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The Electrogenic Chloride Pump of the *Limonium* Salt Gland

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Summary. A general method for analyzing ion transport in a system of complex geometry has been used to study the secretory activity of the *Limonium* salt gland. On the basis of an electron-microscopical study of the cells and their arrangement, a simple model has been set up which predicts qualitatively the changes in inhibitor-sensitive ion fluxes under a range of positive and negative voltage clamps. Comparison with experimental data indicates that sodium ions, although transported against an overall electrochemical gradient, are not pumped in this system but electrically coupled to chloride transport inside the gland complex. The basis of the electrical activity, the salt transport, and the volume efflux from these cells is therefore an electrogenic chloride pump.

The *Limonium* salt gland is a multicellular complex embedded in the leaf surface which is connected to the surrounding cells by plasmodesmata, its function being similar to that in marine reptiles and birds. It secretes salt solution when the plant grows in saline conditions, and can be shown directly to control the ionic activities of the surrounding cytoplasm (Larkum & Hill, 1970). The gland gives rise to a considerable volume efflux during activity, and shows a stable secretory potential and short-circuit current, which are consistently negative. The short-circuit current drawn from a leaf disc is due to the activity of a great number of glands, of which there are about $500/cm^{-2}$. Previous studies with this system have shown quite clearly that halide ions and a range of cations are all actively extruded from the gland against an electrochemical potential gradient (Hill, 1967*a*, *b*). When chloride (bromide or iodide) is excluded from the medium, however, the secretion ceases and the gland cells become electrically inactive: sodium can be replaced by almost any other cation to preserve activity, and this even extends to complex organic cations. In an experiment to determine

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Table 1. The equilibrium ion potential and flux ratio for choline ions[a]

Open circuited	E_m	$-\frac{RT}{zF}\log_e\frac{c_o}{c_i}$
	-5 ± 2	-22 ± 5
Short circuited	E_m	F_{io}/F_{oi}
	0	4.71 ± 2.1

[a] The open circuit results are from 12 experiments, the short circuit results from 4. F_{io} is an efflux, i being the inside surface, o the exterior. E_m is the secretory potential.

the extent to which choline can be said to be actively transported by this system, the flux ratio for ^{14}C-labeled choline was determined under short-circuit conditions across a leaf preparation and the electrochemical potential gradient against which pumping occurs in the open-circuited condition was determined as described previously (Hill, 1967*a*). The results are shown in Table 1 and indicate that by these criteria choline is actively extruded from the gland cells. That this should be so is quite surprising as plant cells cannot readily be thought of as possessing specific transport systems for solutes which play little part in their metabolism or osmotic regulation.

It has been suggested (Keynes, 1969) that the absence of a secretory potential in gallbladder, and the apparent neutral pumping of sodium chloride can be explained by entrainment of chloride ions in an internal electrical gradient, much as water is entrained in an internal osmotic gradient in this system. If the space constant of the lumen is sufficiently short, a sodium pump could be operative in creating a potential over the lumen wall which would drive chloride into the lumen, but this potential would then be attenuated drastically by the time the lumen mouth were reached; the secretory potential will therefore appear to be exceedingly small, which is the case (Diamond, 1962). To transfer this idea to the *Limonium* gland system would provide an excellent explanation of the transport properties; if a chloride pump is operative in a channel where attenuation of the transmembrane potential can occur, and the cation permeability is reasonably high, sodium ions will move down an electrochemical potential gradient into the channel. If the space constant of the channel is not too small, a secretory potential will be observed, a short-circuit current can be drawn from the preparation, and by the criterion of the Ussing flux ratio equation, sodium or indeed any cation will appear to be actively transported; at no point, however, will there be direct coupling of sodium transfer to any other dissipative process, metabolic or diffusive.

Although the fact that the system seems to be strongly chloride-dependent and nonspecific towards cations, indicates that a chloride pump is probably operative in the gland, we were looking for a more concrete electrophysiological demonstration that in the presence of both sodium and chloride ions only chloride pumping occurs. In this paper we describe the effects of several positive and negative voltage clamps on the cyanide-sensitive fluxes of sodium and chloride ions from the gland cells, and interpret them in terms of a model derived below. This model is simply that of an internal channel bordered by the cells of the gland complex and opening onto the leaf surface. This channel is treated as leaky cable with a resistive core, in which the clamping voltage is attenuated towards the closed end of the channel, and into which the solute and negative current are injected in the steady state. This model therefore envisages that the active transport takes place at the plasmalemma of a gland cell, transferring ions and water from the gland cell cytoplasm to the extracellular channel. The gland cell cytoplasm is itself fed with ions from the adjacent symplasm by plasmodesmata which traverse cuticle-free regions in the glandular insulation.

Fine Structure

Fig. 1 is a cross-section of the *Limonium* gland complex at about its mid point, in which the prominent central channel can be seen: at several points where more than two cells are in juxtaposition there are small channels, and it is very likely that salt is pumped into the cell wall region everywhere, through which it moves towards the surface. We wish to stress, therefore, that when a model is later developed in which the extracellular space is represented by a single longitudinal channel of constant cross-section, this merely serves to show how the fluxes of cations and anions behave in such an internal space. No simulation of the exact fluxes under voltage clamp can be attempted because the geometry is far too complicated. In Fig. 2, a section down a longitudinal channel can be seen which exposes invaginations of the cell wall which presumably serve to increase the surface area of the plasmalemma for transport. These are a feature of most plant glands, and are analogous to the intracellular channels and intercellular spaces found in animal cells (Gunning & Pate, 1969). In Fig. 3 this scheme is outlined diagrammatically. The internal channel functions as a standing gradient osmotic system, and the exudate is more or less isotonic or slightly hypertonic to the basal medium (Arisz, Camphius, Heikens & Van Tooren, 1955); inhibitors and low temperature reduce the volume flow but not the emergent concentration.

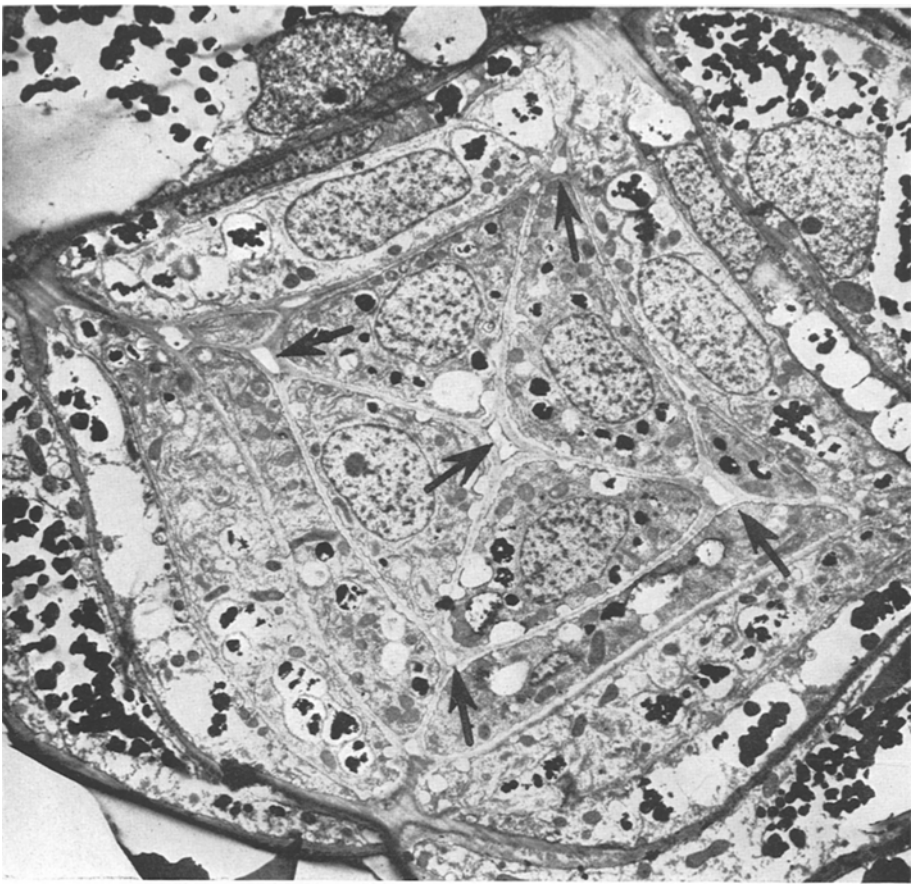

Fig. 1

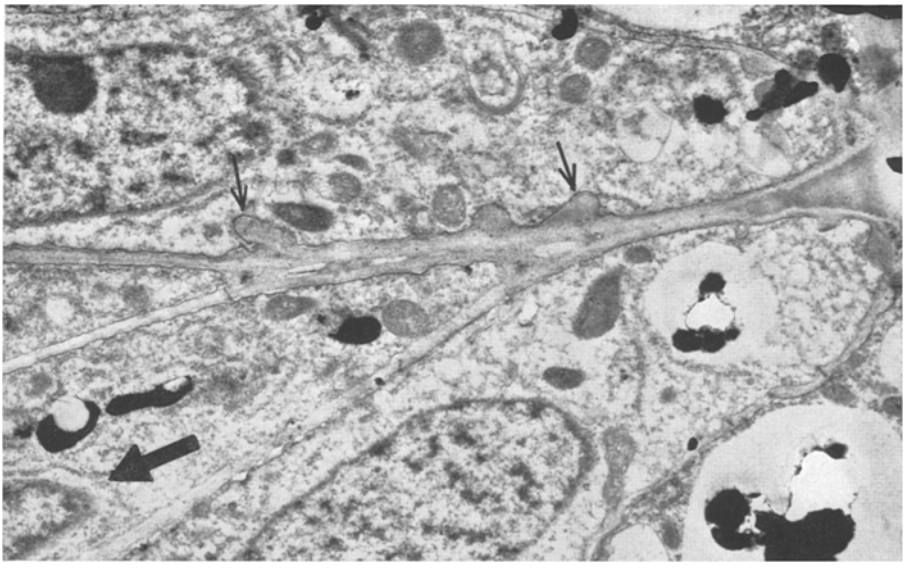

Fig. 2

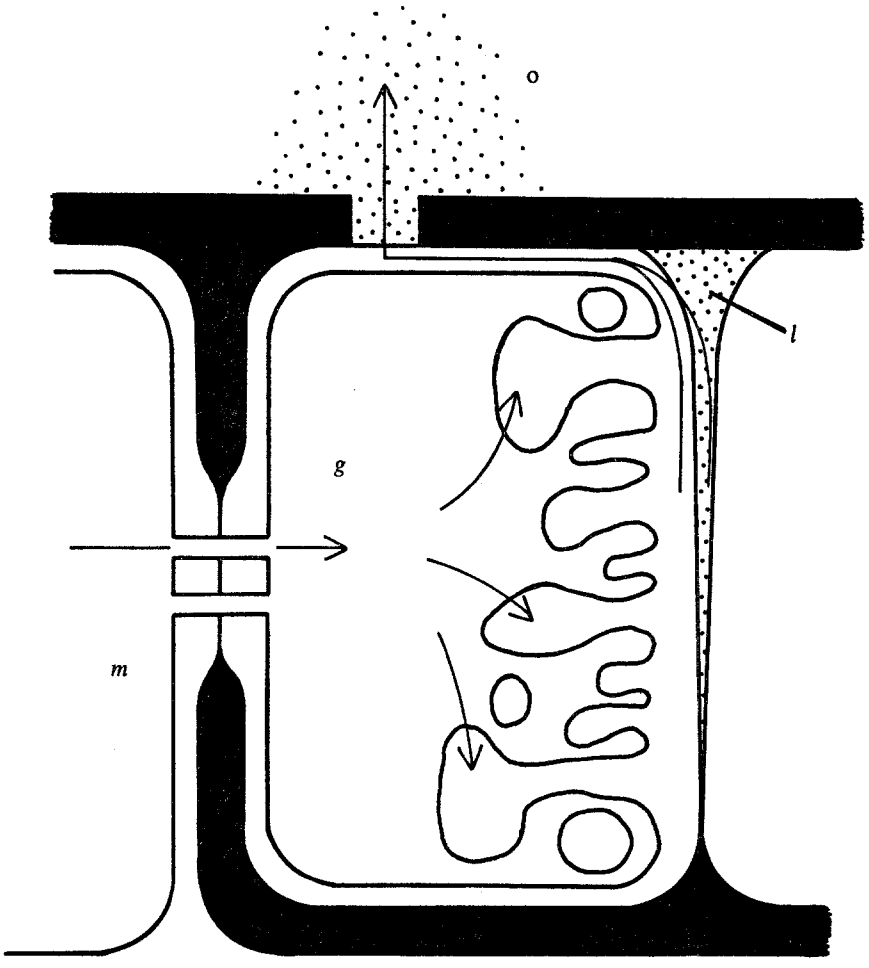

Fig. 3. Diagram of the process of salt extrusion in the gland. Ions enter the gland cell (*g*) via plasmodesmata from the surrounding mesophyll cells (*m*) and are pumped into the plasmalemma invaginations of the gland cell. The invaginations, the intercellular lumen (*l*) and other such channels then function as a coupling space for salt and water, the solution leaving a cuticle pore to the exterior (*o*)

Fig. 1. A cross-section of the *Limonium* salt gland showing the parallel channels (arrows) through which the exudate flows during secretory activity. ×4,500

Fig. 2. A longitudinal section through a gland showing part of a channel with wall intrusions into the cells (small arrows). The large arrow shows the direction of volume flow. ×11,500

Theory

As the secretory potential and the current drawn from a gland are always negative, and under short-circuit conditions the chloride fluxes are always larger than the sodium fluxes, we assume that any model must incorporate an active transport system for chloride ion. There are thus four possibilities for the active transport over the gland cell membranes (Fig. 4): (*i*) separate electrogenic pumping of sodium and chloride ions; (*ii*) a neutral NaCl pump, the negative secretory potential being due to back diffusion (i.e., shunting) of sodium through a relatively high conductance; (*iii*) a stoichiometric coupling of sodium and chloride (say, 2 Cl:1 Na) similar to the coupling of Na–K in red-cells and nerve; and (*iv*) only a chloride pump with no active transport of sodium at all. How these differing systems would react under various voltage clamps can be studied by considering the voltage profile within the channel, and its effect on the active and passive

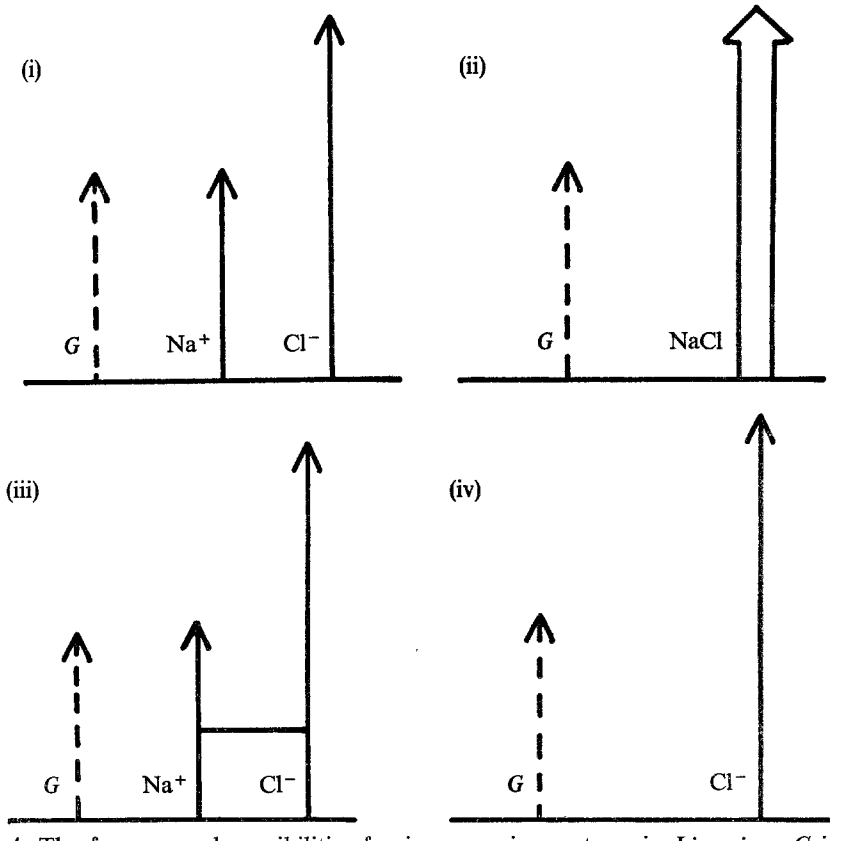

Fig. 4. The four general possibilities for ion pumping systems in *Limonium*. *G* is the passive conductance which in NaCl is probably only a sodium conductance. (*i*) Separate Na^+ and Cl^- pumps. (*ii*) A neutral NaCl pump. (*iii*) Stoichiometric coupling of Na^+ and Cl^- transport. (*iv*) Only Cl^- pumping

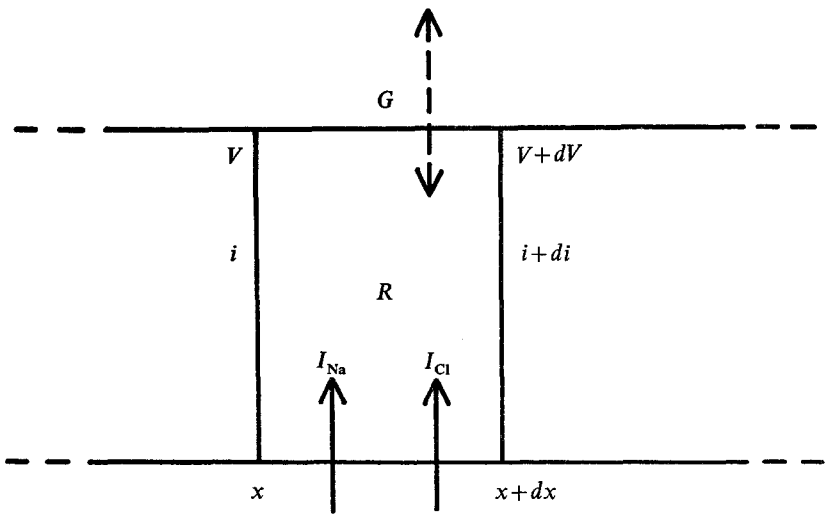

Fig. 5. A small section of an extracellular channel in which G is the wall conductance, R the resistivity of the internal fluid, and $I_{Na,Cl}$ are the active pumps; the voltage and current at any point are V and i, respectively

ion fluxes. Inside the channel there will be a potential profile that is determined by both the clamping voltage at the mouth which attenuates with distance, and the current injection due to electrogenic processes in the channel wall. We have assumed that any pressure gradients in the system have little or no effect upon the ion fluxes because the partial molar volumes of the ions are so small. In addition, any concentration gradients within the channel are assumed to be negligible; i.e., the water permeability of the channel walls is assumed to be very high. This assumption does not qualitatively affect the end result, and as the model presented here allows us to reject certain situations on a qualitative basis alone, concentration effects are not considered any further. A general model is developed in which both ions are pumped into the channel over the whole length of the system, i.e., the channel is semi-infinite and homogeneous for all the parameters; the four possibilities in Fig. 4 are then considered as special cases.

Fig. 5 is a section of the channel in which R is the resistivity of the internal fluid (here assumed constant) and G the conductivity per cm^2 of the wall membrane; I_{Na} and I_{Cl} are the currents flowing into the lumen due to sodium and chloride pumping and V and i are voltage and current, respectively, in the channel at any point. The gland system seems to be highly impermeable to anions, for the glandular secretion during activity contains only traces of other anions when these are added to the NaCl medium. It is therefore assumed here that the passive conductance is solely

due to Na ions, and that $G=G_{Na}$. Any models containing an appreciable chloride conductance, however, lead to the same end result. We obtain the following relations, as a first-order approximation;

$$dV=-Ri\,dx \tag{1}$$

$$di=-GV\,dx+(I_{Na}+I_{Cl})\,dx. \tag{2}$$

The general thermodynamic description of an active transport process at constant affinity is one in which the flux through the active element is composed of two parts, one a constant fraction linked to metabolism, the other a fraction dependent on the prevailing electrochemical potential gradient. In current terms

$$I_{Na}=i_{Na}-c_{Na}V \tag{3}$$

and

$$I_{Cl}=i_{Cl}-c_{Cl}V \tag{4}$$

where i_{Na} and i_{Cl} are the metabolic components of the ion flux and the c's are the conductances of the pumps.

Then

$$\frac{di}{dx}=-GV+\sum i-\sum cV \tag{5}$$

where

$$\sum i=i_{Na}+i_{Cl}, \quad \sum c=c_{Na}+c_{Cl}.$$

From Eqs. (1) and (5)

$$\frac{d^2V}{dx^2}=-\frac{R\,di}{dx}=V(RG+R\sum c)-R\sum i.$$

Writing $m^2=RG+R\sum c$, we obtain

$$\frac{d^2V}{dx^2}=m^2V-R\sum i \tag{6}$$

which has the general solution

$$V=A\sinh mx+B\cosh mx+R\sum i/m^2. \tag{7}$$

To evaluate the constants with the boundary conditions of this system from Eq. (1) we proceed as follows;

$$i=-\frac{1}{R}\frac{dV}{dx}=-\frac{1}{R}(Am\cosh mx+Bm\sinh mx)$$

and at the channel end $x=0$, $i=0$; i.e., the channel has an impermeable end-wall. This assumption merely simplifies the mathematics without adding anything to the final results (Diamond & Bossert, 1967). Thus,

$$A=0. \tag{8}$$

When $x=a$, where a is the channel length, $V=V_a$ so that

$$B=\left(V_a-\frac{R\sum i}{m^2}\right)\frac{1}{\cosh m a} \tag{9}$$

and

$$V=\left(V_a-\frac{R\sum i}{m^2}\right)\frac{\cosh m x}{\cosh m a}+\frac{R\sum i}{m^2}. \tag{10}$$

We now wish to concentrate attention upon the partial flux of sodium (i.e., radiosodium) into the lumen at any particular clamping voltage V_a, using the Goldman-Hodgkin-Katz theory. The passive influx at a point x is given by

$$F_p=\frac{zFV_{m,n}Pa_0}{RT(\exp(zFV_{m,n}/RT)-1)} \tag{11}$$

where P is the wall permeability to sodium, a_0 is the cellular activity and V_m and V_n are the potentials at the point x in the absence and presence of inhibitor, respectively. V_m is given by Eq. (10), but under inhibition $\sum i=0$ so that

$$V_n=V_a\frac{\cosh m x}{\cosh m a}. \tag{12}$$

Combined influx into the channel is therefore given by

$$F=\frac{V_mPa_0}{25.3(\exp(V_m/25.3)-1)}-\frac{V_nPa_0}{25.3(\exp(V_n/25.3)-1)}+(i_{Na}-\phi c_{Na}V_m) \tag{13}$$

where RT/zF is taken as 25.3 mV at 20 °C and V is also in millivolts. The last term in brackets in Eq. (13) represents the active transport of radiosodium into the channel as described by Eq. (4) where ϕ is the valency of the complex by which sodium crosses the membrane. As this last term is zero under inhibition, Eq. (13) represents the inhibitor-sensitive efflux of radiosodium into the channel at the point x.

The four possibilities for differing transport systems into the channel can now be discussed in terms of this last Eq. (13), and the four cases presented in Fig. 4 are illustrated diagrammatically below. In the first case we must consider a pure sodium pump, for which ϕ is positive and

$$m=\sqrt{R(G_{Na}+c_{Na})}, \quad n=\sqrt{RG_{Na}}.$$

The slope is therefore negative; i.e., a pure sodium pump is rate-reduced by increasing positive clamps, depending upon the pump conductance, however. When $c_{Na}=0$ the slope is zero also. Inasmuch as the potential gradient is one of the thermodynamic forces against which an electrogenic pump must transfer charge, no such pump can in reality be insensitive to changes in potential. The factors controlling c_{Na} are the pump valency and

the size of the free energy change of the metabolic driving reaction. In the second case, that of a neutral salt pump, the pump must be insensitive to voltage as it does not transfer charge, which also implies that $c_{Na}=0$, $m=n$ and $\sum i=i_{Na}+i_{Cl}=0$ which leads to

$$\frac{dF}{dV_1}=0.$$

The third case represents a stoichiometric coupling which is electrogenic, but in this case $\phi=-1$, because the actual flux of sodium via such a mechanism is stimulated by positive voltages due to the fact that the sodium is crossing the membrane as part of a negatively charged complex. In the fourth case where the active transport of sodium is absent, the passive sodium transfer alone is apparent. As the negative clamp is increased the interior of the channel becomes more negative, and the chloride pump is repressed. The chloride pump however, is contributing towards the negative channel potential, and there must therefore be a clamp voltage when the mean internal potential in the poisoned and unpoisoned channel and hence the sodium fluxes are identical (i.e., strictly the integral of the fluxes over the whole channel are identical) and their difference is zero. In the region of positive clamping, the sodium fluxes are soon reduced to low values and the difference between the fluxes in the presence and absence of inhibitor becomes undetectably small. The inhibitor-sensitive sodium efflux should therefore cut off in both the negative and positive planes. These four cases are shown diagrammatically in Figs. 6, 7 and 8, for different values of the several parameters involved, although they are plotted as relative flux curves; i.e., the values are relative to those obtained at zero voltage clamp. Not only does this reflect our experimental practice, for this relative flux is fairly insensitive to biological variation, but it is also very insensitive to position within the channel. Computer graphing of the relative flux curves at various positions in the channel failed to detect any significant differences, at any rate within the range of values of the parameters used in the study; these were chosen to approximate quite closely to the observable parameters of the *Limonium* gland.

We should in fact expect the passive sodium effluxes to obey a displaced "constant-field" equation (Goldman, 1943), displaced because the internal voltage at any point within the channel changes more slowly than the clamping voltage. This is in fact the case, as shown in Fig. 9. A full treatment of the system would require the solution of the Nernst-Planck equation down a leaky cable, to calculate the specific activity profile; we consider that this would so complicate matters as to become counter-productive.

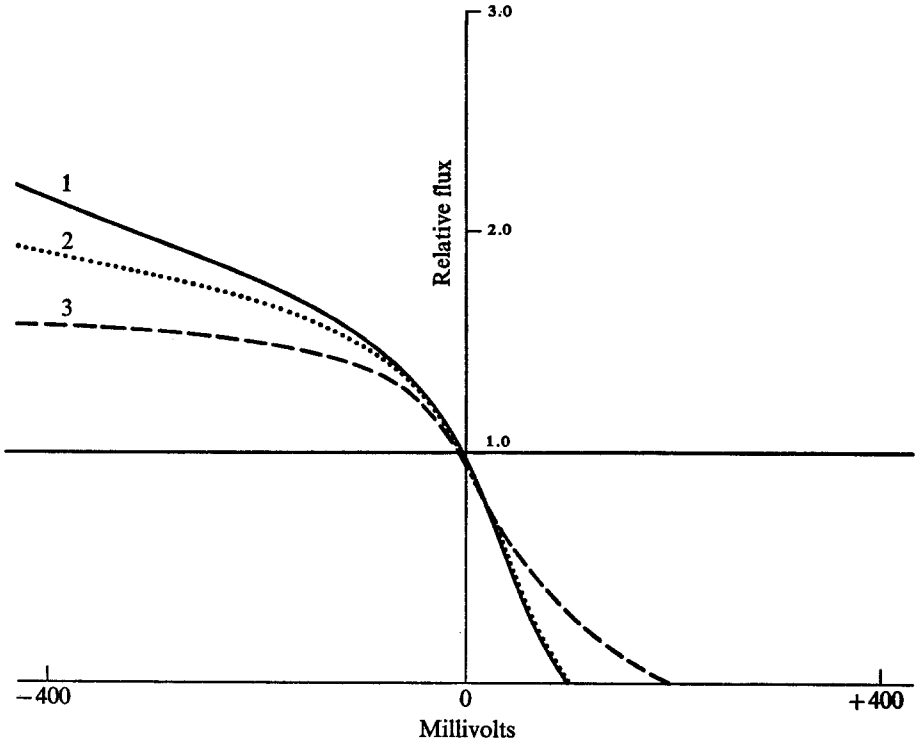

Fig. 6. A graphical print-out of the inhibitor-sensitive sodium efflux when $\phi = +1$; i.e., when sodium is transported into the channel by a pure sodium pump, according to Eq. (13). In these graphs and in those of Figs. 7 and 8, the three curves are calculated according to the following constants: Curve $1 - R = 5.0 \times 10^9$ Ω/cm, $a = 20.0 \times 10^{-4}$ cm, $\sum i = 2.0 \times 10^{-5}$ amps/cm, $G = 1.6 \times 10^{-6}$ mhos/cm, $c = 3.2 \times 10^{-6}$ mhos/cm; Curve $2 - R = 3.0 \times 10^6$ Ω/cm, $a = 20.0 \times 10^{-4}$ cm, $\sum i = 2.0 \times 10^{-9}$ amps/cm, $G = 9.0 \times 10^{-7}$ mhos/cm, $c = 1.35 \times 10^{-5}$ mhos/cm; Curve $3 - R = 4.0 \times 10^9$ Ω/cm, $a = 12.0 \times 10^{-4}$ cm, $\sum i = 2.0 \times 10^{-6}$ amps/cm, $G = 1.0 \times 10^{-6}$ mhos/cm, $c = 1.0 \times 10^{-5}$ mhos/cm. For glandular frequencies in the range 300 to 500 per cm^2 these constants all give short-circuit currents of between 1 and 2 μamps/cm^2 of tissue surface, as is observed in experiment. Ranges of G and c (approximately 10^{-6} and 10^{-5} mhos/cm, respectively) were calculated from the transglandular resistances before and after induction, i.e. in the virtual absence and presence of chloride pumping; $\sum i$ was then chosen to give an overall short-circuit current in the right range. The hypothetical level of active sodium transport linked to metabolism (i_{Na}) was set at a value which doubled the passive component at a clamp voltage of 0 [*Cf.* Eq. (13)]. The length of most gland channels varies from 10 to 20 μ, and the channel resistances R were calculated assuming they are filled with 0.1 M NaCl solution. The fluxes are relative to those at zero clamp voltage, and the voltage span is from −400 to +400 mV. The straight line represents the case of neutral salt pumping

Fortunately, we can say quite simply on the results of the above analysis that (*i*) if sodium transport is purely passive the inhibitor-sensitive sodium efflux should cut off in both the negative and positive voltage planes.

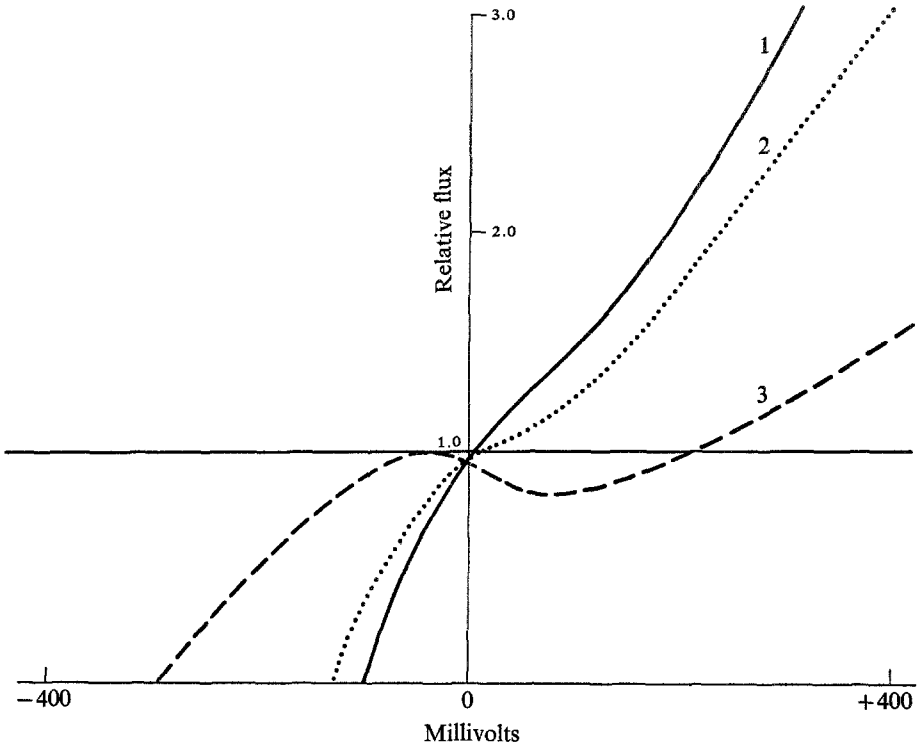

Fig. 7. The inhibitor-sensitive sodium efflux when $\phi = -1$, i.e. when sodium is transported across the channel walls by way of a negatively charged complex. The constants are identical to those in Fig. 6. The fluxes are relative to those at zero voltage clamp. The straight line represents the case of neutral salt pumping

(*ii*) Any pumping of sodium into the channel must manifest itself clearly at either a sufficiently high positive or negative clamping potential, depending upon the nature of the active sodium transfer.

Materials and Methods

The preparation of discs containing glands and measurement of radioactive effluxes has been described in detail previously (Hill, 1967*b*). ^{22}Na and ^{36}Cl were counted by liquid scintillation counting as aliquots withdrawn from the outer disc chamber. The clamp was applied by biasing the input of a Keithley 604 electrometer in a feedback circuit. The accuracy of the clamp was monitored continuously with an additional electrometer. In the ^{14}C-choline experiments, the fluxes of choline were followed in both directions, and the open circuit secretory concentrations were determined by scintillation analysis of the secreted fluid, collected as described in an earlier paper (Hill, 1967*a*). All inhibitions were produced with 10 mM cyanide ions in the inner chamber, under low light intensity. The biological variability between discs was overcome by measuring any particular flux in addition at a clamp voltage of zero. Relative fluxes could then be determined.

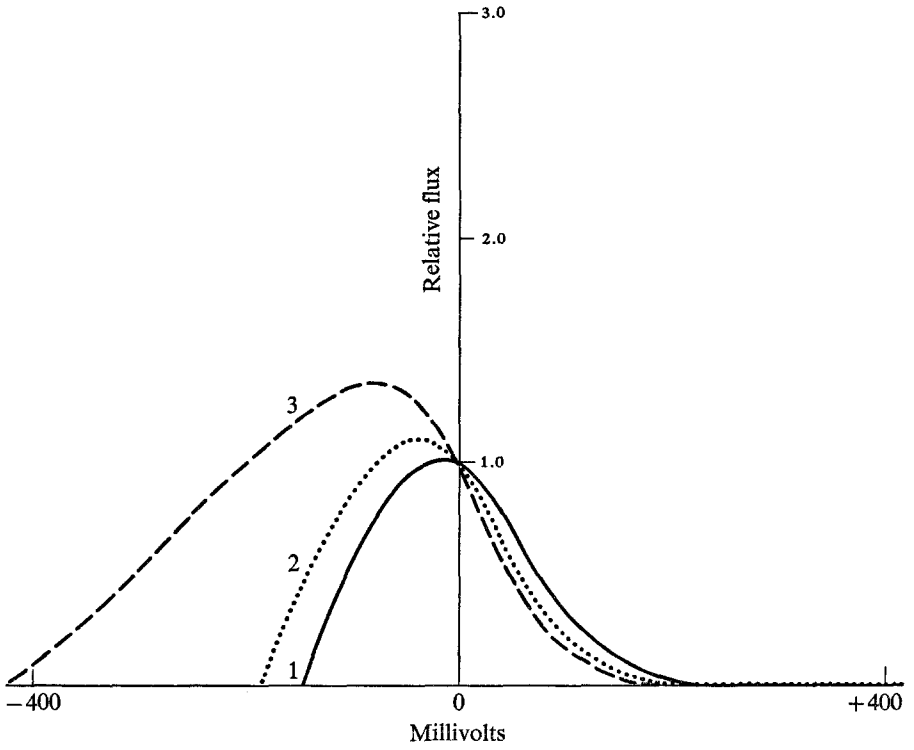

Fig. 8. The sodium efflux when both ϕ and i_{Na} are zero [Eq. (13)], i.e. the sodium crosses the channel walls by a passive mechanism only. The curve is again a relative flux curve. The two parameters controlling the sharpness with which the flux cuts off in the negative voltage sector are the channel length a, and the pump conductance c. Increasing a or decreasing c pushes the cut-off point towards higher negative voltages

Results and Discussion

In Fig. 10 the inhibitor-sensitive sodium flux is plotted as a function of the clamping voltage, relative to the flux at a clamping voltage of 0 mV; in addition, the inhibitor-sensitive chloride flux is plotted over a range of negative voltages. Two points are immediately noticeable. First, the flux pattern is that expected from case (*iv*) (Fig. 8), in which the sodium transport into the internal spaces of the gland is passive, and no sodium pumping is operative. An interesting feature of the results is that in accord with most solutions of Eq. (13), they show that the curve attains a maximum in the negative clamping sector. This maximum can be greatly increased in the results drawn from the model by assuming greater current injection per unit channel wall towards the closed channel end; i.e., an inhomogenous system. The internal gland spaces are, of course, highly complex. Secondly, the chloride flux cuts off in what is apparently the same voltage region,

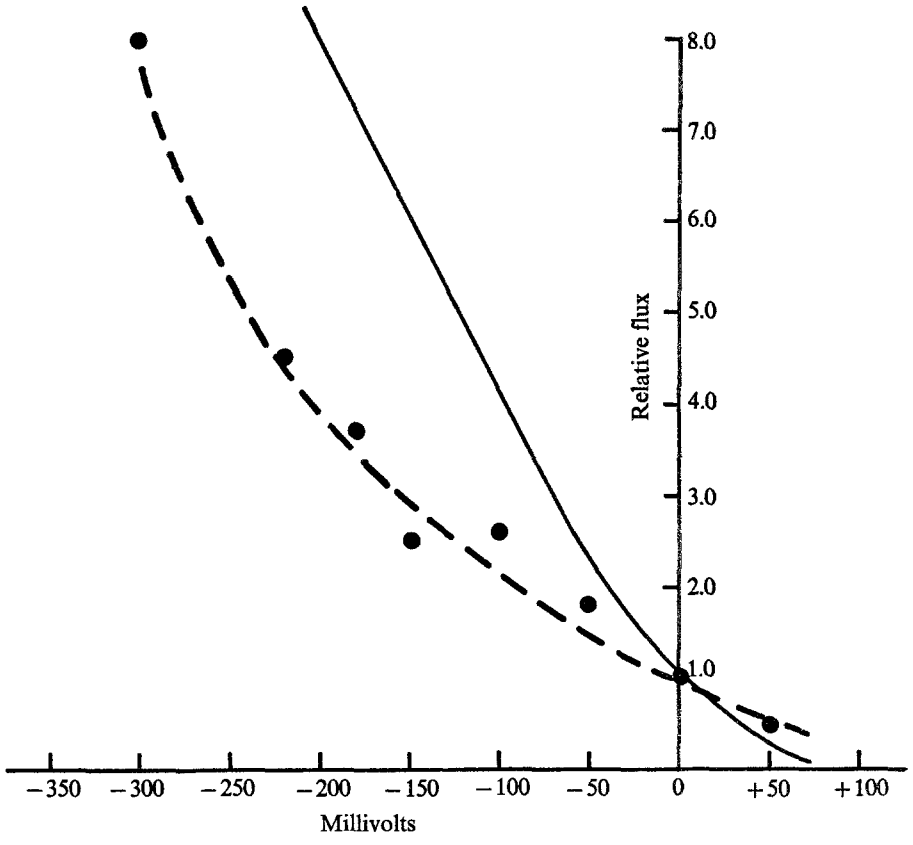

Fig. 9. The passive efflux of sodium via the gland system; each point represents the efflux of ^{22}Na from cyanide-treated *Limonium* discs at a particular clamping voltage (2 determinations, 3 discs, broken line). The solid line is the flux predicted by the constant field equation. Both flux curves are drawn relative to the flux at zero clamp voltage. The experimental curve is displaced to the left voltage axis, as would be expected if the clamp voltage were attenuated inside a narrow channel

which underlines that the sodium flux is somehow linked, or a function of the chloride transport. This would also be in accord with cases (*ii*) and (*iii*), but in these the sodium flux should be insensitive or elevated by higher positive clamps. In addition, these cases are both stoichiometric, and as can be seen there is no apparent stoichiometry between the fluxes. It thus seems quite clear that during activity the *Limonium* gland cells are pumping chloride ions only.

This conclusion accords well with all that we know of the behavior of this system: the dependence of the short-circuit current and secretory potential upon the presence of chloride in the medium, the catholic attitude towards cations, and the apparent low anion permeability. Both bromide

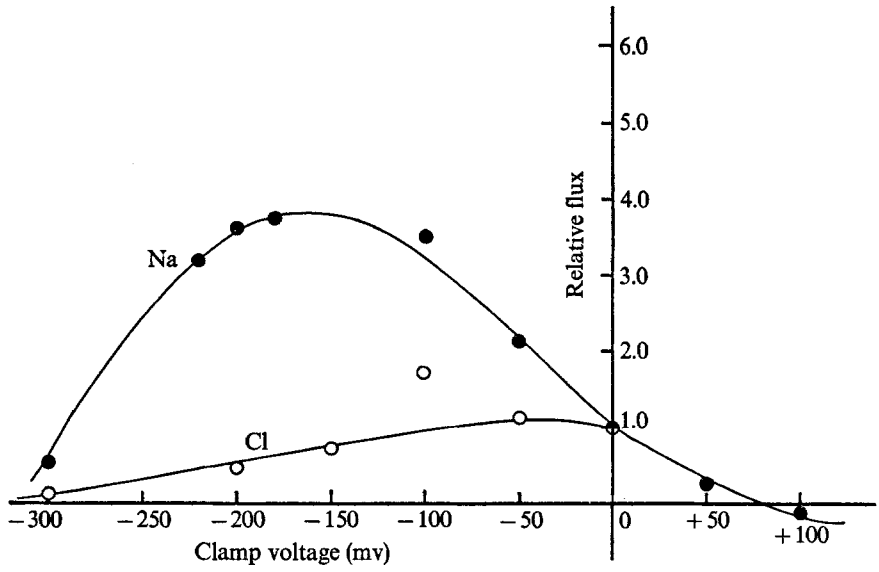

Fig. 10. The cyanide-sensitive effluxes of sodium and chloride ion from the disc as a function of clamp voltage over the gland. Chloride fluxes were not followed in the positive plane. Each point represents the results of 3 or 4 separate experiments. The fluxes are all relative to those at zero voltage clamp

and iodide ions seem to be able to maintain considerable electrical activity of the gland cells in chloride-free media.

In this paper we have rather neglected to discuss the rest of the leaf tissue in which the gland complex is embedded, and have assumed that the membranes of the surrounding cells to which the gland cells are coupled by plasmodesmata play no part in the process of ion tranport, or that the clamping voltage is acting over the gland cell membranes as envisaged in Fig. 3. In reality, of course, neither of these assumptions can be absolutely true. The fact, however, that all the clamp current flows in through a minute membrane area, for the gland cells are very small, and out over the considerable membrane area of the plasmalemma of the connected mesophyll, means that virtually all the potential drop will occur over the gland membrane due to its enormous comparative resistance. The salt pumping in this tissue is not always active, for it has been shown previously that the transport system is inducible, and seems to be brought into play by induction with a salt load (Shachar-Hill & Hill, 1970), much as is salt transport in the avian salt gland (Fletcher, Stainer & Holmes, 1967). Before this induction occurs the whole tissue can be preloaded with NaCl for 24 hr at low temperature, or up to $1\frac{1}{2}$ hr at room temperature directly, without any detectable electrical activity developing. Only when glandular extrusion

begins does a secretory current or potential become apparent. It thus seems that no measureable electrical signal can be picked up from the mesophyll and that ion transport and electrical activity are exclusively glandular in origin, and complementary. Membrane vesiculation has been invoked to explain salt transport in glands, as opposed to direct membrane transport (Thomson, Berry & Liu, 1969), the proposition being that salt is accumulated in microvesicles in the cytoplasm of the gland cells and discharged to the extracellular channels at the plasmalemma. Such a system would presumably function as a neutral salt pump discharging into the channel, and the above demonstration of electrogenic chloride pumping would seem to rule out this mechanism in *Limonium*.

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