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A NEW TECHNIQUE FOR MEASURING WATER TRANSPORT ACROSS THE SEAWATER EEL INTESTINE

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SUMMARY

A new technique was developed for measuring net water flux across the eel intestine *in vitro*. The new perfusion method was suitable for long duration experiments because of continuous oxygen supply to both the external and the perfusion medium. Net water flux was calculated directly from the difference between the rates of effluent and perfusate flow without measuring the concentration of marker substances. The calculated value of net flux appears to be reliable because: its direction is always from mucosa to serosa in both everted and non-everted intestine; ouabain diminishes it to zero; it is identical with the standard water flux obtained under zero perfusion; and it is identical with the value obtained by means of [^{14}C]PEG following the previous perfusion method. The net water fluxes obtained by the new method were steadier than those obtained with the previous complicated perfusion method. In this new experimental system, the net water flux and the transepithelial potential difference (PD) decreased gradually with time, and were not restored by application of adrenergic agonists or cortisol. These parameters were stimulated by 5 mmol l^{-1} L-alanine, but not by D-glucose, L-valine or L-glycine, indicating a specific action of L-alanine.

INTRODUCTION

It is well known that marine teleosts ingest sea water and absorb water together with monovalent ions from the intestine to compensate for water loss from the body (Smith, 1930). Such solute-linked water transport has been demonstrated in the isolated seawater eel intestine *in vitro* (Oide & Utida, 1967; Utida *et al.* 1972; Ando, 1975, 1980, 1981, 1983, 1985), where the net water flux was measured gravimetrically using an intestinal sac (Wilson & Wiseman, 1954). However, for elucidating mechanisms of water transport more precisely by altering ionic composition, this method has the defects that the inside of the intestinal sac is not stirred and the ionic concentration of the inside solution tends to change gradually with time.

Key words: water transport, alanine, eel intestine.

In highly water-permeable epithelia such as small intestine, the relatively small net water flux can hardly be obtained from the difference between larger unidirectional fluxes of labelled water. Besides the gravimetric method, two other methods have been employed. One method involves monitoring the water level inside a small capillary tube connected to one side of the Ussing chamber, using either optical measurements (Rüphi, De Sousa, Favrod-Coune & Posternak, 1972; Van Os, Michels & Slegers, 1976; Eldrup, Frederiksen, Møllgård & Rostgaard, 1982) or changes in electrical capacitance (Wiedner, 1976; Van Os, Wiedner & Wright, 1979). However, in these systems oxygen supply is limited and stirring may be insufficient, since one compartment of the Ussing chamber must be closed completely.

The other method is to measure the net water flux in the perfusion system, where the net water flux ($J_{net}^{H_2O}$) is calculated from the ratio of the concentrations of reference substances, such as [^{14}C]polyethylene glycol (PEG) or [^{3}H]inulin, between the perfusate and effluent as follows:

$$J_{net}^{H_2O} = V_i - V_e = V_e(C_e/C_i - 1),$$

where V_i and V_e are the rates of perfusate and effluent flow; C_i and C_e are the concentration in the perfusate and effluent, respectively (Mullen, Muller & Van Bruggen, 1985). However, it must be noticed that this equation is obtained from assuming $V_iC_i = V_eC_e$. In relation to this point, there are some reports that PEG is an unreliable marker substance in the intestine (Worning & Amdrup, 1965; Barmada, Elder & Tomlin, 1983), probably because of insufficient mixing of the marker (Worning & Amdrup, 1965). Bunce & Spraggs (1982) also reported that the recovery of [^{14}C]PEG from the intestine is incomplete, i.e. $V_iC_i \neq V_eC_e$.

In the present study, by improving the perfusion method, the rate of net water flow is directly calculated from the difference between the rates of perfusate and effluent flow ($J_{net}^{H_2O} = V_i - V_e$) in the perfusion system, where both media are continuously supplied with sufficient oxygen. Using this apparatus, it is clearly demonstrated that water transport across the seawater eel intestine is stimulated by L-alanine but not by D-glucose.

MATERIALS AND METHODS

Japanese cultured eels, *Anguilla japonica*, weighing about 200 g, were obtained from a commercial supplier and kept in seawater aquaria at 20°C for more than 1 week before use. They were decapitated. The intestine was excised and the outer muscle layers were stripped off following our previous method (Ando & Kobayashi, 1978). A cylindrical polyester mesh (NBC Kogyo, T. No 80T) 6 cm long was inserted into the middle part of the intestinal tube and both ends of the intestine were tied to polyethylene tubes (4 mm o.d.) with silk thread. After filling the inside of the intestinal tube with Ringer solution (approx. 1·1 ml), the preparation was set horizontally in a trough (30 ml) which was placed in a water jacket maintained at 20°C.

Ringer solution was pumped into the tube *via* a damper (to eliminate pulsations) using a pump (FMI, RP-G6-0, usually; Iwaki, MM-1, otherwise) led from a

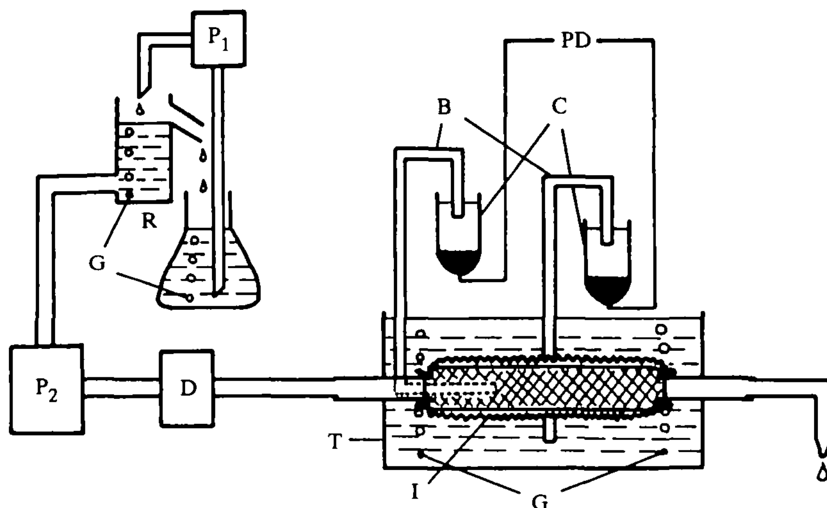

Fig. 1. Schematic diagram of a new apparatus for simultaneous measurement of net water flux and transepithelial potential difference (PD). I, intestine supported with polyester mesh; T, trough with water jacket (not shown) for temperature regulation; P_1, levelling pump; P_2, main pump; R, reservoir with water jacket (not shown) for temperature regulation; D, damper with water jacket (not shown); B, Ringer–agar bridges; C, calomel electrodes; G, 95% O_2–5% CO_2 gas mixture for bubbling and stirring.

constant-level reservoir. The damper was made from a three-way tube with one end closed as an air cell. The reservoir was filled by a peristaltic pump (EYELA, MP-3C). All connections were made with Teflon tubing (1 mm i.d.). The temperature of the perfusate was adjusted to 20°C by placing the reservoir and the damper in water jackets maintained at 20°C.

Effluent was collected with fraction collector (EYELA, DC-20) with droplets of about 7 μl. The collected water volume was measured gravimetrically by using a balance (Sartorius, 1601MP8) connected to a computer (NEC, PC-8801 mkII), and was printed out automatically (NEC, PC-8826) at intervals. After measuring the rate of effluent flow (V_e) through the intestine, the perfusion rate (V_i) of the pump itself was measured by collecting fluid directly from the damper. Net water flux was calculated by subtracting the perfusion rate from the rate of effluent flow; $J_{net}^{H_2O} = V_e - \bar{V}_i$, where $\bar{V}_i$ is the mean of each V_i value. Perfusion could be maintained at a constant rate for more than 4 h (Fig. 2). Therefore, changes in the rate of effluent flow can be regarded as changes in the net water flux across the intestine.

According to the previous perfusion method, net water flux was also calculated simultaneously by using [^{14}C]PEG (New England Nuclear) which was counted with a liquid scintillation counter (Aloka, LSC-701). Collection of the effluent was started after a 90-min equilibration period.

Measurements of the transepithelial potential difference (PD) were made using 2% Ringer–agar bridges, calomel electrodes and a preamplifier (Nihon Kohden, MEZ-7101) connected to a polyrecorder (Toadempa, ERP-200A).

The standard Ringer solution contained ($mmol\,l^{-1}$): 118·5 NaCl; 4·7 KCl; 3·0 $CaCl_2$; 1·2 $MgSO_4$; 1·2 KH_2PO_4; 24·9 $NaHCO_3$; 5·0 glucose (pH 7·3). Glucose-free Ringer was identical to the standard Ringer solution with the omission of glucose. Both sides of the intestine were bathed with identical Ringer solutions and bubbled continuously with 95% O_2–5% CO_2 gas mixture throughout the experiments. Most experiments were started when the PD had almost reached a steady level. Ouabain (Merck), L-alanine, L-valine or L-glycine (Katayama Chemical Co., Osaka, Japan) was added to either side as indicated. At the end of the experiments, the intestine was cut longitudinally and spread on graph paper; the surface area of the intestine was measured using a planimeter (Ushikata, 220L). All data are expressed as mean ± S.E.

RESULTS

Net water flux across perfused intestine

When the lumen of the non-everted intestine was perfused, the rate of effluent flow was less than the perfusion rate by 20 μl/10 min (negative water gain in Fig. 3), indicating that this amount of water was absorbed through the seawater eel intestine (mucosa to serosa). After everting the intestine and perfusing the serosal side, on the other hand, the net water flux from mucosa to serosa was 70 μl/10 min. This difference in rate was also observed when measurements were made on the everted intestine before the non-everted intestine. The serosa-negative PD was nearly equal in both preparations. Since mucus secreted from the mucosa frequently stuck in the

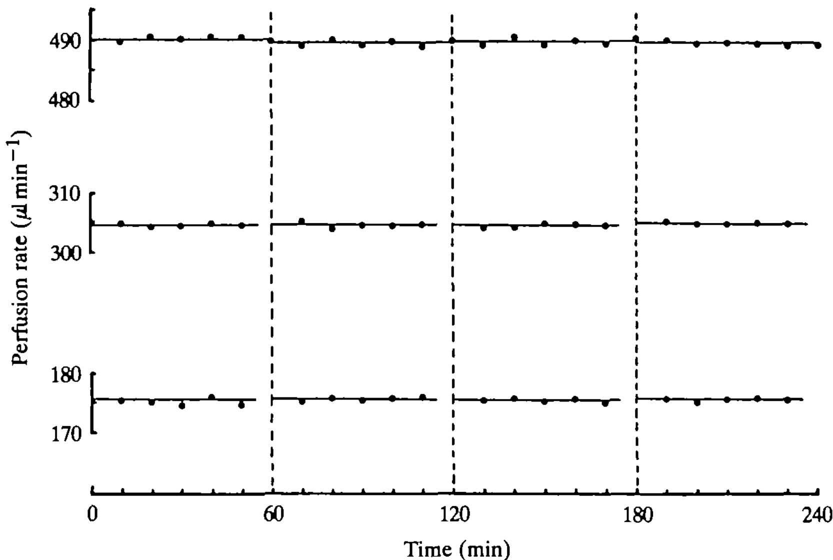

Fig. 2. Monitors of three perfusion rates. Perfusion rate was measured by collecting fluid directly from the damper every 10 min for 50 or 60 min at each perfusion rate. Horizontal lines represent mean values at each period. At the times indicated by vertical dotted lines, the main pump was switched off and then on again. Collection of the perfused fluid was started about 10 min after switching on.

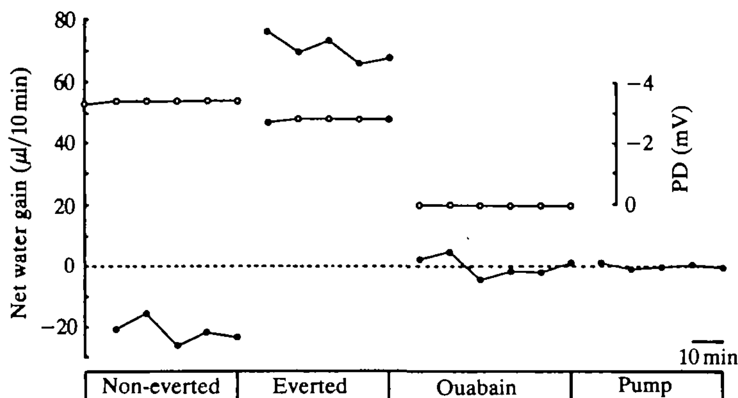

Fig. 3. A typical representation of net water gain (●) and the PD (○) across the seawater eel intestine. After finishing the experiments, the intestine was removed and perfusion rate was measured every 10 min for 50 min (pump period); the mean ± S.E. being 1746·3 ± 0·4 μl/10 min. By subtracting this mean value (dotted horizontal line) from each rate of effluent flow, the increase in the effluent (net water gain) was obtained. Negative sign means fluid lost by passing through the intestine. After perfusing the lumen of the correctly oriented intestine for 50 min (non-everted period), the intestine was removed from the apparatus and everted. The serosal side was then perfused for another 50 min (everted period). Ouabain (10^{-3} mol l^{-1}) was applied to the serosal side of the everted intestine for 1 h.

outlet tube in the non-everted intestine, the following experiments were all done only in the everted intestine.

The values of net water flux appear reliable for two reasons. First, net water flux and PD were abolished by the addition of 10^{-3} mol l^{-1} ouabain to the serosal fluid for 1 h, confirming previous results obtained in the sac preparation (Ando, 1981). Secondly, the net water flux was independent of perfusion rate (Fig. 4).

Even when perfusion was stopped completely by switching off the main pump and clamping the inlet tube into the intestine, effluent from the everted intestine was collected at a nearly constant rate (68·2 ± 7·7 μl cm^{-2} h^{-1}; $N = 7$), corresponding to net water flux from mucosa to serosa. Values in Fig. 4 are expressed as a percentage of this level (initial standard flux). Even when perfusion rate was increased stepwise, the mean net water flux obtained at each perfusion rate, $\bar{V}_e - \bar{V}_i$, was almost identical with the initial standard water flux if the perfusion rate was below 500 μl min^{-1}. However, when the perfusion rate was increased above 500 μl min^{-1}, the net water flux was decreased drastically, presumably due to a rise in hydraulic pressure of intestinal fluid. After these experiments, water flux was measured again under zero perfusion (final standard water flux), and those preparations whose final standard water flux was less than 70 % of the initial one were discarded. Less than 20 % of all preparations were adopted in this way. In most cases, net water flux reduced gradually with time, accompanied by a slight drop in the PD.

Comparison of the present and previous methods

To compare this new method with the previous perfusion method, which uses a marker substance such as PEG, simultaneous measurements of flow rates and PEG

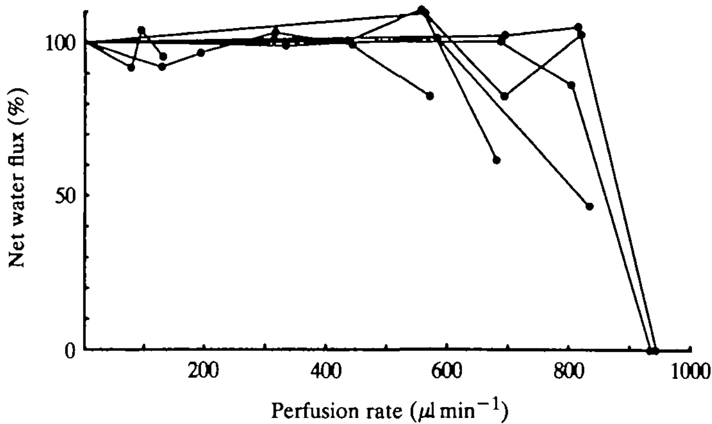

Fig. 4. Relationship between net water flux and perfusion rate in the everted intestine. Net water flux was obtained from the difference between the mean rates of effluent and perfusate flow, measured every 4 min for 20 min at each perfusion rate; $J_{net}^{H_2O} = \bar{V}_e - \bar{V}_i$. By increasing the perfusion rate progressively, the net water flux was plotted against the perfusion rate. Points obtained from an individual preparation are connected with a line. The net water flux is expressed as the percentage of the initial standard water flux (100 %) obtained after stopping perfusion completely ($V_i = 0$).

concentration in each flow were made (Table 1). The concentration of PEG in the effluent was lower by 333·3 c.p.m./50 μl than that in the perfusate, and the total amount of PEG (the product of the concentration of PEG and the rate of effluent flow) was almost equal to that in the perfusate flow, i.e. $V_eC_e = V_iC_i$, if mean values obtained from 10 successive measurements during 100 min were used. Therefore, the mean water flux obtained by the present new method (202·9 μl/10 min) was close to that calculated from the ratio of PEG concentrations (210·7 μl/10 min), according to the previous perfusion method. When net water fluxes were calculated every 10 min, however, the values of net water fluxes obtained from the previous method were erratic, while relatively constant net water fluxes were obtained by the present new method (Fig. 5).

Table 1. *Simultaneous measurement of two flow rates (effluent and perfusate) and (PEG) concentration*

Flow	Rate (μl/10 min)	Concentration of PEG (c.p.m./50 μl)	Total PEG (×10³ c.p.m./10 min)
Perfusate	1734·0 ± 1·7	3063·4 ± 9·8	106·24 ± 0·26
Effluent	1936·9 ± 3·9	2730·1 ± 11·5	105·75 ± 0·40

Mean ± S.E. ($N = 10$).

After bathing the intestine with the standard Ringer solution containing 5 mmol l⁻¹ L-alanine, the effluent or perfusate was collected every 10 min for 100 min, and the concentration of [^{14}C]PEG was determined in each collected sample.

PEG, polyethylene glycol.

Factors affecting net water flux and PD

Net water flux and PD continued to decrease during perfusion, and were not increased after addition of adrenaline (10^{-8}–10^{-4} mol l^{-1}), noradrenaline (10^{-8}–10^{-4} mol l^{-1}), phenylephrine (10^{-6}–10^{-5} mol l^{-1}), naphazoline (10^{-6}–10^{-5} mol l^{-1}), dopamine (10^{-6}–10^{-4} mol l^{-1}), cortisol (50 μg ml^{-1}) or insulin (1 μg ml^{-1}) into the serosal fluid (data not shown).

Addition of 5 mmol l^{-1} L-alanine into the mucosal fluid increased both the net water flux from mucosa to serosa and the serosa-negative PD, after latent periods of 20–30 min for net water flux and about 10 min for the PD (Fig. 6). Further addition of L-alanine (10 mmol l^{-1}) did not add to these increases, and 1 mmol l^{-1} alanine did not induce any enhancements in these parameters (data not shown), implying an all-or-nothing type of response with a threshold concentration of about 5 mmol l^{-1}. Although an effect of serosal L-alanine (5 mmol l^{-1}) is not demonstrated in Fig. 6, a slight enhancement of the net water flux and the PD was induced by serosal L-alanine (5 mmol l^{-1}) in other preparations.

Since mucosal alanine might stimulate Na^+ transport from mucosa to serosa and thus stimulate water transport, the effects of another stimulant of Na^+ transport were examined and compared with those of L-alanine (Fig. 7). After the PD had reached a steady level in glucose-free Ringer solution, 5 and 10 mmol l^{-1} glucose was added into the mucosal fluid, but no enhancement of net water flux or PD was observed.

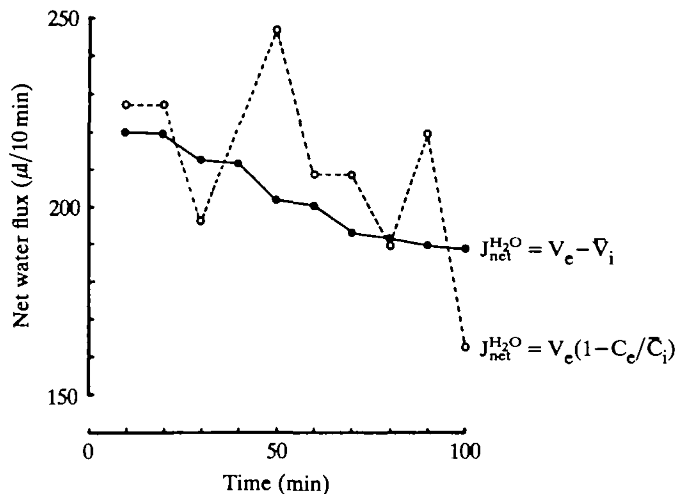

Fig. 5. Time course of the net water fluxes obtained by two perfusion methods. After bathing the intestine with standard Ringer solutions containing 5 mmol l^{-1} L-alanine, the effluent was collected every 10 min for 100 min, and the rate and the polyethylene glycol (PEG) concentration of the effluent were measured simultaneously. The net water fluxes were calculated every 10 min from difference between the rates of perfusate and effluent flow ($J_{net}^{H_2O} = V_e - \bar{V}_i$, solid line), or from ratio of the concentration of PEG ($J_{net}^{H_2O} = V_e[1 - C_e/\bar{C}_i]$, dotted line). $\bar{V}_i$ and $\bar{C}_i$ indicate mean values of the flow rate and the PEG concentration in the perfusate, respectively. Each value was obtained from the same data used in Table 1.

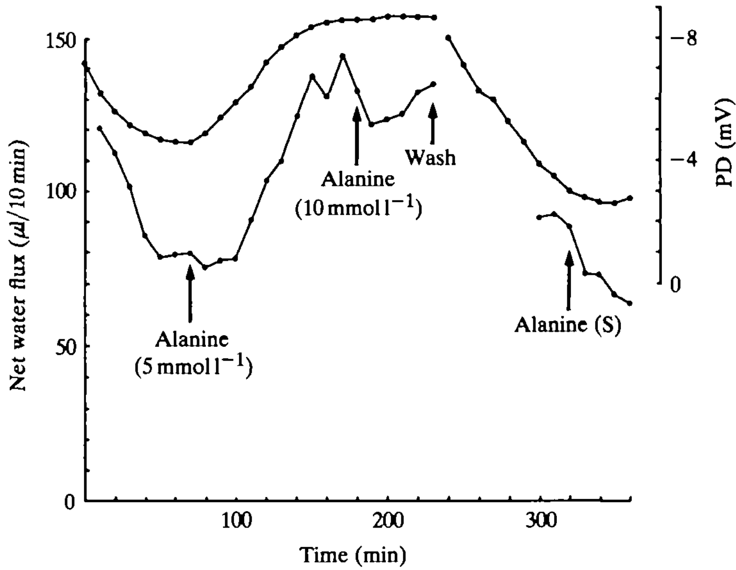

Fig. 6. Effects of L-alanine on the net water flux (●) and the PD (○) in the standard Ringer solution. Measurement of net water flux was started 50 min after setting up the preparation, and $5\,\text{mmol}\,l^{-1}$ L-alanine was added to the mucosal fluid after the PD reached a nearly steady level (first arrow). At the second arrow $10\,\text{mmol}\,l^{-1}$ L-alanine was applied to the mucosal fluid. Perfusion rate was $219{\cdot}9 \pm 0{\cdot}1\,\mu l\,\text{min}^{-1}$. (S) denotes that $5\,\text{mmol}\,l^{-1}$ L-alanine is added to the serosal fluid.

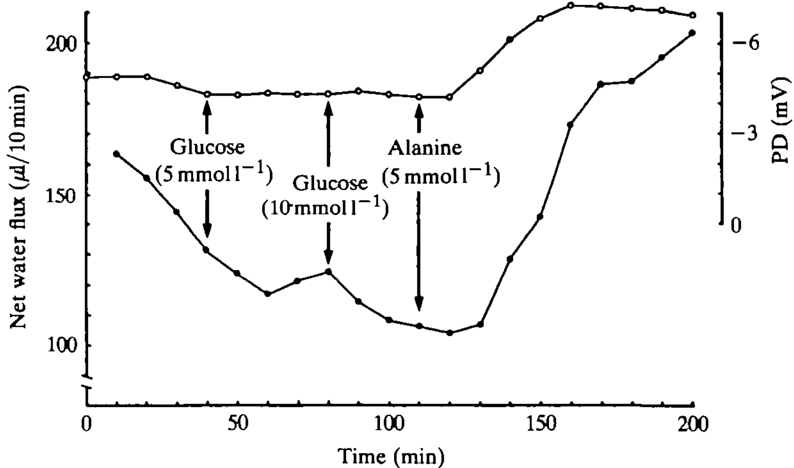

Fig. 7. Effect of D-glucose on the net water flux (●) and the PD (○) in glucose-free Ringer solution. After bathing the intestine with glucose-free Ringer solution, $5\,\text{mmol}\,l^{-1}$ D-glucose was added to the mucosal fluid (first arrows) and an additional $5\,\text{mmol}\,l^{-1}$ at the second arrows ($10\,\text{mmol}\,l^{-1}$). $5\,\text{mmol}\,l^{-1}$ L-alanine was added into the mucosal fluid at the third arrows. Perfusion rate was $219{\cdot}2 \pm 0{\cdot}2\,\mu l\,\text{min}^{-1}$.

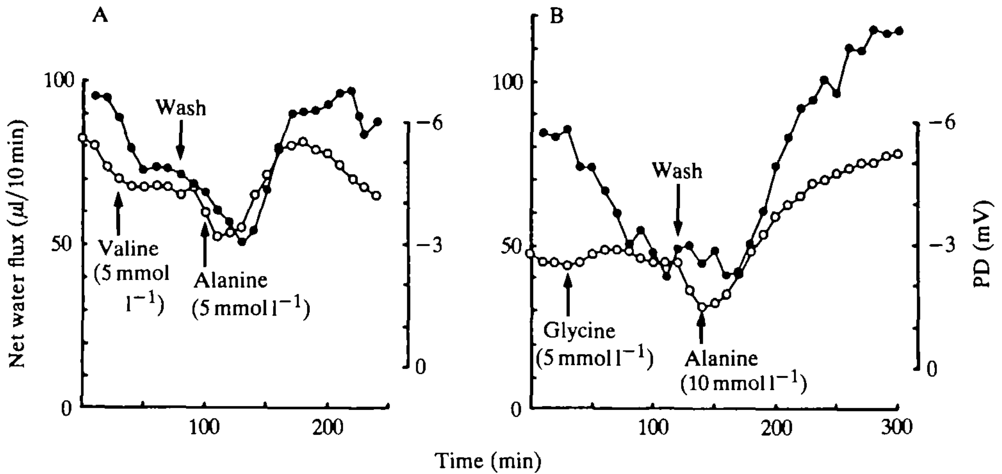

Fig. 8. Effect of other neutral amino acids on the net water flux (●) and the PD (○) in glucose-free Ringer solution. (A). Effect of L-valine. L-Valine (5 mmol l^{-1}) was added to the mucosal fluid at the first arrow, and 5 mmol l^{-1} L-alanine at the third arrow. Perfusion rate was 172·6 ± 0·3 µl min^{-1}. (B) Effect of L-glycine. L-Glycine (10 mmol l^{-1}) was added to the mucosal fluid at the first arrow, and 10 mmol l^{-1} L-alanine at the third arrow. Perfusion rate was 218·5 ± 0·1 µl min^{-1}.

When 5 mmol l^{-1} was added into the mucosal fluid, however, the net water flux and the PD increased gradually, with latent periods as cited above.

To examine the specificity of the effect of L-alanine, other neutral amino acids, L-valine and L-glycine, were applied. These amino acids are known to be absorbed across the fish intestine (for a review, see Ferraris & Ahearn, 1984). As shown in Fig. 8, mucosal addition of 5 mmol l^{-1} L-valine or 10 mmol l^{-1} L-glycine did not enhance the net water flux significantly, and tended to increase the PD only negligibly. Responses to L-alanine were subsequently observed in these preparations.

Table 2 summarizes the effects of D-glucose (5 mmol l^{-1}) and L-alanine (5 mmol l^{-1}) on the PD and the net water flux. When both sides of the seawater eel intestine

Table 2. *Effects of* D-*glucose and* L-*alanine on the transepithelial potential difference (PD) and the net water flux ($J_{net}^{H_2O}$) across the perfused intestine of the seawater eel*

Preparation	Nutrient Glucose (mmol l^{-1})	Nutrient Alanine (mmol l^{-1})	No. of eels	PD* (mV)	$J_{net}^{H_2O}$* (µl cm^{-2} h^{-1})
Perfused (everted)	0	0	16	−3·2 ± 0·3	58·1 ± 3·5
	5	0	9	−4·4 ± 0·4	60·7 ± 7·4
	0	5	8	−6·8 ± 0·6	84·0 ± 6·0
	5	5	7	−7·4 ± 0·3	84·9 ± 3·3
Sac (non-everted)†	5	0	35	−6·0 ± 0·2	49·6 ± 2·7

* Mean ± S.E.

† For comparison, previous results obtained in the sac preparation (Ando, 1980) are given.

In both preparations, muscle layers were stripped off and each value was obtained under a nearly-steady state, more than 1 h after bathing the preparation in each experimental solution.

were bathed with nutrient-free Ringer solution, serosa-negative PD ($-3{\cdot}2 \pm 0{\cdot}3$ mV) and the net water flux ($58{\cdot}1 \pm 3{\cdot}5\,\mu l\,cm^{-2}\,h^{-1}$) from mucosa to serosa were observed, although they tended to decrease with time. Similar results were obtained even in the presence of 5 $mmol\,l^{-1}$ D-glucose. On the other hand, when the Ringer solution contained 5 $mmol\,l^{-1}$ L-alanine, higher serosa-negative PD ($-6{\cdot}8 \pm 0{\cdot}6$ mV) and net water flux ($84{\cdot}0 \pm 6{\cdot}0\,\mu l\,cm^{-2}\,h^{-1}$) were obtained, and they stayed at a nearly steady level for more than 4 h. Further addition of 5 $mmol\,l^{-1}$ D-glucose to the alanine-containing bathing solutions did not significantly enhance these two parameters.

DISCUSSION

The present study reports a new technique for measuring net water flux across the intestine *in vitro*, which overcomes the defects of previous methods (see Introduction). In this new technique, net water flux was calculated directly from the difference between the rates of effluent and perfusate flow ($J_{net}^{H_2O} = V_e - V_i$) without measuring the concentration of a marker substance. The net water flux obtained in this way appears to be reliable: its direction is always from mucosa to serosa in both everted and non-everted intestines; ouabain, an inhibitor of ions and water transport across the seawater eel intestine (Ando, 1981, 1985), reduces it to zero (Fig. 3); it is almost identical with the standard water flux obtained under zero perfusion (Fig. 4); and it is identical with the net water flux obtained by the previous perfusion method, if mean values for 100 min are compared (Table 1). However, the values of net water flux obtained by the previous perfusion method were erratic, if they were calculated every 10 min (Fig. 5), presumably due to insufficient mixing of PEG as reported by Worning & Amdrup (1965). In contrast, the net water fluxes calculated every 10 min by this new method were relatively steady (Fig. 5). Therefore, it may be reasonable to conclude that the present simple method is more accurate than the previous complicated perfusion method. The new method is also convenient for controlling the ionic condition and oxygen supply to both sides of the intestine.

The net water flux was always much lower in the non-everted intestine than in the everted one (Fig. 3). With the non-everted intestine, the polyester mesh in the lumen may prevent solutes and water from arriving at the mucosal membrane of the epithelium; in the everted intestine, the boundary layer on the mucosal membrane is thinner, allowing transporting substances to reach the mucosal membrane more readily. The experiments with the non-everted intestine were also complicated by the accumulation of mucus in the outlet tube. (This intestine, compared with that in mammals, has numerous goblet cells: Ando & Kobayashi, 1978.)

The net water flux and the serosa-negative PD (explained by an active Cl^- transport from mucosa to serosa: Ando, Utida & Nagahama, 1975) were found to be insensitive to adrenergic agents or cortisol, although these substances are known to stimulate NaCl and fluid absorption across the mammalian intestine (reviewed by Berridge, 1983; Tapper, 1983; Turnberg, 1983). Instead, the PD and the water flux were stimulated by L-alanine. It is possible that the gradual decline in these parameters observed in standard saline may be due to the loss of L-alanine from the tissue.

L-Alanine had a greater effect on net water flux when it was applied on the mucosal rather than the serosal side. This suggests that Na^+ transport stimulated by mucosal L-alanine may enhance water transport since transport of L-alanine and D-glucose are coupled with Na^+ transport in the marine fish intestine (Eveloff, Field, Kinne & Mure, 1980; Bogé & Rigal, 1981) as in the mammalian intestine (see Schultz, 1979). However, D-glucose did not enhance the net water flux in the seawater eel intestine (Fig. 7; Table 2), although D-glucose is transported by the intestine of many fish (for a review, see Ferraris & Ahearn, 1984) including moray eel intestine (Ferraris & Ahearn, 1983). Moreover, other neutral amino acids such as L-valine and L-glycine, which are transported in the intestine of goldfish (Kitchin & Morris, 1971) and rainbow trout (Bogé, Rigal & Pérès, 1979), respectively, do not enhance the net water flux across the seawater eel intestine (Fig. 8). In addition, the enhanced serosa-negativity in the PD after application of L-alanine cannot simply be explained by the stimulation of Na^+ transport from mucosa to serosa. Therefore, the enhancement of the net water flux induced by L-alanine does not appear to be due to a stimulation of Na^+ transport, but seems to be due to a specific action of L-alanine.

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