment. Chloride cells represent the only cell type with appreciable numbers of mitochondria in the tilapia opercular membrane and can be specifically stained with 2 μ*M* DASPEI (*7*). With higher concentrations of DASPEI (0.1 m*M*), chloride cells are still the only cells that are stained in fluorescence microscopy and they appear yellow with normal bright-field illumination, thereby permitting positive identification under the present experimental conditions. We used DASPEI to confirm the identity of chloride cells only at the end of each experiment since in vitro exposure to even low concentrations (2 μ*M*) of DASPEI has an inhibitory effect on I_{sc}. (Bottom) The two chloride cells studied in this transect are separated by 127 μm (center to center) and have been stained with DASPEI (0.1 m*M* at the end of the experiment) to confirm their identities. The photograph was taken with Kodak Tech Pan 2415 with illumination through a blue filter (Kodak 45). (Top) Vibrating probe output (one pole–filtered with a time constant of 1 second), converted to current density, obtained at 11.8-μm intervals as the tip of the probe was moved in a straight line between two chloride cells. Additional determinations were made at and near the chloride cells. With the probe tip 20 μm above the tissue, current density peaks correspond to 9.8- and 7.3-nA epithelial surface sources of negative charge flow into the apical solution. Although the apparent cell diameters when viewed from above are almost 20 μm, an apical crypt 4 to 5 μm in diameter represents the contact of the cell with the external medium (*2*, *7*); thus, one would not expect peak current flow to be recorded until the tip is immediately over the apical crypt, presumably near the center of the cell when viewed from above, as observed. The probe was moved along the horizontal component of probe vibration; thus, the asymmetry of the peaks (that is, a steeper slope on the left side as opposed to the right side of the cells) is as predicted because of the horizontal component of tip vibration. This opercular membrane was isolated from a tilapia adapted to seawater for 7 weeks.

over the centers of the chloride cells and rapidly falls off as the probe is moved into nonchloride cell areas.

The decrease in the probe signal as the tip is moved a short distance away from a chloride cell is the same as that predicted from calculations of the expected current density distribution about an isolated current source on a planar surface. It can be calculated that the contribution to the probe signal from a unit current source immediately under the probe is about twice that of an equivalent current source 10 μm away from that point and about ten times that of a unit current source 30 μm away. This calculated resolution does not mean that the probe ignores the total contribution from all current sources more than 30 μm away. The negative current density at the position marked "150 μm" in Fig. 1 is too large to be accounted for as current spread from the two chloride cells on the transect but can be accounted for as current spread from all the chloride cells within 100 μm of that position.

This nonzero current density between chloride cells complicates efforts to determine whether nonchloride cells are pumping a small current. The current density reported by the probe over areas of the epithelium devoid of chloride cells is not measurably different from zero; the uncertainty in measurement is small enough to allow the conclusion that, if the nonchloride cells in these areas are pumping any current under short-circuit conditions, the current is at least two orders of magnitude smaller (per cell) than the current pumped by chloride cells. Our belief that the nonzero probe signal measured between chloride cells is entirely due to current spread from chloride cells is also supported by the observation that this signal is more negative when measured with larger tip probes (20 μm), which have a lower spatial resolution and consequently detect more current density from distant chloride cells. Therefore, it appears that under short-circuit conditions there is current flow across the opercular membrane only through chloride cells and that small amounts of current may be detected over nonchloride cell areas because of insufficient spatial resolution of the vibrating probe.

On the basis of data obtained from limited numbers of chloride cells, the probe-measured short-circuit currents per chloride cell (I_{sc}^{cc}) are in excellent agreement with those determined earlier by indirect methods. Thus, probe-measured I_{sc}^{cc} for tilapia adapted to seawater for 2 and 3 weeks were 2.4 and 3.3 nA per cell (measurement error ≤ 50 percent), respectively, as compared with 2.4 and 3.0 nA per cell determined indirectly by dividing I_{sc} by the total number of chloride cells (measurement error ≤ 15 percent) (*7*). Earlier work indicated that I_{sc}^{cc} was still increasing after 3 weeks of adaptation to seawater (*7*); the present data suggest that long-term adapted fish do have higher I_{sc}^{cc}, ranging from 4.6 to 9.8 nA per cell for 7-week seawater-adapted fish.

Figure 2 depicts typical instantaneous current/voltage (*I*/*V*) relations obtained over chloride cells and nonchloride cells. The dependency of the current density upon voltage was so small over nonchloride cells (< 2 fA μm^{-2} mV^{-1}) that it was usually necessary to clamp the tissue to large voltages to detect any cur-

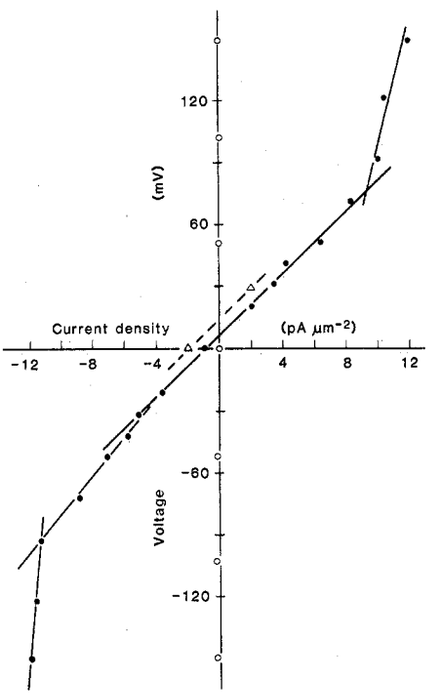

Fig. 2. Instantaneous current-voltage (*I*/*V*) relationships for a chloride cell and a nearby nonchloride cell. Transepithelial voltage was stepped from 0 mV to symmetrical positive and negative voltages for 0.5 second. The probe signal was one pole–filtered with a time constant of 25 to 250 msec. Since the conductance (the inverse slope of the *I*/*V* relationship) for the nonchloride cell is exceedingly low (0.06 mS cm^{-2}), only the data points (open circles), and not the line representing the *I*/*V* relation, are shown. Chloride cell slope conductance is much higher (13.42 mS cm^{-2}), although it typically decreases at high positive and lower negative voltages. The complete chloride cell *I*/*V* relationship in this experiment was determined with the probe tip over the edge of cell (closed circles). Also shown are two *I*/*V* determinations after subsequent movement of the tip to the center of the cell to maximize the current density (triangles). The conductance at the center appears to be the same as over the edge of the cell. This opercular membrane was from a 7-week seawater-adapted tilapia.

rent flow through these areas. It is even possible that this small current represents interference from current flow through chloride cells (see above). In any case, it is clear that nonchloride cells offer high resistance to ionic flow. In contrast, chloride cells represent sites of much higher conductance. The rectified nature of the chloride cell *I/V* relationship is similar to that observed for the entire opercular membrane (*8*), demonstrating that *I/V* characteristics of chloride cells determine these parameters for the entire tissue. The chloride cell conductance (G^{cc}) begins to decrease at -20 ± 3 mV (N = five tissues) and $+76 \pm 10$ mV ($N = 5$). These voltage-dependent decreases of G^{cc} may be caused by conductance or transport pathways that are voltage-sensitive or saturable, or both. However, their exact cause and anatomical location are unknown. Probe-measured G^{cc} from five cells in five tissues, determined from the slopes of the *I/V* relationships over the linear range, are similar for 2- and 3-week seawater-adapted fish, 0.05 and 0.04 microsiemens (μS), respectively. These values compare well with indirect estimates based on total tissue conductance and the total number of chloride cells, that is 0.09 and 0.15 μS for 2- and 3-week seawater-adapted fish, respectively (*7*). Adaptation of fish to seawater for 7 weeks appears to result in an enhancement of probe-measured G^{cc} (0.35 to 1.14 μS).

We have provided direct evidence that chloride cells are the only significant electrogenic and conductive elements in the tilapia opercular membrane. The large negative current densities observed over chloride cells represent net chloride extrusion by these cells under short-circuit conditions since transepithelial I_{sc} appears to be carried exclusively by chloride (*7*). The basis of the branchial salt extrusion mechanism is also an active chloride secretion process (*10*). Nonchloride cells do not appear to be involved in net electrogenic ion transport and, in view of their high resistance, probably serve as relatively impermeable barriers to passive conductive ionic flow. These results validate the cumulative correlative data that have strongly implicated the chloride cell as the one responsible for branchial salt secretion (*1*, *2*). Furthermore, it is now clear that Ussing chamber studies of opercular membrane electrophysiology under the conditions of our experiments represent electrophysiological studies of chloride cell biology. The vibrating probe technique should have successful application in other epithelia in which the heterogeneous nature of the cell types comprising the epithelium has made the assignment of transport functions ambiguous.

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Liposomes as Gene Carriers: Efficient Transformation of Mouse L Cells by Thymidine Kinase Gene

Abstract. *Stable transformation of mouse L cells deficient in thymidine kinase was achieved by liposome-mediated transfer of a recombinant plasmid carrying the thymidine kinase gene. Ten percent of the recipient cells expressed thymidine kinase activity. The transformed phenotype (for example, 200 out of 10^6 cells) was stable under selective and nonselective conditions. The liposome technique is compared with other methods currently used for gene transfer.*

A variety of techniques for introducing nucleic acids into eukaryotic cells in vitro has been developed. These techniques include incubation of the recipient cells with coprecipitates of DNA and calcium phosphate (*1*); direct injection of genes into the nucleus of the recipient cell (*2*); and use of viral vectors to carry genes into cells (*3*). Because these methods have some limitations, the use of liposomes as vehicles for gene transfer has been investigated. Liposomes could offer certain advantages, such as simplicity, low toxicity, and higher efficiency, and they could, perhaps, be used in vivo.

Recently, viral SV40 DNA (*4*) and a prokaryotic gene coding for β-lactamase (*5*) were introduced into mammalian cells by liposomes and their expression has been shown. However, SV40 DNA is replicated extrachromosomally in permissive cells, and it is not known whether the liposome-mediated transfer of eukaryotic genes will lead to stably transformed cell lines. We have studied this question and have further evaluated the liposome method of gene transfer using the thymidine kinase gene as a model system. The transfer of this gene to mutant mouse cells deficient in thymidine kinase was previously accomplished with the calcium phosphate method (*6*). A newly acquired thymidine kinase activity can be assayed immediately, the transformed cells can be selected by a special culture medium, and the covalent integration of the TK gene in chromosomal DNA is a prerequisite for stable expression (*7*). We report here the liposome-mediated delivery of the TK gene to LTK^- cells and the stable expression of thymidine kinase in transformants. We found that the efficiency of transformation is comparable to that of other transfection methods.

For the preparation of DNA-loaded liposomes (*8*) we used the method of reverse phase evaporation, which yields large unilamellar vesicles with high efficiency of entrapment (*9*). As one of the lipid components we chose phosphatidylserine, since a high percentage of negatively charged phospholipid favors the binding capacity of liposomes to cells and their cellular uptake (*10*); as another component we used cholesterol, since a high content of cholesterol increases the size of the vesicles (*11*), reduces liposomal leakage, and stabilizes the vesicles in the presence of serum proteins (*12*). Short sonication of the two-phase system of lipid-ether and DNA-buffer produces inverted micelles which, by evaporation of ether, reassemble into lipo-

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