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The Effect of Poly-L-Lysine, Amiloride and Methyl-L-Lysine on Gill Ion Transport and Permeability in the Rainbow Trout

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Summary. The action of poly-L-lysine (PLL) on Na and Cl transport across freshwater fish gills was studied. Low concentrations (10^{-6}M) were added to the external medium for brief periods (1–5 min), then removed. During the next 20 min there was a rapid net loss of Na (117 ± 17 μEquiv[100 g]$^{-1}$hr^{-1}) and Cl (129 ± 17 μEquiv[100 g]$^{-1}$hr^{-1}). Both values are an order of magnitude larger than unidirectional effluxes in control fish. The efflux of both ions decreased to control values within 60 min after application and removal of PLL. In contrast, unidirectional influxes (J_{in}^{Cl} and J_{in}^{Na}) were inhibited by about 40% and showed no sign of returning to the original rates for 3 hr. Thus, PLL has two independent actions, causing a large increase in gill permeability which is reversible within an hour and a partial inhibition of influx which showed no sign of reversing for 2–3 hr. When PLL was applied for a longer period (60 min, the results were qualitatively similar but the permeability change was larger and persisted longer. These effects were compared with those of the small organic amines, amiloride and methyl-L-lysine. The latter inhibited J_{in}^{Na}, but there was no other similarity to PLL. Neither affected sodium efflux, nor did they have any effect on Cl movements, in or out, across the gill. Inhibition of J_{in}^{Na}, was rapidly and completely reversible, amd amiloride was shown to act by competing with Na for an entry site.

Much recent work on sodium transport across a number of epithelia has focussed on the external membranes, those in contact with a tubular lumen or the environmental medium. Although we are far from a complete understanding of the functional characteristics of these membranes, some of their properties have been described. Sodium entry into the epithelial cells is mediated by a saturable system in isolated frog skin (Biber & Curran, 1970; Cereijido *et al.*, 1973) and the same thing has been observed for sodium uptake in intact frogs (Greenwald, 1971) and fish (Kerstetter, Kirschner & Rafuse, 1970). Sodium uptake by *in vivo* systems involves exchange for another cation such as H^+ or NH_4^+ (Kerstetter *et al.*, 1970; Maetz, 1971), although this has not been noted in isolated prepara-

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tions (Frazier, 1974). Significantly, high external concentrations of either H^+ or NH_4^+ can inhibit sodium transport in intact animals (Shaw, 1960; Maetz, 1971). The diuretic drug amiloride (N-amidino-3, 5-diamino-6-chloropyrazinecarboxamide) which contains three amino groups per molecule, is also a potent inhibitor of sodium uptake both *in vitro* and *in vivo* (Bentley, 1968; Kirschner, Greenwald & Kerstetter, 1973). In surface epithelia (gills, skin) from several freshwater vertebrates and invertebrates, amiloride has been shown to inhibit the Na^+ for H^+ exchange system (Kirschner *et al.*, 1973).

It was recently observed that polyamines such as poly-L-lysine (PLL) and protamine are capable of inhibiting fluid absorption from isolated renal tubules (Sato & Ullrich, 1971). Since tubular fluid reabsorption is coupled to sodium transport, these polycations might be interfering with Na transport by tubular cells. Other laboratories, however, have presented evidence that the effect of PLL on epithelia involves disruption of membrane integrity (Mamelak *et al.*, 1969; Quinton & Philpott, 1973). Indeed, Sato & Ullrich (1975) now feel that such an effect may have been responsible for their results. Some preliminary experiments on trout gill indicated that PLL might cause both permeability changes and inhibition of active Na uptake (Kirschner, 1973).

Thus, it appears that NH_4^+, some low molecular weight amino compounds, and possibly basic polypeptides all inhibit Na^+ influx. However, the mechanisms are not well-characterized, nor is it known whether the mode and site of action is the same for low molecular weight compounds and polymers. Therefore, we undertook to compare the effects of representative compounds on ion fluxes across the gill epithelium of the rainbow trout.

Materials and Methods

Rainbow trout (*Salmo gairdneri*), weighing 100–400 g, were obtained from a commercial hatchery near Soap Lake, Washington. The trout were held in dechlorinated tap water at 8 °C before being used in experiments at 13 ± 1 °C. All measurements were made using the gill irrigation procedure described by Kerstetter *et al.* (1970). This involves recirculating cold, aerated solutions past the gills of anesthetized fish. The animal is suspended upside-down in a plastic hammock, and the gill irrigation solution is pumped from a reservoir into the mouth and buccal cavity. After passing the gills it leaves the operculum and is returned to the reservoir. The solution contains sufficient neutralized tricaine methane sulfonate (TMS) to keep the fish from struggling.

Nearly all of the experiments followed a similar protocol. The trout was allowed to equilibrate for one hour on the apparatus. Then the irrigation solution was replaced

with 100 ml of fresh 0.7 mM NaCl containing about 0.02% TMS. This solution also contained 0.3 μCi of ^{22}Na and 1 μCi of ^{36}Cl. Samples (5 ml) were taken at 0, 20, 40 and 60 min. When Na and Cl fluxes were to be determined simultaneously 1 ml of each sample was counted in a well-type, gamma scintillation counter to determine ^{22}Na in the solution. Another 1-ml aliquot was counted in a gas flow counter to determine the combined ^{22}Na and ^{36}Cl counts. The ^{36}Cl count rate was obtained by subtracting the ^{22}Na counts from the total after correcting for the efficiency difference between the beta and gamma counters. Ion influxes were calculated from the rate at which the isotopes entered the trout. The samples were also analyzed for Na concentration by atomic absorption spectrophotometry and Cl concentration by electrometric titration. Concentration changes were used to calculate net ion fluxes. Unidirectional Na and Cl effluxes were then calculated from the conservation equation $J_{net}=J_{in}-J_{out}$. These methods for determining ion fluxes in intact animals have been discussed by Kirschner (1970).

The first hour of measurement was considered a control or pre-exposure period. At the end of this hour, the irrigation solution was replaced with one containing the substance to be tested. Its pH was adjusted as necessary with tris-hydroxymethyl aminomethane (TRIS). In many experiments with the polymers the solution was in contact with the gill for only a short time (1–10 min), and flux measurements were made after it was removed. In some experiments with polymers and with the low molecular weight amines, fluxes were measured with the compounds present in the bathing solution. Such differences in experimental protocol will be described with the eperimental results.

Poly-L-lysine (MW 100,000), protamine sulfate, poly-L-glutamic acid, were purchased from the Sigma Chemical Company, St. Louis. The amiloride was generously provided by Dr. John Baer, Merck Institute for Therapeutic Research.

Results

Polypeptides

When poly-L-lysine was added to the irrigation solution for as little as one minute there occurred a large net loss of Na after its removal. However, detailed examination of its action revealed a number of interesting points. Fig. 1 shows the results of a 1-min exposure of the gill to PLL at a concentration 50 μg/ml (about 5×10^{-7} M). The fish was in a steady state during the control period, but it lost about 100 μEquiv during the first 20 min after exposure. Net loss continued but was much slower, amounting to only about 10 μEquiv, during the last 40 min of this period. The upper panel shows that isotope uptake was also suppressed during this period, but influx values could not be calculated for reasons given below. When the solution was changed and measurements made for a second hour, inhibition of tracer uptake persisted, although net loss of Na from the animal was slow.

Brief exposure of the gill to PLL had the same effects on chloride movement. As shown in Fig. 2, there was rapid net loss during the

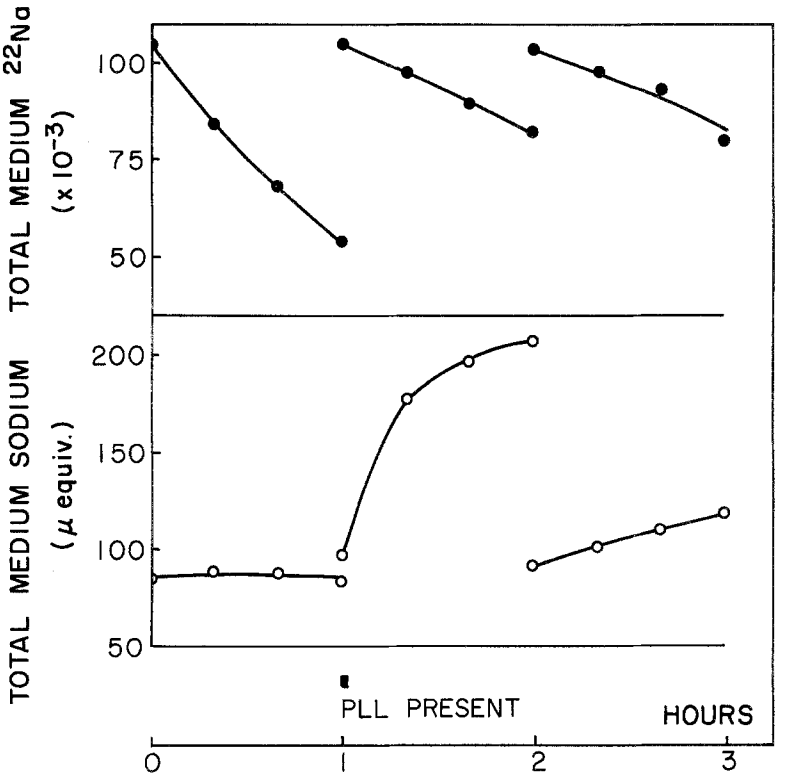

Fig. 1. The effect of brief exposure to PLL on Na fluxes across trout gill. *Upper panel*: Total quantity of tracer in the irrigation (external) medium. Influxes are calculated from tangents to these lines and the medium specific activity (Kirschner, 1970). *Lower panel:* Total quantity of sodium in the medium. Net fluxes are calculated from tangents to these lines. Although the abscissa suggests that the three hours of measurements were unbroken, about 5 min elapsed between the end of the first period and the beginning of the second. The PLL was applied (black bar), then removed, and the gill rinsed with PLL-free solution during this interval. About 2 min elapsed between the end of the second period and beginning of the third

first 20 min after exposure followed by reversion to a lower rate which continued during the next hour. In contrast, inhibition of isotope uptake persisted for at least two hours after PLL was removed.

The results seen in Figs. 1 and 2 were noted at all concentrations between 3 and 100 μg/ml and with exposure times varying between 1–10 min. A dose-response relationship was not determined, but the threshold concentration appeared to be 0.1–1.0 μg/ml, and six fish exposed to concentrations between 0.01 and 0.1 μg/ml showed no effect. Table 1 shows mean values for the net loss of Na and Cl from a group of animals exposed briefly (first two lines) to concentrations between 10–

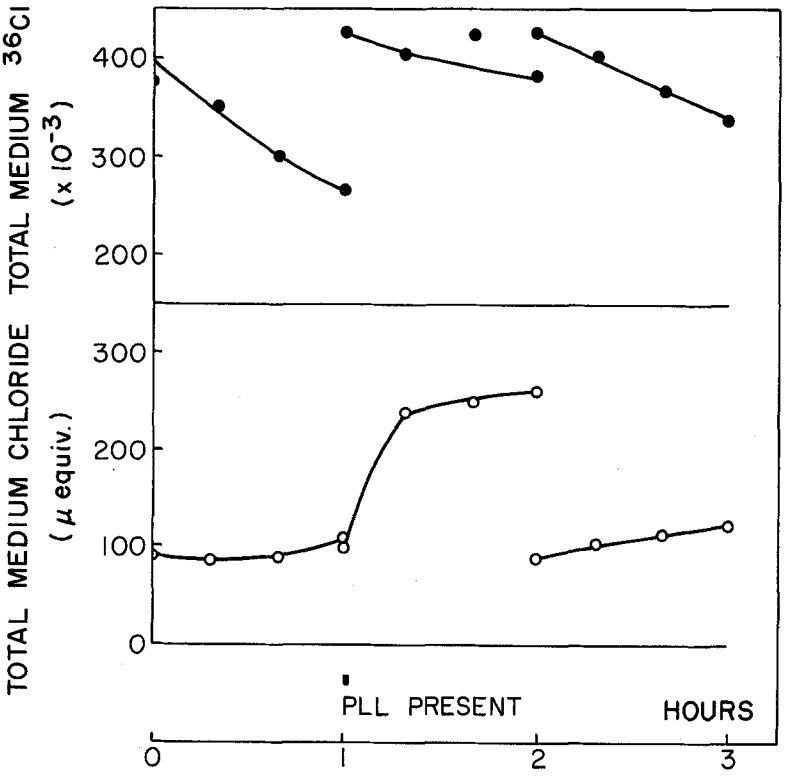

Fig. 2. The effect of brief exposure to PLL on Cl fluxes across trout gill. Chloride measurements made simultaneously with Na on the same fish shown in Fig. 1

Table 1. Net ion movement after PLL

Ion	*n*	Control hour	First hour		Second hour
			0–20 min	40–60 min	
			Short exposures[a]		
Na	8	−1.8±3.0	−117±17	−12.9±4.5	−7.1±3.0
Cl	10	−1.6±2.9	−129±17	−13.0±3.5	–
			Long exposure[b]		
Na	5	−0.2±1.8	−236±45	−71±28	−43±21
Cl	4	+2.5±4.9	−237±57	−99±44	−47±25

Loss rates are in μEquiv $(100\,g)^{-1}hr^{-1}$ ± SEM. Positive sign for net uptake.

[a] PLL was applied for 1–10 min after the control hour, then removed. Net fluxes are shown for two successive hours after its removal.

[b] PLL was added after the control hour and remained in the medium through the next ("first") hour. It was then removed and a PLL-free medium used for the "second" hour.

Table 2. Unidirectional ion fluxes after PLL

Ion	n	Control hour		Second hour	
		J_{in}*	J_{out}*	J_{in}*	J_{out}*
Na	10	16.1±1.4	16.2±3.2	9.7±0.8	14.5±2.3
Cl	6	16.7±2.5	22.8±6.0	9.1±3.7	21.7±2.6

* μEquiv $(100\,g)^{-1}hr^{-1}$.

100 μg/ml. During the first twenty minutes following exposure the average loss rate was more than $100\,\mu Equiv\ (100\,g)^{-1}hr^{-1}$, which is about 7 times the mean unidirectional efflux in the same animals during the control period. Unidirectional fluxes were not estimated during the first post-exposure period because the specific activity, Na concentration, and J_{net} all changed too rapidly. Although isotope uptake was lower than in the control period there is no assurance that total Na influx was suppressed because the isotope was diluted so rapidly during this period. But even if Na influx were completely inhibited this could not account for the rapid net loss, and the latter can be explained only if a massive increase in efflux occurred. Salt loss was slower through a second hour after exposure.

Unidirectional influx and efflux were measured during the second hour after PLL exposure, and the results are shown in Table 2. Influx of both Na and Cl was inhibited by an average of 40%, and in a number of experiments remained depressed for at least two more hours. Reversal of the inhibition was never noted. Efflux during the second and subsequent hours differed little from the controls. Thus, the slow net loss shown in the last panels of Figs. 1 and 2 is due primarily to the suppressed influx.

Poly-D-lysine and protamine both have the same effect, hence the actions described are neither stereochemically constrained nor specific to a lysine polymer. On the other hand, poly-L-glutamate was inactive showing that a net positive charge is required.

In the experiments described above the PLL was applied briefly, then removed before any measurements were made. However, the results were changed only quantitatively when it was allowed to remain in the medium. Fig. 3 shows an experiment in which PLL was present for an hour during which isotope and chemical quantities of Na were measured. It was then removed and measurements were continued for a second

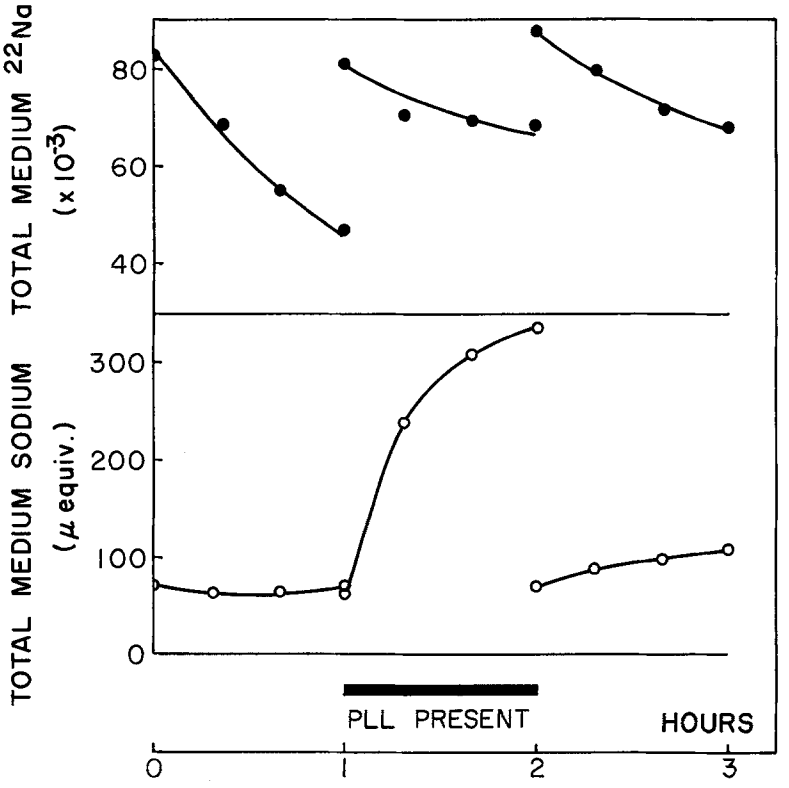

Fig. 3. The effect of prolonged exposure to PLL on Na fluxes across trout gill. PLL present throughout the second hour (black bar). All notations as in Fig. 1

hour. There was an initial rapid loss of Na, but this was markedly reduced during the last part of the period and was further reduced during the next hour. The third line of data in Table 1 shows the results of several such experiments on net Na loss. All loss rates are higher than after brief exposures, but it is clear that the permeability change reverses even in the presence of the PLL.

Fig. 4 shows that PLL causes the same striking increase in mannitol efflux and, as with the ions, the effect was transient. Expressed as a plasma clearance mannitol efflux during the control period was 0.053 ml $(100\ g)^{-1}hr^{-1}$. This rose to 0.33 ml $(100\ g)^{-1}hr^{-1}$ during the first 15 min of a one-hour exposure, but fell markedly during the last 30 min. Following removal of the PLL the clearance rate again was 0.053 ml $(100\ g)^{-1}hr^{-1}$, close to the control value. Sodium and chloride clearances in similar experiments (from Table 1) averaged about 1.5 ml $(100\ g)^{-1}hr^{-1}$ during and 0.25 ml $(100\ g)^{-1}hr^{-1}$ after exposure to PLL.

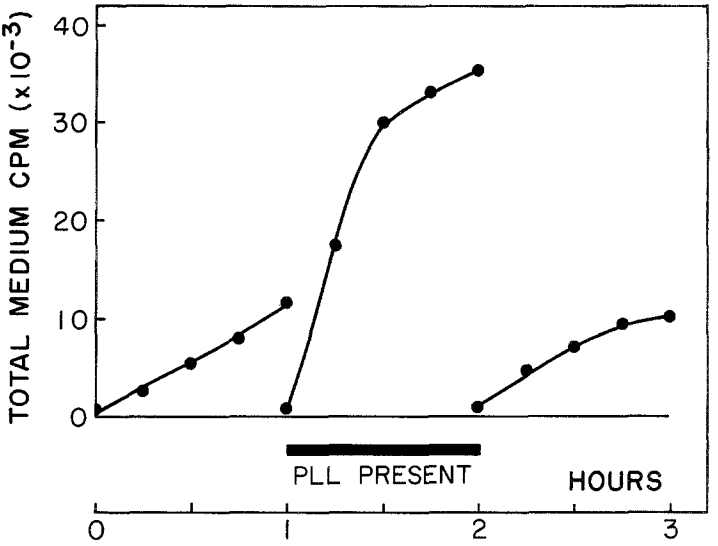

Fig. 4. The effect of prolonged exposure to PLL on mannitol efflux across trout gill. Ten μCi of ^{14}C-mannitol (50 μCi/μmole) were injected into the peritoneal cavity. The vehicle was 1 ml Ringer's solution. One hour was allowed for equilibration before the initial medium sample was taken (time zero). A blood sample was taken at the end of the experiment

Thus, the leak pathway temporarily opens to a relatively large nonpolar compound as well as to the smaller ions.

Lysine, Methyl-lysine and Amiloride

In most of the PLL experiments the concentration of lysine residues was 0.1–1.0 mM. In this concentration range lysine had no effect on ion movement across the gill. At 10 mM lysine stimulated influx by nearly 50% but had little effect on efflux as shown in Table 3. Further experiments were done using lysine methyl ester. By blocking the carboxyl group, the possibility of ionic interaction between the epsilon amino group of one lysine molecule and the carboxyl of another is eliminated. In this regard, lysine methyl ester more closely resembles PLL in which the carboxyl is "blocked" by the peptide linkage. However, the ester should have one more amino group per lysine residue available for possible reaction with anionic sites on the gill. Lysine methyl ester had

Table 3. Low molecular weight amines and Na fluxes

Compound	Conc. (mM)	n	First hour*		Second hour*		Third hour*	
			J_{in}	J_{out}	J_{in}	J_{out}	J_{in}	J_{out}
L-lysine	10	5	15.2±2.5	13.9±1.5	22.2±3.7	14.1±3.6	18.9±2.9	12.6±1.5
Methyl lysine	10	6	21.9±2.2	17.2±3.1	9.8±2.2	16.4±2.4	25.2±2.7	17.7±2.5

Fluxes in μEquiv $(100\ g)^{-1} hr^{-1}$.

* The test compound was present only during the second hour.

no effect on sodium efflux across the gill (Table 3), but it did cause a 55% reduction in sodium influx. This inhibition of influx was completely reversible. Thus, methyl lysine resembles amiloride in its action on the gill, and its action differs markedly from that of PLL.

Since it had been shown previously (Kirschner *et al.*, 1973) that amiloride inhibits only sodium influx and not chloride transport, we considered the possibility that amiloride inhibition of sodium influx was competitive. To test this, influx was determined for 10 fish as a function of external [Na] in the presence or absence of 5×10^{-5} M amiloride (5 controls and 5 experimentals). Reciprocal fluxes are shown as a function of reciprocal concentrations in Fig. 5. The lines were fitted by least squares. The ordinate intercepts ($1/J_{in}^{max}$) are not significantly different (0.044 ± 0.021 and 0.036 ± 0.005), while the slopes (K_m/J_{in}^{max}) differ by factor of about six (0.015 ± 0.001 and 0.094 ± 0.006). From these data a K_I of 10^{-5} M can be calculated for amiloride. The mechanism of inhibition by methyl lysine was not explored, but the similarity of its action to that of amiloride suggests a common mode of action.

Although the actions of PLL and of amiloride differ in several regards it seemed worth considering that both might combine with anionic sites on the outer surface of the gill, possibly the same sites. This possibility was examined by attempting to block or reverse the PLL effect with amiloride. Table 4 shows the results of one type of competitive experiment. The animals involved were exposed for 15 min to amiloride (10^{-3} M; 10 times the concentration needed for complete inhibition of Na influx), then for 10 min to amiloride plus PLL. After removing the inhibitors net and unidirectional Na fluxes were measured as in Fig. 1 and Table 1. The data in Table 4 show that these behave as if PLL were present alone. In addition, when influx was inhibited by PLL the inhibition could not be reversed by exposing the gill to 10^{-3} M amiloride.

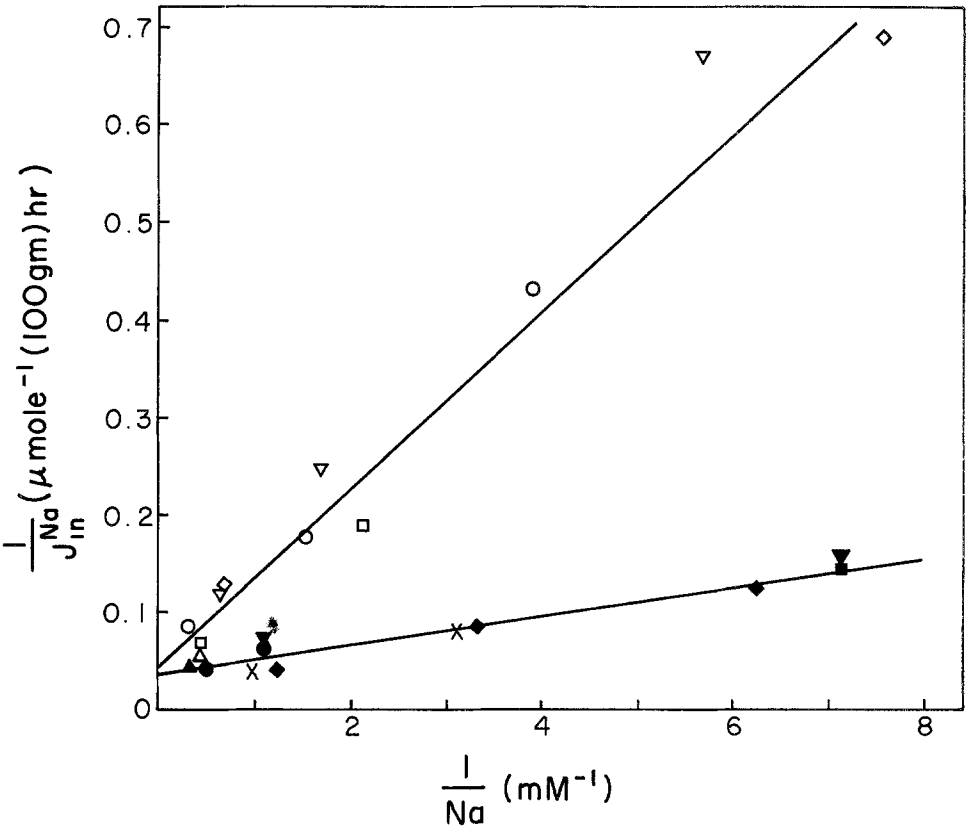

Fig. 5. Lineweaver-Burke plot of Na influx in the presence and absence of amiloride (5×10^{-4}M). Because of the length of time necessary for each flux determination no more than three measurements were made in any one animal. Identical symbols (e.g. three open circles) show values obtained from one animal. Control and experimental measurements were made on different groups of fish; no control fluxes were measured for fish exposed to amiloride (e.g. a filled circle and an open circle represent different animals). Use of two different groups of animals can be justified on the basis of the reproducibility of data in such experiments. For example J_{max} and K_m from this control set are 27.7 μEquiv (100 g)$^{-1}$hr^{-1} and 0.42 mM compared with 33.3 μEquiv (100 g)$^{-1}$hr^{-1} and 0.46 mM from Kerstetter *et al.* (1970). Measurements made in the presence of amiloride are represented by open symbols

Table 4. PLL and amiloride on Na fluxes

n	Control		After PLL+Amiloride		
	J_{in}	J_{out}	0–20 min	60–120 min	
			J_{net}	J_{in}	J_{out}
6	15.6±2.8	15.3±1.0	−77.1±12.6	8.4±1.4	13.8±1.3

Fluxes in μEquiv (100 g)$^{-1}$hr^{-1}.

Discussion

PLL, added to the medium bathing the gill, clearly has two distinct effects. Even very brief exposure causes a gross increase in permeability. Na and Cl loss is initially very rapid, amounting to the clearance of about 0.5 ml plasma $(100\ g)^{-1}hr^{-1}$. The leak appeared to be nonselective; Na, Cl and mannitol were all lost rapidly at high rates. The change was transient, and if exposure to PLL was brief, efflux of Na and Cl reverted to control values within an hour. Even when the inhibitor remained in the medium during flux measurements, ion loss decreased markedly, although it remained above control values.

The other effect of PLL was inhibition of influx of both Na and Cl. We did not estimate the extent of inhibition during the first hour following brief exposure, but during the second and subsequent hours transport of both ions appeared to be irreversibly inhibited by about 40%.

Several characteristics of these actions are worth noting. They developed very rapidly, so that a 1-min exposure (10^{-6} M) was nearly as effective as treatment for an hour. There seems to be little specificity involved beyond the requirement that the compound be a polycation; poly-L-glutamate was completely inactive. Nor did the size of the compound appear to be critical. Ply-L-lysine with molecular weights ranging from 4,000–100,000 were all effective. These observations suggest that anionic sites with little specificity are involved as was suggested by Mamelak *et al.* (1969). They noted that when PLL was added to the outside (mucosal side) of the toad bladder, passive efflux (serosa-mucosa) doubled and active influx fell. Qualitatively their results resembled ours, though with significant differences in detail. Using ^{14}C-labeled PLL they showed that the compound was attached to the mucosal surface of the epithelium, and that many of the cells appeared lysed at concentrations used in our work. They attributed the permeability increase to either membrane damage or to shedding of a mucus coat. Membrane damage has been shown to cause leakage of low molecular weight compounds from a number of plant tissues (Drew & McLaren, 1970; Osmond & Laties, 1970). However, in the toad bladder radioactive PLL was found on the mucosal membranes, and if confined there in the gill, the basolateral membranes should have remained intact. This means that transcellular solute loss from the blood should occur at rates determined by the permeability of the intact serosal surfaces. In our experiments rates of Na, Cl and mannitol loss were of about the same order of magnitude.

It seems unlikely that serosal permeabilities to the three solutes are similar, suggesting that the rapid efflux may not have been transcellular. An alternative explanation is that PLL also caused transient damage to the tight junctions thereby opening intercellular pathways for solute loss to the external medium. However, we cannot exclude the possibility that damage was done to both mucosal and serosal membranes rendering both abnormally permeable to all three solutes and opening the transcellular route. Whether the PLL effect is on cell membranes or intercellular junctions (or both), the mechanism by which the damage is repaired, even in the presence of the polycation, is unknown and worth further investigation.

The mechanism by which PLL suppresses influx for at least 2–3 hr is also obscure. Our initial hypothesis was that this was merely another example of the ability of substituted ammonium compounds to inhibit Na transport. However, the effects of PLL and compounds such as amiloride and methyl lysine differ markedly. The low molecular weight compounds inhibit only Na uptake and have no effect on Cl influx, while PLL inhibits both. The action of amiloride and methyl lysine is reversible, and at least for the former, is apparently competitive with Na for binding sites, while the PLL block lasts for several hours after the compound is removed. And finally, amiloride was unable to block the action of PLL even in concentrations ten times higher than that needed to saturate the binding sites. The data indicate that anionic sites accessible from the mucosal medium are involved in inhibition by both classes of compounds, but that different anionic groups must be involved. It is also curious that lysine itself did not inhibit Na influx. Lysine has two free amino groups, one of which may interact with its alpha carboxyl group. It is not clear from these studies why NH_4^+, lysine methyl ester, amiloride (3 NH_3^+ per molecule), but not lysine, can apparently compete with sodium for transport sites on epithelia.

One other point is worth noting. In the first paper describing the inhibitory effect of amiloride on Na influx on isolated toad bladder it was noted that the inhibition did not appear to be competitive (Bentley, 1968). Yet inhibition in fish gill is clearly competitive. This is another in a lengthening list of differences between the functions of transport epithelia *in vivo* and *in vitro*.

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