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Effects of Cd^{++} on Short-Circuit Current across Epithelial Membranes

II. Studies with the Isolated Frog Skin Epithelium, Urinary Bladder, and Large Intestine

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Summary. Cadmium ion (Cd^{++}) was found not to inhibit active sodium transport across the isolated frog skin when added in 10^{-3} M concentration to the basal-lateral surface. The same Cd^{++} concentration similarly had no effect on Na^+ transport across the isolated epithelial cell layer from the frog skin, although this dose of Cd^{++} did inhibit Na^+ transport across the frog urinary bladder and large intestine. When 10^{-3} M Cd^{++} was added to the apical surface of the isolated frog skin or to the isolated epithelial cells from the frog skin, sodium transport was reversibly stimulated, in contrast to the irreversible inhibition noted above. If equimolar cysteine was added with Cd^{++} to the apical surface of the skin, however, active Na^+ transport was irreversibly inhibited. In conjunction with the inhibition produced by equimolar Cd^{++} and cysteine, isotopic Cd^{++} permeation into the tissue was three times higher when added with cysteine than in the absence of cysteine. Thus, the effects of Cd^{++} on epithelial Na^+ transport is variable according to the epithelium studied and the presence of potential carrier molecules.

In an earlier study (Hillyard & Gonick, 1976) cadmium ion (Cd^{++}) added to the outer (apical) surface of the isolated frog skin was found to stimulate active sodium transport, as measured by short-circuit current (SCC). Cd^{++} added to the inner (basal-lateral) surface of the skin had no effect on SCC. This result was somewhat unexpected since Cd^{++} is known to inhibit the transport enzyme sodium-potassium adenosine triphosphatase (Na^+-K^+ ATPase) at concentrations as low as 10^{-6} M (Rifkin, 1965) and other inhibitors of this enzyme, such as ouabain and mercuric ion (Hg^{++}) inhibit SCC across the isolated frog skin (Lodin, Janacek & Muller, 1963).

Chronic Cd^{++} exposure in man and experimental animals is known to produce Fanconi syndrome, a disorder in which proximal tubular

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reabsorption of sodium and other solutes is impaired (Gonick, 1978). Thus, Cd^{++} appears to be an inhibitor of epithelial sodium transport in the proximal tubule but not in the frog skin. One possible explanation for the different responses of these tissues is that Cd^{++} becomes trapped in the dermal layers of the frog skin and is prevented from reaching the active transport site in the epithelial cells (Hayashi, Takada & Arita, 1977). A second possibility might be that a barrier exists to the permeation of Cd^{++} into or across the membranes of various epithelial cells and that a carrier might be required to facilitate permeation of Cd^{++} into some, but not all, epithelial tissues.

Cd^{++} is known to stimulate the synthesis of a metal-binding protein, metallothionein (Webb, 1972), in several mammalian species. This protein is known to modify the physiological response to Cd^{++}. For example, when Cd^{++} metallothionein is injected acutely into the rat, there is considerable renal injury but little testicular damage (Gunn, Gould & Anderson, 1968; Nordberg, Royer & Nordberg, 1975). Thus, the kidney and testis appear to have different permeability characteristics for Cd^{++}. There is also evidence that cysteine, the principal amino acid of metallothionein, can serve to enhance Cd^{++} permeability into kidney tissue. Vander (1962) found Cd^{++} to stimulate proximal tubular sodium reabsorption only when injected into the renal artery with an equimolar amount of cysteine. Cd^{++} injected alone was without effect in these experiments.

To explore these possibilities, we studied the effects of Cd^{++}, either alone or with an equimolar amount of cysteine, on SCC across the isolated frog skin, isolated frog skin epithelium, large intestine, and urinary bladder.

Materials and Methods

Measurement of Short-Circuit Current

Short-circuit current was measured across the intact frog skin, the isolated epithelial cell layer from frog skin, urinary bladder, and large intestine of the frog, *Rana pipiens* (Los Angeles Biologicals) by the method of Ussing and Zerahn (1951). Voltage across the different tissues was measured with agar-Ringer's bridges and paired calomel reference electrodes connected to an Orion Research model 801 digital voltmeter. Short-circuit current was applied with Ag–AgCl electrodes via agar-Ringer's bridges to the Ringer's chambers. In all experiments, potential was monitored constantly (open circuit) with SCC applied every 10 min. A Ringer's solution having the composition: 111 mM NaCl, 2.0 mM KCl, 2.4 mM $NaHCO_3$, 1.0 mM $CaCl_2$ and 5.0 mM glucose (standard Ringer's) bathed both sides of the tissues in all experiments unless otherwise specified. Cadmium chloride was added

to the standard Ringer's to give a concentration of 10^{-3} M (Cd^{++} Ringer's), and the effects of this single dosage was compared in the three tissues.

The use of SCC to estimate the rate of active sodium transport has been practiced on anuran skin (Ussing & Zerahn, 1951), urinary bladder (Bentley, 1960), and large intestine (Cofré & Crabbé, 1967). We, therefore, feel that SCC is a valid technique for comparing, qualitatively, the effects of Cd^{++} on active sodium transport across these tissues.

Effects of Cd^{++} on the Inner Surface of the Intact Frog Skin

This study utilized a Ussing-type cell with two chambers so that one area from a piece of skin could serve as a control for a second area and the exposed area in each chamber was 1 cm^2.

After the animals were doubly pithed and the ventral abdominal skin was mounted between the chambers, a 1-hr period was allowed for equilibration before voltage and SCC measurements were begun. When SCC values varied by less than 10% for 30 min, the standard Ringer's bathing the basal-lateral surface of the skin was replaced with Cd^{++} Ringer's and SCC recorded for 60 min. This procedure was followed in nine separate preparations.

Isolated Frog Skin Epithelium

The layer of epithelial cells was isolated from the skin with the collagenase method described by Siegel, Tormay and Candia (1975) and mounted between the halves of a single-chambered Ussing cell which gave an exposed area of 1 cm^2. Once the isolated epithelium was mounted, voltage and SCC were monitored until SCC varied by less than 10% over a 30-min period. At this time, the standard Ringer's bathing the apical surface of the skin was replaced with Cd^{++} Ringer's and SCC monitored for 30 min at which time the Cd^{++} Ringer's was replaced with standard Ringer's. Two additional washings of the apical Ringer's were performed and SCC was allowed to return to values near those of the original control period. When SCC values again varied by less than 10% over a 30-min period, the standard Ringer's bathing the inner surface of the epithelium was replaced with Cd^{++} Ringer's and SCC monitored for a final 60 min. In this manner each piece of epithelium served as its own control for assessing the effects of Cd^{++} on the apical and basal-lateral surfaces. This procedure was followed in five separate preparations. As an additional control, five preparations were treated with Cd^{++} Ringer's at the apical surface and the Cd^{++} effect was monitored for 60 min without a washing procedure. This was done to insure that the reversible nature of apical Cd^{++} treatment was not a transient rise in SCC.

Effects of Cd^{++} and Cysteine on the Intact Skin

Cd^{++} and cysteine were found to precipitate as a complex at pH values above 5.8, making it impossible to use the standard Ringer's solution. Instead, a modified Ringer's solution was prepared by replacing the sodium bicarbonate buffer with 20 mM imidazole-HCl buffer adjusted to a pH of 5.5. Sufficient NaCl was deleted to keep osmolality constant. It was possible to maintain steady SCC values with this more acidic Ringer's bathing the apical surface of the skin, but not the basal-lateral surface. It was, therefore, necessary to bathe the basal-lateral surface with an imidazole-HCl Ringer's solution buffered to

pH 7.0. Thus, the effects of the Cd^{++}-cysteine complex could be tested only on the apical surface of the skin. Control SCC with imidazole Ringer's averaged 33.0 ± 8.7 μA (mean ± 1 SE, $n=10$) as contrasted with 43.6 ± 9.5 μA with the standard Ringer's (mean ± 1 SE, $n=9$).

Experimentally, the intact skin was mounted in the two-chambered cell described earlier, with a 1-hr equilibration period observed before recording PD and SCC. Initially, the apical surfaces of both chambers were bathed with the imidazole-HCl Ringer's having a pH of 5.5, while the pH of the solution bathing the basal-lateral surfaces was 7.0. When SCC values varied by less than 10% over a 30-min period, an imidazole-HCl Ringer's solution containing 10^{-3} M cysteine was added to the chamber bathing the apical surface of one of the chambers. The apical Ringer's solution in the other chamber was replaced with an imidazole-HCl Ringer's containing equimolar 10^{-3} M cysteine and Cd^{++}. In all cases the pH of the apical solution was carefully maintained at 5.5. SCC was monitored for 60 min after the addition of cysteine or cysteine and Cd^{++} at which time the additions were washed out with fresh imidazole-HCl Ringer's pH 5.5, to see if the effects of the Cd^{++} and cysteine could be reversed.

As an additional control. 10^{-3} M Cd^{++} was added to the apical surface of another series of five skins in order to insure that the reduced pH of the imidazole-HCl Ringer's did not produce any qualitative changes in the effects of Cd^{++} on the tissue.

$^{109}Cd^{++}$ Permeation into the Frog Skin

The movement of Cd^{++} into and across the frog skin and the modification of this movement by cysteine was determined with $^{109}CdCl_2$. The skin was placed between the chambers of a single-chambered Ussing cell, each having a volume of 3 ml and giving an exposed surface area of 1 cm^2. The apical surface of the skin was bathed with imidazole-HCl Ringer's, pH 5.5, while the pH of the basal-lateral Ringer's was 7.0. In one series of five skins 1–1.5 μCi of $^{109}CdCl_2$ alone was added to the apical Ringer's, while in another series of five skins 10^{-3} M cysteine plus 1–1.5 μCi of $^{109}CdCl_2$ was added to the apical Ringer's. The specific activity of our $^{109}CdCl_2$ was 0.1 mCi/ml so that 10–15 μl of this solution was added to the apical Ringer's. Immediately after the isotope was added, a 1-ml aliquot from the basal-lateral chamber was removed and counted for 1 min in a Packard deep well gamma counter. This sample was then returned to the basal-lateral chamber. At 10-min intervals thereafter, 1-ml aliquots were removed, counted for 1 min, and returned to the basal-lateral chamber. After 1 hr the entire 3-ml volume of the apical and basal-lateral chambers was removed and replaced with nonradioactive imidazole-HCl Ringer's (apical pH 5.5; basal-lateral pH 7.0). After 10 min this initial washing was removed and counted, and fresh Ringer's was again replaced in the Ussing chambers. This procedure was followed for six consecutive washings. Finally, the skin was removed from the chamber and trimmed so that the area of the skin exposed to the $^{109}CdCl_2$ could be counted in order to estimate the amount of residual cadmium in the tissue.

The rate of Cd^{++} flux across the tissue was calculated as the amount of radioactivity (cpm) moving into the basal-lateral Ringer's per min. The residual Cd^{++} in the skin itself is expressed as radioactivity (cpm) per cm^2 of the surface area (1 cm^2 in all cases).

Effects of Cd^{++} on the Large Intestine

The large intestine was removed from the animal, cut longitudinally and mounted between the halves of the single-chambered cell described in the isolated epithelium experiment. As before, open circuited conditions were maintained with SCC being monitored

at 10-min intervals. When SCC varied by less than 10% over a 30-min period, the standard Ringer's bathing the luminal or apical surface of the intestine was replaced with Cd^{++} Ringer's and SCC monitored for a second 30-min period. The Cd^{++} Ringer's was then washed out and SCC monitored until values similar to the control level of SCC were obtained. When SCC values again varied by less than 10% over a 30-min period, Cd^{++} Ringer's was added to the serosal or basal-lateral surface of the intestine and SCC monitored for 60 min. In two of the five experiments, the Cd^{++} Ringer's bathing the serosal surface was washed out with standard Ringer's and SCC monitored for a final 30 min.

Effects of Cd^{++} on the Urinary Bladder

The urinary bladder was dissected from the animal and one lobe mounted between the halves of the same Ussing chamber used for the experiments with the large intestine and isolated skin epithelium. The protocol for the experiments with the urinary bladder was identical to that for the large intestine. As with the frog skin epithelium, five bladder preparations were treated with Cd^{++} Ringer's at the apical surface and the stimulation of SCC was monitored for 60 min without a washout procedure.

Treatment of Data

The changes in SCC produced by the experimental treatments were normalized as the percentage change relative to the control value recorded just prior to treatment. Where statistical comparisons were necessary, the paired t test for related data (Dixon & Massey, 1957) was employed.

Results

I. Experiments with Intact Skin and Isolated Epithelium

Cd^{++} (10^{-3} M) applied to the basal-lateral surface of the intact skin failed to produce any significant change in SCC over a 60-min period (Fig. 1). Similar treatment to the serosal surface of the isolated epithelium also failed to produce significant changes in SCC; however, 10^{-3} M Cd^{++} added to the apical surface of the isolated epithelium reversibly stimulated SCC by more than 50% above the control level. In the preparations where Cd^{++} was not washed out, SCC remained significantly elevated over a 60-min period. These results are similar to what we have demonstrated earlier in the intact frog skin (Hillyard & Gonick, 1976).

When the pH of the outer Ringer's was reduced to 5.5 with imidazole-HCl Ringer's containing 10^{-3} M cysteine a stable baseline was obtained, with SCC varying by less than 10% over a 2-hr period (Fig. 2). When Cd^{++} and cysteine were added to the outer Ringer's in equimolar

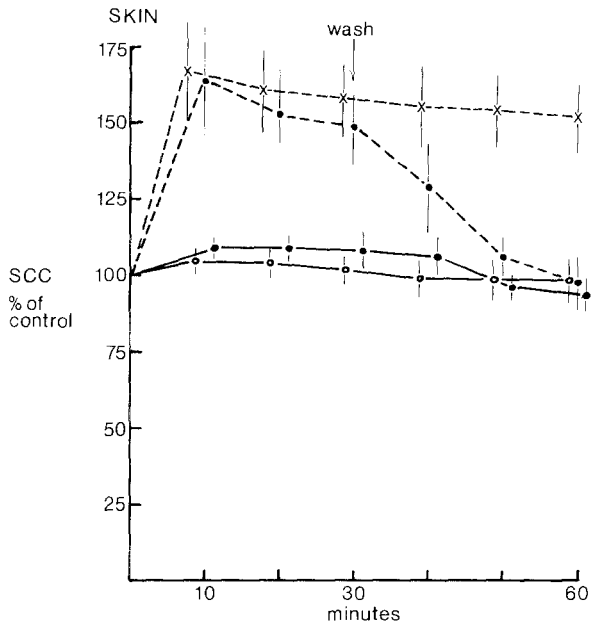

Fig. 1. The effects of 10^{-3} M Cd^{++} added to the apical (●---●) and basal-lateral (●—●) surfaces of the isolated epithelial cell layer from the frog skin on SCC (5 preparations). Also shown is the continued elevation of SCC by apical Cd^{++} treatment, in the absence of a washing with fresh Ringer's (×---×) (5 preparations). The effects of 1 mM Cd^{++} added to the basal-lateral surface of the intact skin is shown for comparison (○—○) (9 preparations). Each point in all of the text figures represents the mean ±1 SE

(10^{-3} M) amounts, SCC was progressively inhibited to less than 30% of the control value after 60 min treatment. This inhibition was only partially reversible by a 60-min washout with imidazole-HCl Ringer's. When 10^{-3} M Cd^{++} alone was added to the imidazole-HCL Ringer's bathing the apical surface of the skin, SCC was stimulated by about 75% above the control values, indicating that the cysteine rather than the reduced pH reversed the effects of Cd^{++} on the apical surface of the tissue.

The rate of isotopic Cd^{++} movement across the skin, as measured by its appearance in the basal-lateral Ringer's was not affected by the presence of 10^{-3} M cysteine in the apical Ringer's (Table 1). In both cases, the average rate of $^{109}Cd^{++}$ movement across the skin over a 60-min period, amounted to only about 0.004% of the total amount of isotope added to the apical Ringer's. The amount of radioactivity remaining in the tissue after the washout procedure, however, was over three times as great when cysteine was added with the $^{109}CdCl_2$.

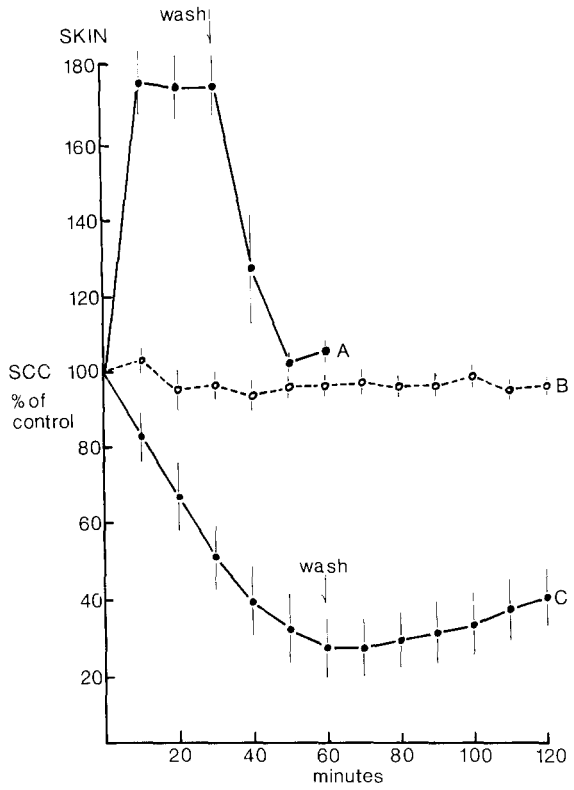

Fig. 2. Inhibition of SCC by equimolar (10^{-3} M) Cd^{++} and cysteine added to the apical Ringer's (*C*) compared with the stimulation produced by 10^{-3} M Cd^{++} alone (*A*). Cysteine alone (B) had no effect on SCC ($n=5$)

Table 1

	(a) Appearance rate of $^{109}Cd^{++}$ (cpm/min)	(b) Residual $^{109}Cd^{++}$ in the tissue (cpm/cm^2)
0 M cysteine	61 ± 31	$52{,}518 \pm 2{,}803$
10^{-3} M cysteine	67 ± 51	$186{,}294 \pm 39{,}691$ [a]

(*a*) The effect of 10^{-3} M cysteine on the movement of $^{109}Cd^{++}$ across the isolated frog skin, measured as the rate of $^{109}Cd^{++}$ appearance in the basal-lateral Ringer's (cpm/min) during the 60-min period following the addition of 1–1.5 μCi of the isotope to the apical Ringer's.

(*b*): The effect of 10^{-3} M cysteine on the amount of ^{109}Cd remaining in the above pieces of frog skin (cpm/cm^2) after six washings with isotope-free Ringer's. The mean value for five preparations $\pm$ one SD is given for each experimental treatment.

[a] Significantly greater than 0 M cysteine value ($P<0.01$).

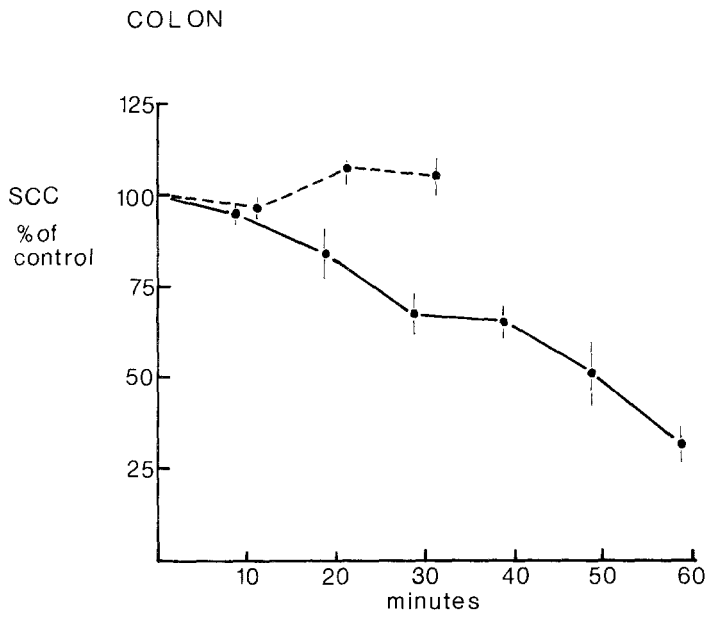

Fig. 3. Inhibition of SCC across the isolated frog large intestine (colon) by 10^3 M Cd^{++} treatment at the basal-lateral Ringer's (●—●). By comparison, treatment at the apical surface (●---●) is without significant effect ($n=5$)

II. Experiments with the Large Intestine

Cd^{++} Ringer's added to the apical surface of the isolated large intestine failed to produce a significant change in SCC over a 30-min treatment period (Fig. 3). The same Cd^{++} Ringer's added to the serosal surface inhibited SCC to about 40% of the control. This inhibition was not reversible by a 30-min washout period with fresh standard Ringer's.

III. Experiments with the Urinary Bladder

When Cd^{++} Ringer's was added to the apical surface, SCC across the urinary bladder was reversibly stimulated by about 50% over the control level (Fig. 4). SCC across the bladders which received continuous exposure to Cd^{++} Ringer's remained significantly elevated over the 60-min experimental period. Cd^{++} Ringer's added to the serosal surface, on the other hand, produced inhibition of SCC, which was irreversible by 30 min washing with fresh standard Ringer's.

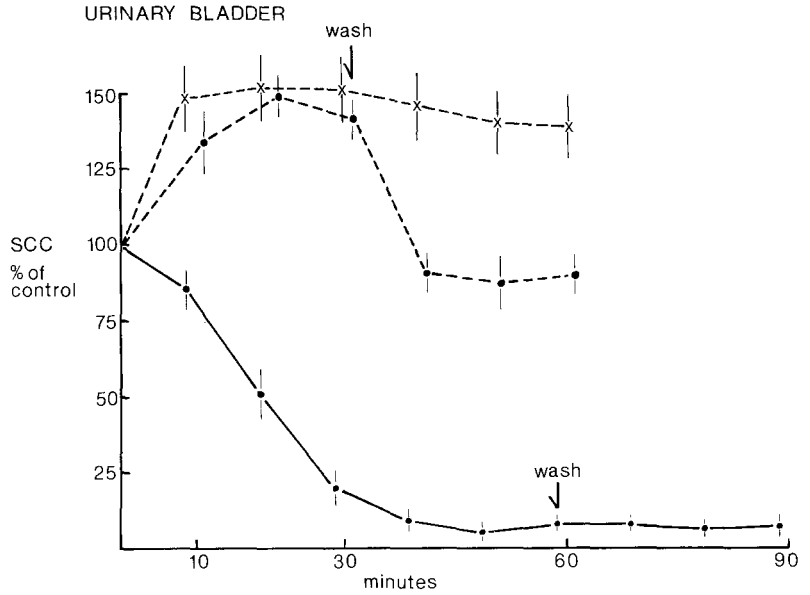

Fig. 4. Stimulation of SCC produced by 10^{-3} M Cd^{++} treatment at the apical surface of the frog urinary bladder (●---●) contrasted to the inhibition of SCC produced by 10^{-3} M Cd^{++} added to the basal-lateral surface (●—●) ($n=5$). Continued elevation of SCC is seen when apical Cd^{++} treatment is not washed with fresh Ringer's (×—×) ($n=5$)

In the washout periods for both the large intestine and urinary bladder, three successive changes of fresh Ringer's were applied in order to minimize residual Cd^{++} in the chambers.

Discussion

The present study verifies our preliminary data (Hillyard & Gonick, 1976) demonstrating that 10^{-3} M Cd^{++} added to the basal-lateral surface of the frog skin does not impair active sodium transport (Fig. 1). Hayashi et al. (1977) similarly found that 2×10^{-3} M Cd^{++} was without effect when added to the basal-lateral surface of isolated skin from the bullfrog, *Rana catesbiana*. They suggested that the failure of basal-lateral Cd^{++} treatment to inhibit SCC may be due to an inability of Cd^{++} to pass through the dermal layers of the skin. In the present study, however, 10^{-3} M Cd^{++} added to the basal-lateral surface of the isolated frog skin epithelium had no effect on SCC. Since the collagenase method employed to isolate the epidermal cells has been shown to separate the dermal elements of the skin from the epidermis (Erlij, 1971), we would

conclude that some characteristic other than the supporting layers of the dermis accounts for the resistance of the frog skin to the inhibitory effects of Cd^{++}. The collagenase method could conceivably alter the cells of the *stratum germanitivum* in such a way that its penetration to the intercellular space surrounding more superficial cell layers would be impaired. Erlij (1971), however, found that the tissue appeared normal, histologically, and that ruthenium red penetrated to the basal surface of the *stratum granulosum* in epidermal sheets prepared with collagenase and hydrostatic pressure.

In both the urinary bladder (Fig. 3) and large intestine (Fig. 4), SCC was inhibited irreversibly by the addition of 10^{-3} M Cd^{++} to the basal-lateral surface of the tissue despite the presence of supporting layers of tissue at the basal-lateral surface of the epithelial cells. The ability of Cd^{++} to inhibit SCC across the bladder and large intestine but not across the skin would suggest that sites on the active sodium transport mechanism which are inhibited by Cd^{++} are accessible to the ion at the outer surface of the basal-lateral membranes of the bladder and small intestine but not in the skin.

At the apical surface of the frog skin, Cd^{++} reversibly stimulated SCC (Fig. 1), an effect which was not qualitatively altered by a reduced pH in the apical Ringer's (Fig. 2). A similar pattern of reversible stimulation of SCC was observed when 10^{-3} M Cd^{++} was added to the apical surface of the urinary bladder (Fig. 3). The reversible nature of Cd^{++} stimulation of SCC suggests that Cd^{++} does not enter into the cells or bind irreversibly with components of the apical membranes which regulate sodium entry into the epithelial cells (Hillyard & Gonick, 1976). For this reason, we investigated the effects of cysteine as a possible carrier which would allow Cd^{++} to enter into or across the apical membranes of the frog skin.

The results of the isotopic flux experiments demonstrated that cysteine did not enhance the transepithelial movement of $^{109}Cd^{++}$ from the apical to the basal-lateral Ringer's solutions (Table 1). Isotopic Cd^{++} movement across the skin was very low in the presence or absence of cysteine with the bulk of Cd^{++} probably moving across the tissue at edge-damaged areas. On the other hand, the amount of isotope which remained in the tissue, after the washout procedure, was over three times greater when cysteine was added with the $^{109}Cd^{++}$ at the apical surface of the skin. It was not possible, however, to localize the Cd^{++} in the cells or to the apical or basal-lateral surface.

In conjunction with the enhanced residual level of Cd^{++} in the tissue

in the presence of cysteine, SCC across the frog skin was irreversibly inhibited, in contrast with the reversible stimulation of SCC produced by apical treatment with Cd^{++} alone (Fig. 2). The inhibition of SCC produced by Cd^{++} and cysteine together could arise from direct inhibition of $Na^+ - K^+$ ATPase located in the basal-lateral plasma membranes of the cells. This possibility is consistent with observations that kidney $Na^+ - K^+$ ATPase activity is reduced in rats which demonstrate the Fanconi syndrome (Gonick *et al.*, 1975) following chronic Cd^{++} exposure. Another possibility is that Cd^{++} in the cell can interfere with energy metabolism. It has been shown, for example, that Cd^{++} inhibits oxidative phosphorylation in mitochondria from lung macrophages (Mustafa *et al.*, 1971). An alternate possibility is that cysteine alters the binding of Cd^{++} by the apical membranes of frog skin epithelial cells which reduces the passive entry of Na^+ into the cells.

The inhibition of SCC produced by equimolar addition of cysteine and Cd^{++} to the apical Ringer's is similar to results obtained by Linderholm (1952) and Lodin *et al.* (1963), who found 10^{-4} M $HgCl_2$ alone to inhibit SCC when added to the apical surface of isolated *Rana temporaria* skin. Using the method of Steinbach (1933), Lodin *et al.* (1963) found that this inhibition of SCC by Hg^{++} treatment was associated with an increase in the resistance of the morphologically outer barrier of the epithelium with little or no effect on the inner barrier.

It would appear, then, that divalent metals can inhibit SCC by either reducing passive entry into epithelial cells, as Lodin *et al.* (1963) observed for Hg^{++}, or by directly inhibiting the active transport mechanism of the basal-lateral membranes, as observed with the bladder and large intestine. Furthermore, the presence of a carrier, such as cysteine or metallothionein, can modify the response at either site of action. Cd^{++} effects on epithelial Na^+ transport must, therefore, be interpreted in terms of which side of the tissue is exposed to this ion.

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