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AMINO ACID METABOLISM AND WATER TRANSPORT ACROSS THE SEAWATER EEL INTESTINE

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Summary

To elucidate how intracellular L-alanine enhances water transport across the seawater eel intestine, effects of various metabolic inhibitors were examined. The L-alanine-induced water flux was inhibited by amino-oxyacetate, an inhibitor of aminotransferase. After blocking the synthesis of pyruvate from L-alanine with this drug, water transport was stimulated with pyruvate, whose effects were inhibited by oxythiamine, an inhibitor of pyruvate dehydrogenase. 2,4-Dinitrophenol (DNP) also inhibited the effects of L-alanine. Furthermore, L-alanine enhanced ouabain-sensitive O_2 consumption in this tissue, and the enhancement in O_2 consumption preceded that in the transepithelial potential difference (PD) and the net water flux. These results indicate that L-alanine is metabolized through the citric acid cycle to produce ATP, and that a metabolic product stimulates ion and water transport. L-Glutamine also seems to be metabolized just like L-alanine because: L-glutamine acted from inside the enterocyte; DNP inhibited the effects of L-glutamine; neither of the effects of L-glutamine and L-alanine were additive but they were mutually complementary; L-glutamine also enhanced ouabain-sensitive O_2 consumption; and the increment in O_2 consumption preceded that in the PD and the net water flux. The effects of L-glutamine on the PD and the net water flux depended on glutamine concentration and the concentration–response curve was of the Michaelis–Menten type, indicating that the rate of L-glutamine uptake into the enterocyte limits the overall rate of L-glutamine metabolism. A regulatory role of amino acids for ion and water transport is discussed.

Introduction

It has been demonstrated that water absorption across the seawater eel intestine is mostly coupled to a $Na^+/K^+/Cl^-$ transport system (Ando & Utida, 1986), but it is not yet clear how this transport is regulated. Recently, we discovered that water transport across the seawater eel intestine decreased gradually with time when the intestine was isolated and the serosal fluid was perfused (Ando *et al.* 1986), and that L-alanine stimulated the water transport by acting from inside the enterocytes (Ando, 1987). Similar stimulatory effects were also observed after application of D-alanine, L-glutamine and L-glutamate, but not other amino acids (Ando, 1987).

Key words: amino acid metabolism, glutamine, water transport, eel intestine.

To elucidate how intracellular L-alanine enhances water transport, various metabolic inhibitors were applied. In addition, L-glutamine metabolism was examined in relation to L-alanine metabolism. Finally, the effects of four kinds of amino acids (L-alanine, D-alanine, L-glutamine and L-glutamate) on oxygen consumption were investigated. The results obtained indicate that all these amino acids are metabolized and their metabolic products, such as ATP, stimulate ion and water transport.

Materials and methods

Japanese cultured eels, *Anguilla japonica*, weighing about 230 g, were obtained from a commercial supplier and kept in seawater aquaria at 20°C for more than 1 week without food. They were decapitated and the intestine was excised. The longitudinal muscle and much of the circular muscle of the intestine was stripped off to improve aeration, following our previous method (Ando & Kobayashi, 1978). After everting the intestine, a cylindrical polyester mesh was inserted into the middle part of the intestinal tube and the serosal side was perfused with standard Ringer's solution at a constant rate (around 173 $\mu l\,min^{-1}$). The perfusate was isolated from the bathing medium by tying off the ends of the isolated and everted intestine. The effluent was collected every 10 min and the net water flux was calculated directly from the difference between the rates of effluent and perfusate flow. Details for simultaneous measurement of net water flux and transepithelial potential difference (PD) were as described previously (Ando *et al.* 1986).

Oxygen consumption rate of the intestine was measured with a Clark oxygen electrode (Yellow Spring Instrument Co., Yellow Spring, OH), whose current output was recorded (Toadempa, EPR-10A); applied voltage was 0·7 V. From the slope of the record, O_2 consumption rate was calculated and expressed as the Q_{O_2} ($\mu l\,mg^{-1}h^{-1}$). The oxygen probe was immersed in a closed vessel (3·2 ml) containing standard Ringer's solution with three small sheets (approx. 1·5 cm × 0·5 cm) of the stripped intestine. The incubation medium was kept at 20°C and stirred gently (approx. 2 rev. s^{-1}). Various substrates or inhibitors were injected into the incubation medium through a small hole using a microsyringe.

The standard Ringer's solution contained (mmol l^{-1}): NaCl, 118·5; KCl, 4·7; $CaCl_2$, 3·0; $MgSO_4$, 1·2; KH_2PO_4, 1·2; $NaHCO_3$, 24·9 (pH 7·3 when bubbled with a 95 % O_2, 5 % CO_2 gas mixture). Amino-oxyacetate, oxythiamine, furosemide (Tokyokasei Co. Tokyo), 2,4-dinitrophenol (DNP), L-alanine, D-alanine, L-glutamine, L-glutamate, sodium glutamate, pyruvate (Katayama Chemical Co., Osaka) or ouabain (Merck) was added to either side of the intestine 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). The dry mass was obtained after drying the tissue at 110°C for 24 h.

Results

L-Alanine metabolism

When 5 mmol l^{-1} L-alanine was added to the mucosal fluid of the intestine, the serosa-negative PD and the net water flux (mucosa to serosa) increased as cited previously (Ando *et al.* 1986, 1987; Ando, 1987). These effects of L-alanine were inhibited by serosal application of 0·5 mmol l^{-1} amino-oxyacetate (an inhibitor of aminotransferase which converts L-alanine into pyruvate) (Fig. 1). Even in the presence of amino-oxyacetate, addition of 5 mmol l^{-1} pyruvate to the serosal fluid enhanced both the PD and the net water flux. These results indicate that L-alanine is converted into pyruvate by aminotransferase. The stimulatory effects of pyruvate were inhibited by oxythiamine (3 mmol l^{-1}), an inhibitor of pyruvate dehydrogenase which converts pyruvate into acetyl-CoA. This suggests that pyruvate acts through acetyl-CoA, which enters the citric acid cycle to produce ATP. Although in this experiment some agents were applied under non-steady-state condition to avoid deterioration due to prolonged incubation, each agent had

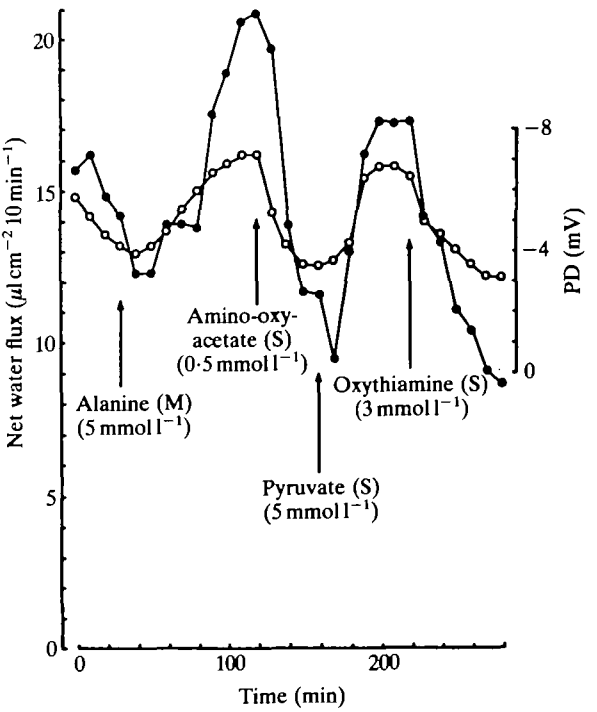

Fig. 1. Effects of L-alanine metabolism on the potential difference (PD, O) and the net water flux (●). After bathing the intestine with standard Ringer's solution, 5 mmol l^{-1} L-alanine was added to the mucosal fluid (first arrow). At the second arrow, 0·5 mmol l^{-1} amino-oxyacetate was added to the serosal fluid, while the mucosal medium contained L-alanine. In the presence of amino-oxyacetate, 5 mmol l^{-1} pyruvate was applied (third arrow). At the fourth arrow, 3 mmol l^{-1} oxythiamine was added to the serosal fluid. M or S in parentheses denotes that each agent is applied to the mucosal side or the serosal side of the intestine, respectively.

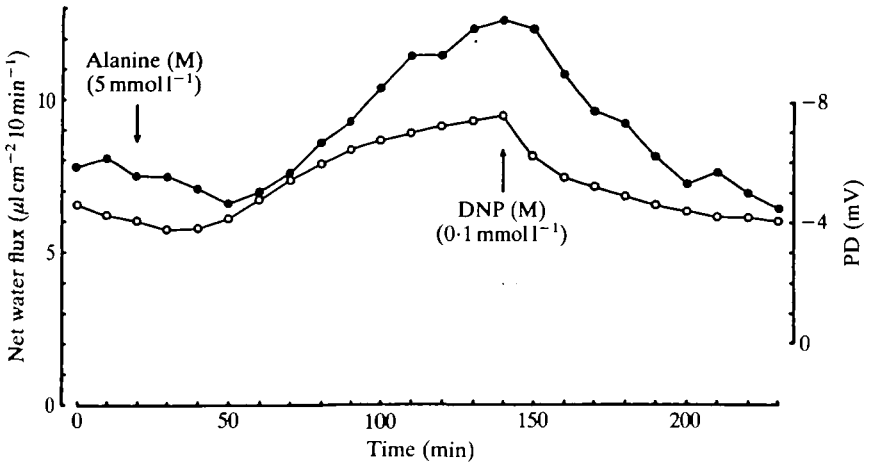

Fig. 2. Effects of 2,4-dinitrophenol (DNP). After the PD (○) and the net water flux (●) had been enhanced by the addition of L-alanine to the mucosal (M) fluid (first arrow), 0·1 mmol l^{-1} DNP was added to the mucosal fluid (second arrow).

the same effects even under steady-state conditions. In a steady state, more than three agents could not be examined in the same preparation, since the experimental period was limited to 250 min. When 0·1 mmol l^{-1} DNP (an uncoupler of respiratory chain phosphorylation) was added to the mucosal fluid, the L-alanine-induced PD and the net water flux were almost completely abolished (Fig. 2).

The PD and net water flux were also stimulated by L-glutamine, L-glutamate or D-alanine. Of these amino acids, L-glutamine had the most immediate and greatest effect, despite a lower concentration (Fig. 3).

L-Glutamine metabolism

When 10 mmol l^{-1} L-glutamine was applied to the serosa, the PD was slightly increased and there was a parallel slight increase in water flux (Fig. 4). In contrast, when added to the mucosal side, L-glutamine produced a marked enhancement of PD and net water flux (Fig. 4). Therefore, it can be concluded that L-glutamine is more effective from the mucosal side than from the serosal side.

With increasing concentration of mucosal L-glutamine, both PD and net water flux showed a progressive increase, with Michaelis–Menten characteristics (Fig. 5). The threshold concentration was less than 0·1 mmol l^{-1}. Similar concentration–response curves were also obtained for L-glutamate (Fig. 5), although the response was less sensitive. The threshold concentration (1 mmol l^{-1}) was 10-fold higher than that of L-glutamine.

To determine whether L-glutamine transport itself drags water or glutamine metabolism participates in ion and water transport, as in the case of L-alanine, the following experiments were performed. After blocking the $Na^+/K^+/Cl^-$ transport with furosemide, addition of L-glutamine (10 mmol l^{-1}) to the mucosal

compartment did not increase the net water flux, and there was a negligible hyperpolarization of the PD (Fig. 6). When the mucosal fluid was replaced with fresh standard Ringer's solution (glutamine-free), however, the PD and the net water flux increased immediately, and the peak levels were much higher than their

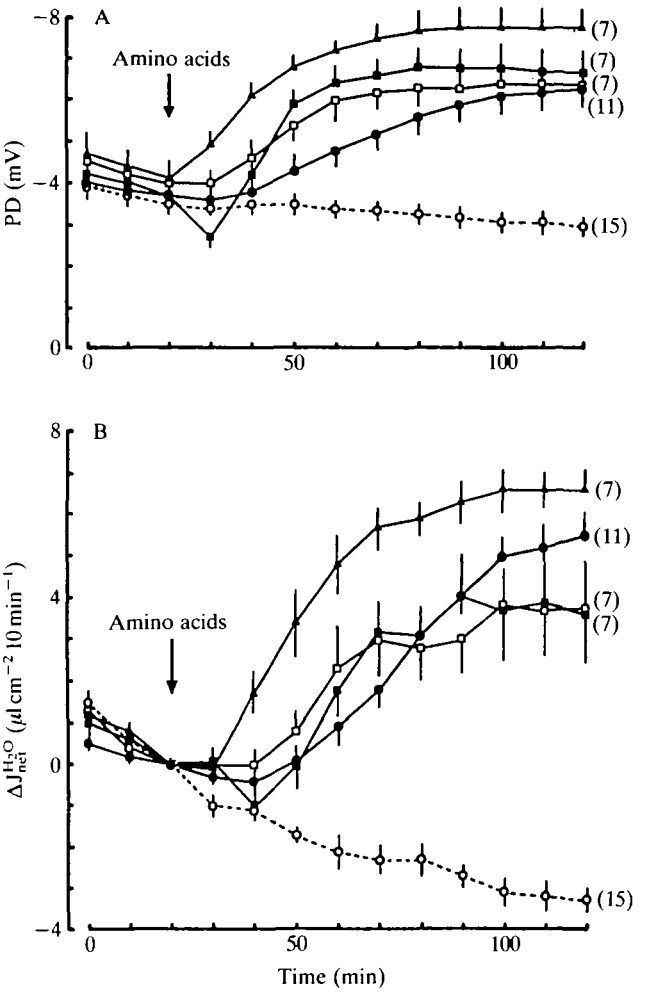

Fig. 3. Time course of the effects of various amino acids. After 30 min in the standard Ringer's solution (arrows), amino acids were added to the mucosal medium. Each point and vertical bar indicate the mean ± S.E., respectively. The number of preparations is shown in parentheses. (A) Effects of L-glutamine (▲, 2 mmol l^{-1}), L-glutamate (■, 10 mmol l^{-1}), D-alanine (□, 10 mmol l^{-1}) and L-alanine (●, 5 mmol l^{-1}) on the PD. As a control, effects of other amino acids (L-glycine, L-valine, L-proline, L-serine, L-methionine, L-norvaline, L-lysine and L-ornithine) which did not enhance the PD and the net water flux are represented as open circles (10 mmol l^{-1} each). (B) Effects of various amino acids on the net water flux ($\Delta J_{net}^{H_2O}$). Each value is expressed as differences from the values at 20 min, immediately before addition of amino acids. All symbols are identical with those in Fig. 3A.

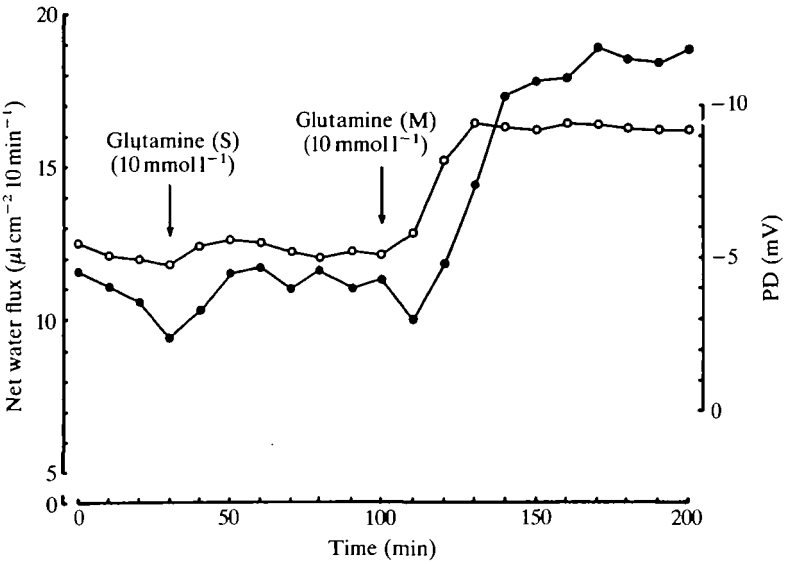

Fig. 4. Comparison of the effects of L-glutamine on the PD (○) and the net water flux (●) when applied to either serosa (S) or mucosa (M). After bathing the intestine with standard Ringer's solutions, 10 mmol l^{-1} L-glutamine was added to the serosal fluid (first arrow). At the second arrow, 10 mmol l^{-1} L-glutamine was added to the mucosal fluid.

initial levels. Since the higher PD and net water flux were obtained in spite of the absence of L-glutamine in the bathing medium, glutamine must act from inside the cell. In other words, L-glutamine has been taken up into the enterocyte during treatment with furosemide and its effect appears after washing out the drug. Therefore, it is unlikely that L-glutamine transport itself drags water, since L-glutamine does not increase the net water flux in the presence of furosemide.

Metabolism of L-glutamine was therefore examined. After stimulating the PD and the net water flux with 2 mmol l^{-1} L-glutamine, the addition of amino-oxyacetate (1 mmol l^{-1}) to the serosal fluid did not inhibit the PD increase and the net water flux (Fig. 7). Similarly, no inhibitory effect of amino-oxyacetate (1–10 mmol l^{-1}) was observed after treatment with L-glutamate or D-alanine (data not shown). As shown in Fig. 7, however, the stimulatory effects of L-glutamine, L-glutamate or D-alanine were completely blocked by DNP, suggesting that these amino acids are also metabolized to produce ATP.

In the next experiment, the effects of L-glutamine were compared with those of L-alanine. After stimulating the PD and the net water flux with 5 mmol l^{-1} L-alanine, the addition of L-glutamine (2 mmol l^{-1}) to the mucosal fluid did not enhance the net water flux, and the PD was hyperpolarized only slightly (Fig. 8A). In the presence of both L-glutamine and L-alanine, amino-oxyacetate (0·5 mmol l^{-1}) did not inhibit the PD and the net water flux, indicating that the stimulated PD and net water flux are not maintained by L-alanine metabolism, but

by L-glutamine metabolism. This means that L-alanine metabolism is replaced by L-glutamine metabolism under this condition. Furthermore, after stimulating the PD and the net water flux with L-glutamine ($2\,mmol\,l^{-1}$), serosal application of pyruvate (an L-alanine metabolite) did not add to the increase induced by L-glutamine (Fig. 8B). Since the effects of L-glutamine and L-alanine are not additive, but mutually complementary, both amino acids may be metabolized through a common pathway, such as the citric acid cycle.

Oxygen consumption

To confirm that both L-glutamine and L-alanine were metabolized, effects of these amino acids on the O_2 consumption (Q_{O_2}) were examined. Fig. 9 demonstrates a typical recording. O_2 consumption was not affected when D-glucose ($10\,mmol\,l^{-1}$) was added to the incubation medium, but was enhanced immedi-

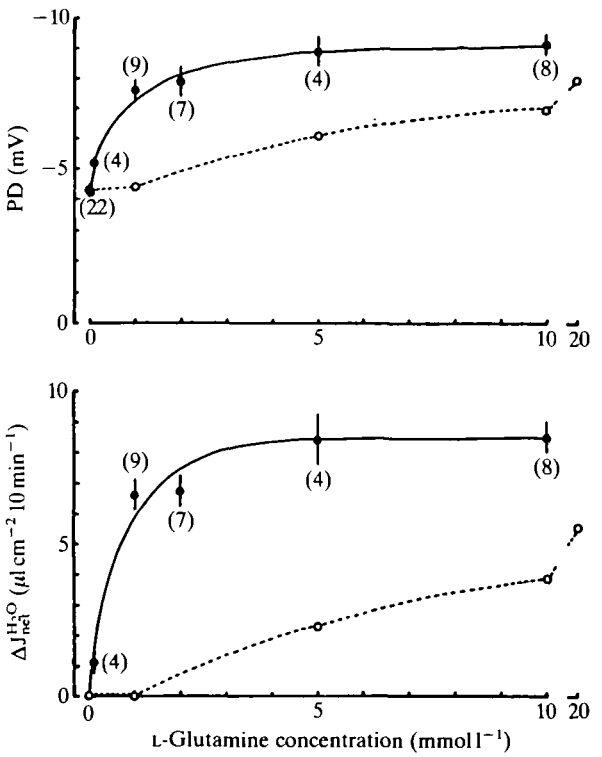

Fig. 5. Concentration–response curve of the effects of L-glutamine on the PD (upper figure) and the net water flux (lower figure). The net water flux is expressed as the difference between the values before and after addition of L-glutamine; $\Delta J_{net}^{H_2O}$. L-Glutamine was added to the mucosal fluid, while the serosal side was kept perfused with the standard Ringer's solution. For comparison, the concentration–response curve of the effects of mucosal L-glutamate on the same preparation is also shown (○). Each point and vertical bar indicate the mean ± S.E., respectively. The number in parentheses denotes sample size.

ately after addition of L-glutamine (2 mmol l^{-1}), and blocked almost completely by NaCN (1 mmol l^{-1}).

All amino acids examined (L-alanine, D-alanine, L-glutamine and L-glutamate) stimulated O_2 consumption (Fig. 10). Although the effects of L-alanine appear to be smaller than those of the other amino acids, this may be due to a long latent period after addition of this amino acid (see Fig. 11).

When one half of the intestine was incubated in the standard Ringer's solution as a control and the other half was incubated with 5 mmol l^{-1} L-alanine, the O_2 consumption in L-alanine was much greater than in the control (Fig. 10). In the presence of ouabain, O_2 consumption was unaffected by L-alanine, having a value of 0·8 μl mg^{-1} h^{-1} under both conditions. In other words, L-alanine enhances only ouabain-sensitive O_2 consumption. The same ouabain-insensitive O_2 consumption was also obtained after treatment with L-glutamine. The depressed O_2 consumption after treatment with ouabain was enhanced again when 0·1 mmol l^{-1} DNP was added (data not shown). This confirms that DNP acts as an uncoupler.

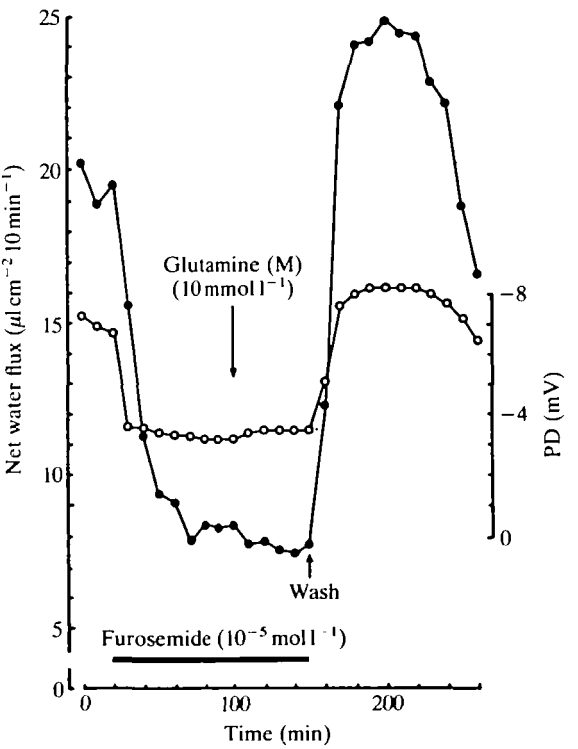

Fig. 6. Effects of L-glutamine on the PD (○) and the net water flux (●) in the presence of furosemide. After bathing the intestine with standard Ringer's solutions, 0·01 mmol l^{-1} furosemide was added into the mucosal (M) fluid (at 20 min). In the presence of furosemide (open bar), 10 mmol l^{-1} L-glutamine was applied to the mucosal fluid (first arrow). At the second arrow, both glutamine and furosemide were removed from the bathing medium by washing the intestine twice with the standard Ringer's solution.

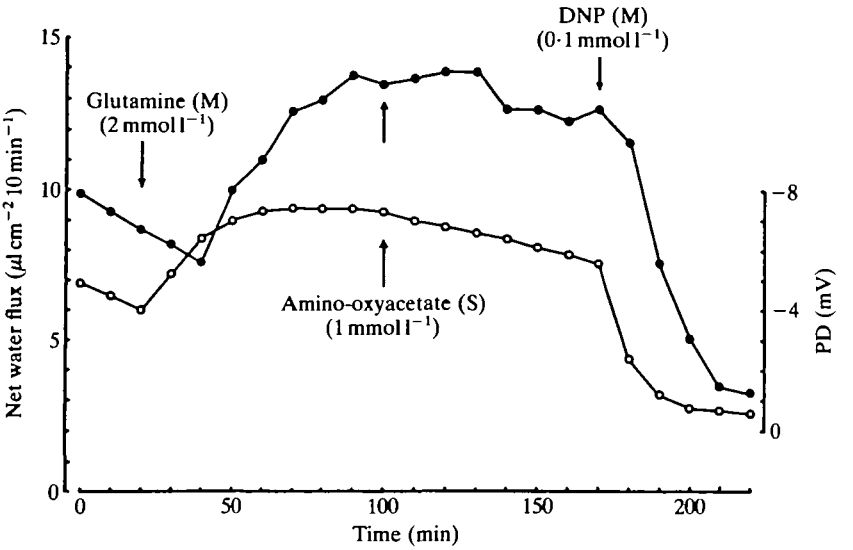

Fig. 7. Effects of amino-oxyacetate and 2,4-dinitrophenol (DNP). After enhancing the PD (○) and the net water flux (●) by addition of 2 mmol l^{-1} L-glutamine to the mucosal (M) fluid (first arrow), 1 mmol l^{-1} amino-oxyacetate was added to the serosal (S) fluid (second arrows). At the third arrow, 0·1 mmol l^{-1} DNP was applied to the mucosal fluid.

All four amino acids examined produced first an increase in O_2 consumption, then in serosa-negativity of the PD, and finally in water absorption (Fig. 11). L-Glutamine stimulated O_2 consumption most quickly, the latent period being 1·4 ± 0·4 min, and L-alanine was the slowest (14·7 ± 2·3 min). Surprisingly, D-alanine also stimulated O_2 consumption and its stimulatory action was much faster (4·3 ± 0·8 min) than that of L-alanine.

Discussion

The present study indicates that amino acid metabolism stimulates water absorption across the seawater eel intestine. L-Alanine appears to be metabolized through the citric acid cycle to produce ATP because: (1) the increments in the PD and the net water flux observed after addition of L-alanine are inhibited by amino-oxyacetate, an inhibitor of aminotransferase which converts L-alanine into pyruvate; (2) pyruvate restores these parameters even after blocking the synthesis of pyruvate from L-alanine with amino-oxyacetate; (3) the stimulatory effects of pyruvate are inhibited by oxythiamine, an inhibitor of dehydrogenase which converts pyruvate into acetyl-CoA (which is known to enter the citric acid cycle to produce ATP); (4) the stimulatory effects of L-alanine are also inhibited by DNP, an uncoupler of respiratory chain phosphorylation; (5) L-alanine enhances ouabain-sensitive O_2 consumption in the intestinal tissue; and (6) the enhancement in O_2 consumption precedes the increase in the serosa-negative PD and the water transport.

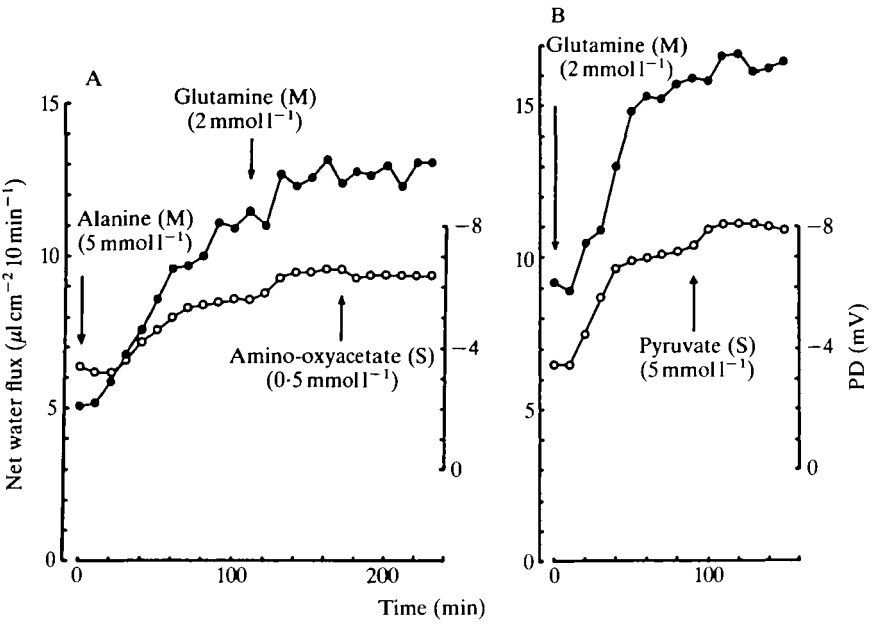

Fig. 8. Comparison of the effects of L-alanine and L-glutamine. (A) Effects of L-glutamine after treatment with L-alanine. After enhancing the PD (○) and the net water flux (●) by addition of 5 mmol l^{-1} L-alanine to the mucosal (M) fluid (first arrow), 2 mmol l^{-1} L-glutamine was added to the mucosal fluid (second arrow). At the third arrow, 0·5 mmol l^{-1} amino-oxyacetate was added to the serosal fluid. (B) Effects of pyruvate (an L-alanine metabolite) after treatment with L-glutamine. After stimulating the PD (○) and the net water flux (●) with 2 mmol l^{-1} L-glutamine (first arrow), 5 mmol l^{-1} pyruvate was added to the serosal (S) fluid (second arrow).

Combining these results with a model for ion and water transport across the seawater eel intestine (Ando & Utida, 1986) leads us to suggest that ATP produced from L-alanine stimulates the ouabain-sensitive Na^+/K^+-ATPase in the basolateral membrane, and thus increases intracellular K^+ concentration. An accumulation of K^+ would enhance the serosa-negativity of the PD, since this PD is thought to be mainly due to the K^+ gradient across the mucosal membrane, as in the flounder intestine (Halm *et al.* 1985). In addition, a protein phosphorylation process might be involved to increase the K^+ permeability across the brush-border membrane. A tendency for K^+ to diffuse across the mucosal membrane might raise the K^+ concentration in the microclimate of this membrane, which could then stimulate the $Na^+/K^+/Cl^-$ transport system, and thus enhance water absorption.

L-Glutamine also seems to be metabolized like L-alanine because: (1) L-glutamine enhances the PD and the net water flux by acting from inside the enterocyte; (2) the stimulatory effects of L-glutamine are inhibited by DNP; (3) effects of L-glutamine and L-alanine are not additive but mutually complementary;

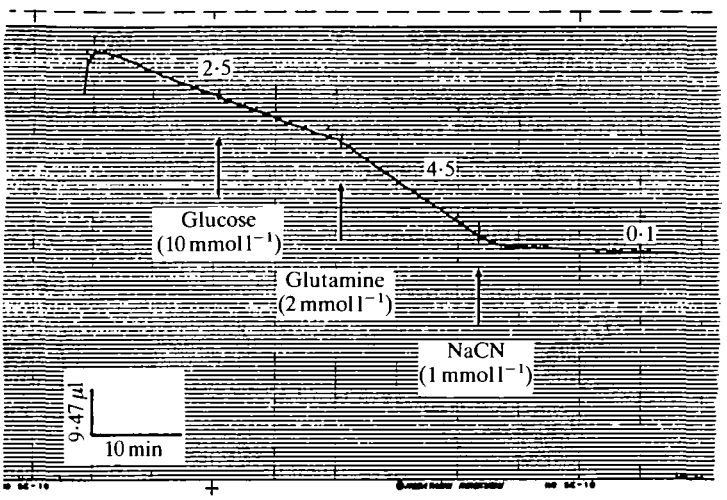

Fig. 9. A typical recording of oxygen consumption. Oxygen consumption rate was obtained from the slope of the straight line and expressed as Q_{O_2} (μl mg^{-1} h^{-1}). Each value on the line denotes a Q_{O_2} value. At each arrow, 10 mmol l^{-1} D-glucose, 2 mmol l^{-1} L-glutamine or 1 mmol l^{-1} NaCN was added to the incubation medium.

(4) L-glutamine also enhances ouabain-sensitive O_2 consumption; and (5) the increment in O_2 consumption precedes that in other parameters.

Although the response induced by L-alanine was of the all-or-nothing type (Ando *et al.* 1986), the dose–response curve of L-glutamine was of the Michaelis–Menten type. This suggests that L-alanine metabolism has a much slower rate than the uptake across the brush-border membrane, and thus limits the final rate of ATP production. The long latent period to enhance O_2 consumption after addition of L-alanine also supports this suggestion. In contrast, metabolism of L-glutamine may proceed at a faster rate than its uptake, so that the rate of end-product formation may be limited by this uptake. In fact, O_2 consumption increased immediately after addition of L-glutamine, indicating that the intracellular content of the metabolic products, such as ATP, is controlled by extracellular L-glutamine. Therefore, it is possible that L-glutamine may act as a regulator for ion and water transport across the seawater eel intestine by controlling intracellular ATP content.

Since the dose–response curve for L-glutamate was of a similar type to that for L-glutamine, the rate of L-glutamate uptake may also limit the overall rate of L-glutamate metabolism. This amino acid also enhances O_2 consumption immediately. These similarities between the effects of L-glutamine and L-glutamate suggest that both amino acids are metabolized through a common pathway. However, a much higher concentration of L-glutamate was required to attain the same response as for L-glutamine. The discrepancy in efficacy might be due to a difference in their uptake rate: L-glutamine might be taken up much faster than

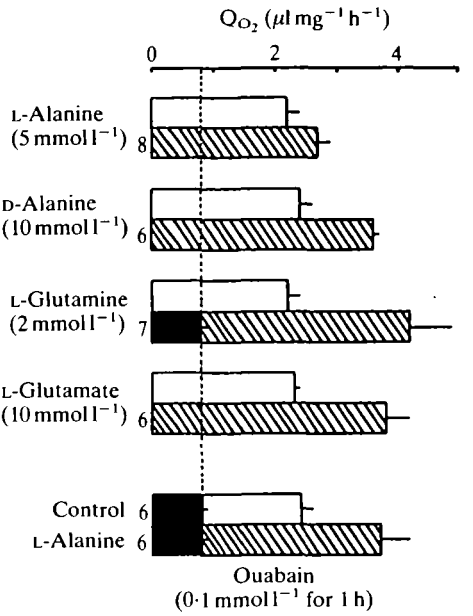

Fig. 10. Effects of L-alanine, D-alanine, L-glutamine and L-glutamate (sodium salt) on O_2 consumption (Q_{O_2}). After measurements of Q_{O_2} in the standard Ringer's solution (open columns), the amino acids were added to the incubation medium. The hatched columns show the Q_{O_2} in the presence of amino acids. The filled column indicates the Q_{O_2} obtained after treatment with 0·1 mmol l^{-1} ouabain for 1 h. Whereas the upper four experiments were carried out on the same preparation, the lowest experiment was performed in preparations where the intestine was divided into two parts: one half as control and the other half incubated with 5 mmol l^{-1} L-alanine from the beginning. All values are expressed as mean ± S.E. The concentration of each amino acid and the number of preparations are indicated in parenthesis and beside the column, respectively.

L-glutamate, as found in mammalian intestine (Peraino & Harper, 1962; Windmueller & Spaeth, 1975).

It is well established that L-glutamine is metabolized into L-glutamate and then into α-ketoglutarate. Although the conversion of L-glutamate into α-ketoglutarate is usually thought to be catalysed by aminotransferase, such a metabolic pathway does not seem to work in this tissue, since amino-oxyacetate (an inhibitor of the aminotransferase) does not inhibit the effects of L-glutamine or L-glutamate. In the seawater eel intestine, L-glutamate may be converted into α-ketoglutarate by glutamate dehydrogenase, not aminotransferase, as indicated by Kovacevic & McGivan (1983).

Surprisingly, D-alanine also appears to be metabolized, since D-alanine enhances O_2 consumption, the serosa-negative PD and the net water flux. Although D-alanine is commonly thought to be converted into L-alanine by racemase, the following results argue against this possibility. First, D-alanine enhanced O_2 consumption more quickly than L-alanine did. Second, amino-

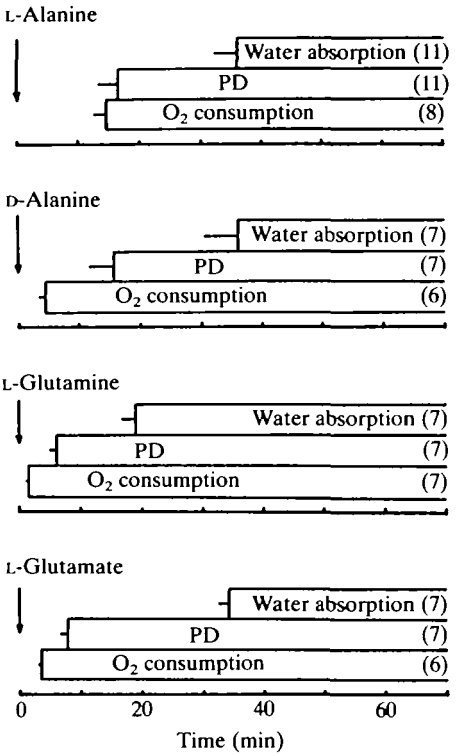

Fig. 11. Sequence of events after application of various amino acids. L-alanine ($5\,mmol\,l^{-1}$), D-alanine ($10\,mmol\,l^{-1}$), L-glutamine ($2\,mmol\,l^{-1}$) and L-glutamate ($10\,mmol\,l^{-1}$) were applied to the seawater eel intestine at time zero. Each column indicates the latent period from the application of amino acid until the onset of an increase in O_2 consumption, serosa-negativity of the PD and the net water flux. Times are obtained from the experiments shown in Figs 3 and 10, and expressed as mean ± S.E.

oxyacetate which inhibited the effects of L-alanine did not inhibit the effects of D-alanine. The metabolic pathway of D-alanine may be distinct from that of L-alanine in the seawater eel intestine. In brackish-water bivalves, it has been observed that D-alanine, as well as L-alanine, contribute to cell volume regulation after hypertonic stress (Matsushima *et al.* 1984). Therefore, D-alanine might be an amino acid which plays a specific role in seawater adaptation of animals.

Although the reason why the seawater eel intestine cannot use D-glucose as a metabolic fuel is not yet clear, a similar result is obtained in rabbit ileum *in vitro*, where water transport is enhanced by $20\,mmol\,l^{-1}$ L-glutamine but not by $20\,mmol\,l^{-1}$ D-glucose (Love *et al.* 1965). Starvation might be involved in the alteration from D-glucose metabolism to L-glutamine metabolism, since L-glutamine uptake and its metabolism by the gut are enhanced with fasting in both conscious dogs (Cerrosino *et al.* 1986) and *in vivo* rats (Windmueller & Spaeth, 1978). However, even in the intestine of fed rats, amino acids seem to be

metabolized in preference to D-glucose (Windmueller & Spaeth, 1980). Therefore, it is likely that in some animals the intestine prefers L-glutamine to D-glucose as a metabolic fuel.

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