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EPITHELIAL GLUCOSE TRANSPORT BY LOBSTER ANTENNAL GLAND

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Summary

Transport of D-[^{3}H]glucose into lobster (*Homarus americanus*) brush-border membrane vesicles (BBMVs) prepared by Mg^{2+} precipitation from antennal gland labyrinth–coelomosac tissue was examined. Influx of D-glucose occurred primarily by a phlorizin-sensitive, Na^+-dependent carrier similar to that found in vertebrate renal epithelium. An inwardly directed Na^+ gradient drove concentrative D-glucose uptake, whereas similar gradients of Li^+ and K^+ did not. Stimulation by the Na^+ gradient was further enhanced by the imposition of an inside-negative potential difference and also by increases in the pH of the vesicle and incubation media. An analysis of *cis* inhibition of D-glucose uptake by a number of sugars and sugar derivatives indicated that the transporter requires (a) that the sugar substrate be a D-pyranose in the C1 chair conformation and (b) that the hydroxyl groups at C2 and C3 of the ring be unmodified and equatorial. Apparent kinetic parameters for glucose uptake were determined under zero-*trans*, short-circuited conditions. Maximal influx of D-glucose into vesicles was estimated to be 96 pmol mg^{-1} protein s^{-1}. Half-maximal influx was determined to occur at 0.20 mmol l^{-1} D-glucose. The relationship between external Na^+ concentration and glucose influx was sigmoidal, and the stoichiometry of Na^+-dependent glucose transport found to be 3 Na^+:1 glucose using the static head method.

Key words: glucose, transport, epithelium, lobster, *Homarus americanus*, Na^+ gradient, antennal gland, cotransport, BBMV, vesicle, kidney.

Introduction

The renal system of the adult lobster (*Homarus americanus*) consists of the paired antennal glands, which rest in the basal antennal segment of the cephalothorax. Like the vertebrate kidneys, these organs function in maintaining volume and in regulating concentrations of divalent ions, nutrients and other solutes (Mantel and Farmer, 1983). The decapod antennal gland is very simple in structure compared with the nephronal vertebrate kidney. Rather than being functionally subdivided into several microscopic filtration/reabsorption/secretion units in parallel, it is composed of four macroscopic substructures arranged in series. In a way, it is like a single giant nephron: a coelomosac, derived from the vestigial coelom of the animal, and intimately associated with the gland labyrinth, filters hemolymph delivered by the antennal artery. The cells comprising the coelomosac are elongated epithelial cells with podocytic processes. Next, the filtrate passes into a labyrinth, a single tubule with innumerable anastomoses, made up of cells having well-defined apical brush-border membranes. The basolateral membranes of these cells are extensively infolded and are associated with numerous mitochondria (Riegel and Cook, 1975). These characteristics suggest that the labyrinth may have an active role in absorptive and/or secretory processes. The fluid next enters a distal tubule, which is thought to function in water absorption and secretion (Riegel, 1972). Lastly, a bladder receives and stores the urine and in some groups, including crabs and crayfish, is a site of urine modification (Gross, 1967; Holliday, 1978; Riegel, 1970). In these animals, the bladder tissue is clearly modified for transcellular movement of solutes (Miller, 1989).

The purpose of the present study is to examine the mechanisms behind the reclamation of circulating glucose in the lobster. The work of Burger (1957) showed that the lobster antennal gland was able to mediate the reabsorption of glucose injected into the hemolymph at concentrations up to five times normal without spillover into the urine. Until recently, the cellular transport mechanisms that are ultimately responsible for nutrient reclamation in lobsters and other crustaceans have not been studied directly, although many whole-animal studies and some transepithelial experiments have been performed. Previous investigations have demonstrated a common, saturable glucose reabsorption pathway sensitive to phlorizin, a competitive inhibitor of the Na^+-dependent D-glucose transporter found in many epithelia (Binns, 1969; Burger, 1957; Holliday, 1978; Riegel and Cook, 1975). This report focuses on the ionic requirements, apparent substrate specificity and transport stoichiometry of antennal gland Na^+-dependent D-glucose transport at the level of the epithelial brush-border membrane, across which all reabsorbed nutrients

must pass before returning to the circulation. Labyrinth tissue was chosen for study because it is thought to be a major site of urine modification in the lobster (Binns, 1969), although it has been the least-studied component of the antennal gland. Membranes isolated from this tissue have already been shown in the lobster to have a high capacity for the transport of at least one amino acid, L-proline (Behnke *et al.* 1990).

Materials and methods

Intermolt lobsters (*Homarus americanus*), weighing 0.45–0.68 kg, were purchased from a Honolulu commercial source. The animals were maintained unfed for up to 6 days in a refrigerated filtered seawater aquarium kept between 5 and 8 °C. The lobsters were killed by severing the ventral nerve cord, and the antennal glands were then dissected from the thoracic cavity and the bladders removed, leaving only labyrinth and some coelomosac tissue. Brush-border membrane vesicles (BBMVs) were prepared by a method previously described for lobster antennal gland epithelium (Behnke *et al.* 1990). Briefly, tissue was homogenized, then unwanted membrane fractions were removed by $MgCl_2$ precipitation and low-speed centrifugation. Brush-border membranes remaining in the supernatant were pelleted by high-speed centrifugation, resuspended in defined internal medium, repelleted and finally resuspended in a small volume of internal medium.

A small amount of coelomosac epithelium is inseparable from the labyrinth structure even after dissection. However, the coelomosac cells of crustaceans do not appear to be modified for the purpose of reclamation of small solutes from the urine, although they have been shown to be actively endocytotic and exocytotic (Riegel, 1972). The apical membranes of the coelomosac cells are not amplified into brush borders, so they would not be strongly represented in a membrane preparation in which this fraction were enriched. In addition, the amount of coelomosac epithelium included in the preparation is a very small fraction of the total tissue used. Therefore, we consider our BBMV preparation to be made up primarily of labyrinth tissue.

Transport experiments were conducted with external solutions held at 15 °C. Unless otherwise stated, all solutions were adjusted to pH 7.4 with Tris or Mes. The osmolarities of internal and external solutions were measured and balanced with mannitol if necessary. Sampling was performed as follows. For time courses longer than 1 min, 20 µl of ice-cold vesicle suspension was added to 180 µl of test medium containing D-[^{3}H]glucose. Samples (20 µl) were transferred into 2 ml of ice-cold 'stop' solution. Details of the solutions are given in the figure legends. The reaction mixture was then rapidly filtered over a 0.65 µm Millipore filter. An additional 5 ml of cold stop solution was poured over the filter to wash the retained vesicles. Short-term time courses and brief incubations were performed using a rapid exposure apparatus (Inovativ Labor, AG). Each sample was prepared individually, with 95 µl of external test medium placed at the bottom of a polystyrene tube and 5 µl of vesicle suspension carefully placed on the side of the tube out of contact with the bead of test medium. The tube was then agitated (and the two beads in the tube mixed) for a short time at the start of the incubation period. After a defined incubation period, the reaction was arrested by the injection of 2 ml of ice-cold stop solution, and the mixture was poured over the filter followed by a wash with an additional 5 ml of stop solution. Using this methodology, initial rates of D-[^{3}H]glucose influx were determined by measuring the uptake of the sugar over time intervals ranging from 3 to 15 s (data not shown). Uptake over this time period was a linear function of time, and a 5 s uptake was therefore selected for all influx determinations. To determine the amount of D-[^{3}H]glucose accumulated by binding to the vesicles and to the filter disk, a sample of vesicle suspension was plunged into ice-cold stop solution, a sample of the labelled test medium was then added to the mixture, and the entire solution was immediately filtered.

Each filter was placed in a vial and dissolved with Beckman Ready Solv HP scintillation cocktail. The vials were then counted for tritium in a Beckman LS-8100 scintillation counter. The experiments were designed so that at least four replications of each point taken could be made. Results are presented as means ± S.E.M. Each experiment was repeated 3–5 times, and representative data are shown.

Chemicals were obtained from Sigma Chemical, except tetramethyl ammonium chloride (TMA-Cl), which was purchased from Chemical Dynamics. Radioactive materials were purchased from ICN Radiochemicals and New England Nuclear.

Results

Fig. 1 shows the time course of uptake of 0.05 mmol l^{-1} D-[^{3}H]glucose from NaCl-, LiCl-, KCl- or mannitol-containing test medium into mannitol-loaded antennal gland BBMVs. The initial condition of an inwardly directed glucose gradient results in the influx of glucose through a combination of specific and non-specific processes until equilibrium is reached, the point at which there is no further movement of labelled sugar into or out of the vesicles. The effect of an inwardly directed transmembrane NaCl gradient on glucose uptake into BBMVs was compared with that of similar transmembrane KCl and LiCl gradients to determine the ability of the other cations to stimulate transport. Data are presented as total uptake, non-specific binding was not subtracted. Fig. 1 shows that these membranes have a high capacity for Na^+-dependent D-glucose transport. When a transmembrane NaCl gradient is imposed, there is a transient accumulation of D-[^{3}H]glucose at a level more than twice that of equilibrium after a 1 min incubation period. No such concentrative uptake was seen when NaCl was replaced by KCl or LiCl; glucose accumulation increased hyperbolically towards equilibrium. As in most renal tissues studied, apical Na^+-dependent D-glucose transport appears to be cation-specific in the lobster antennal gland tissue.

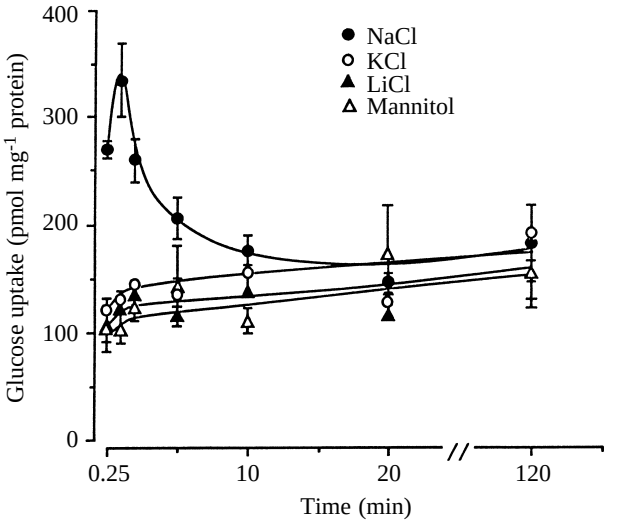

Fig. 1. Long-term time course of D-[^{3}H]glucose accumulation into brush-border membrane vesicles (BBMVs) in the presence of NaCl, KCl and LiCl. Vesicles were preloaded with 500 mmol l^{-1} D-mannitol and 20 mm Hepes. Samples (20 µl) were incubated in 180 µl of external solution containing 20 mmol l^{-1} Hepes, 0.05 mmol l^{-1} D-[^{3}H]glucose and 250 mmol l^{-1} KCl, NaCl or LiCl. Values are means ± S.E.M., *N*=4.

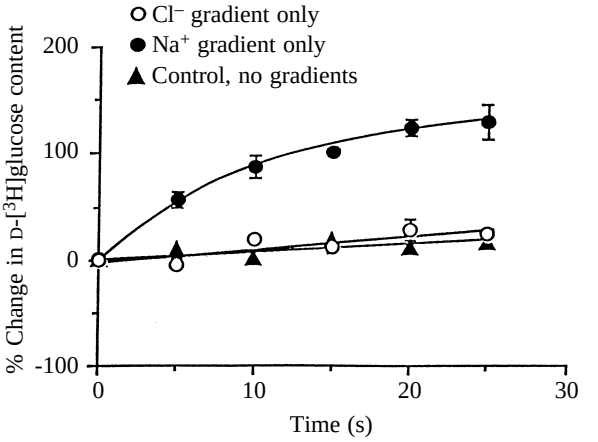

Fig. 2. The effects of Na^+ and Cl^- gradients on D-[^{3}H]glucose uptake. Brush-border membrane vesicles (BBMVs) were loaded with 100 mmol l^{-1} potassium gluconate, 20 mmol l^{-1} Hepes, 0.05 mmol l^{-1} D-[^{3}H]glucose, 50 µmol l^{-1} valinomycin and 250 mmol l^{-1} of one of the following: (a) NaCl (control); (b) TMA-Cl (inward Na^+ gradient); (c) sodium gluconate (inward Cl^- gradient). Samples (5 µl) of the vesicle suspension were incubated with 95 µl of a solution of 100 mmol l^{-1} potassium gluconate, 20 mmol l^{-1} Hepes, 0.05 mmol l^{-1} D-[^{3}H]glucose and 250 mmol l^{-1} NaCl. Transport was stopped with an ice-cold solution identical to the external medium but without D-glucose. Values are means ± S.E.M., *N*=3.

A transmembrane Cl^- gradient has been shown synergistically to drive uptake of nutrients such as β-alanine (Turner, 1986) and L-glutamate (Balon and Ahearn, 1991) into apical membranes from dog renal and lobster digestive epithelia, respectively. To analyze the independent effects of transmembrane Na^+ and Cl^- gradients in effecting the overshoot in D-glucose transport seen in Fig. 1, the net gain or loss of labelled D-glucose loaded into vesicles was observed in the presence of an inwardly directed transmembrane gradient of either Na^+ or Cl^-. All other solutes, including D-[^{3}H]glucose, were held at equal concentration inside and outside the vesicles. Vesicles were treated with the K^+-specific ionophore valinomycin, and potassium gluconate was included in equal amounts in the media on both sides of the vesicles to prevent the development of a vesicle membrane potential by electrogenic transport of sugar. A Na^+ gradient in the presence of equilibrated Cl^-, but not a Cl^- gradient in the presence of equilibrated Na^+, drove net influx of glucose into vesicles (Fig. 2). Because there was no inwardly directed glucose gradient in either condition, this confirms that the stimulatory effect of Na^+ in the external medium seen in Fig. 1 is due to the energetic coupling of transport to a Na^+ gradient rather than to allosteric activation of transport by Na^+ binding. In addition, it demonstrates that stimulation is due entirely to the transmembrane Na^+ gradient and that Cl^- has no role as an energizer in this system.

Na^+-dependent glucose transport has been shown in all other systems studied to be an electrogenic process. Therefore, one would expect an inward-negative transmembrane potential to augment the concentrative accumulation of glucose into vesicles. To test this, mannitol-, potassium-gluconate-, valinomycin-treated vesicles were incubated in external medium lacking K^+ such that an inside-negative K^+ diffusion potential was established. Fig. 3 shows that the potential

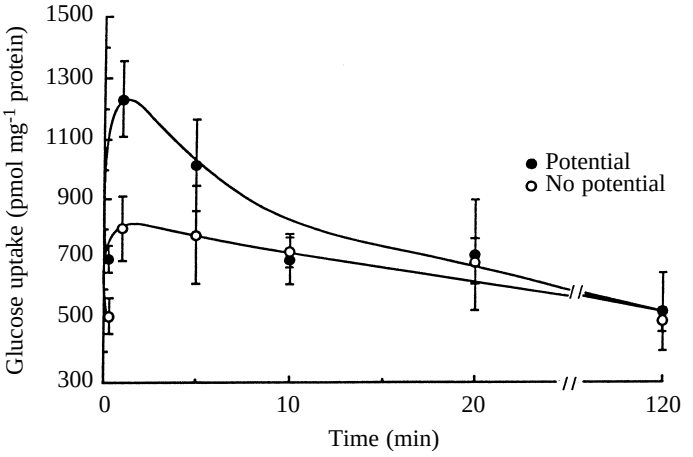

Fig. 3. The effect of an inward-negative potential difference on D-[^{3}H]glucose uptake. Brush-border membrane vesicles (BBMVs) were loaded with 500 mmol l^{-1} mannitol, 50 mmol l^{-1} potassium gluconate, 20 mmol l^{-1} Hepes and 50 µmol l^{-1} valinomycin. Samples (20 µl) were then added to 180 µl of external medium containing 250 mmol l^{-1} NaCl, 20 mmol l^{-1} Hepes and either 50 mmol l^{-1} potassium gluconate or 50 mmol l^{-1} TMA-gluconate. Samples (20 µl) of the reaction mixture were taken at each time point. Transport was stopped in ice-cold solution containing 500 mmol l^{-1} mannitol, 50 mmol l^{-1} potassium gluconate, 20 mmol l^{-1} Hepes and 1 mmol l^{-1} phlorizin. Values are means ± S.E.M., *N*=4.

difference had a stimulatory effect on Na^+-dependent D-glucose uptake. Glucose accumulation into vesicles is significantly greater (P<0.05) after 1 min in the condition in which a transmembrane potential was present.

In many crustaceans, the reclamation of D-glucose from the urine can be blocked by the administration of phlorizin. To test whether the transporters present in the labyrinth BBMVs are phlorizin-sensitive, and are thus involved in the reclamation of glucose *in vivo*, the uptake of subsaturating levels of labelled glucose into vesicles was observed as a function of external phlorizin concentration. Short-circuited vesicles were prepared in potassium gluconate/mannitol solution to which valinomycin had been added (final $[K^+]$ equal on both vesicle surfaces). Transport was measured at two concentrations of external glucose to establish the type of inhibition exerted by phlorizin. Fig. 4 is a Dixon plot of the transport data. The fact that the lines of the Dixon plot intersect above the abscissa is consistent with phlorizin acting as a competitive inhibitor of D-glucose transport. The point at which the lines intersect reflect a K_i of 3.4 μmol l^{-1} phlorizin, a value comparable with that of Na^+-dependent glucose transport in preparations of rabbit renal cortex and outer medulla, which have demonstrated K_i values for phlorizin of 7 μmol l^{-1} (Aronson and Sacktor, 1975) and 1 μmol l^{-1} (Turner and Moran, 1982*a*), respectively. That D-glucose transport in the lobster membranes is very sensitive to the inhibitor suggests that the glucose transporters present in BBMVs are those that transport D-glucose from the tubule lumen *in vivo*.

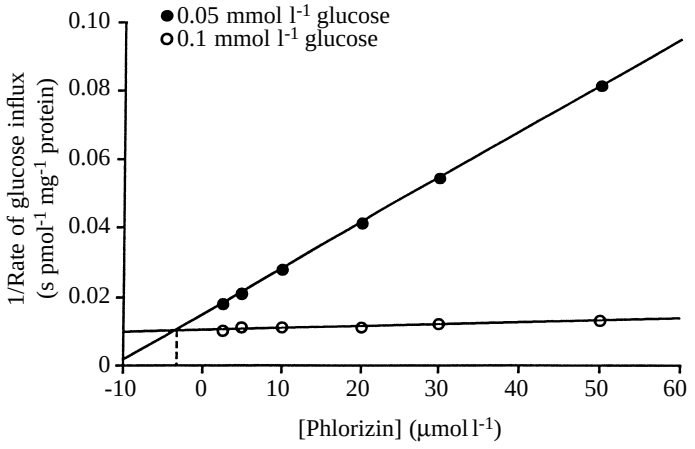

Fig. 4. Inhibition of Na^+-dependent D-[^{3}H]glucose transport by phlorizin. Brush-border membrane vesicles (BBMVs) were preloaded with 500 mmol l^{-1} D-mannitol, 20 mmol l^{-1} Hepes, 50 mmol l^{-1} potassium gluconate and 50 μmol l^{-1} valinomycin. Samples (5 μl) were then incubated for 5 s in 95 μl of external medium containing 0.05 mmol l^{-1} or 0.1 mmol l^{-1} D-[^{3}H]glucose and 250 mmol l^{-1} NaCl, 20 mmol l^{-1} Hepes, 50 mmol l^{-1} potassium gluconate and phlorizin at concentrations ranging from 0 (control) to 50 μmol l^{-1}. Transport was stopped with ice-cold solution containing 500 mmol l^{-1} mannitol and 20 mmol l^{-1} Hepes. Values shown are means ± S.E.M., but all the error bars are smaller than the symbols (N=3).

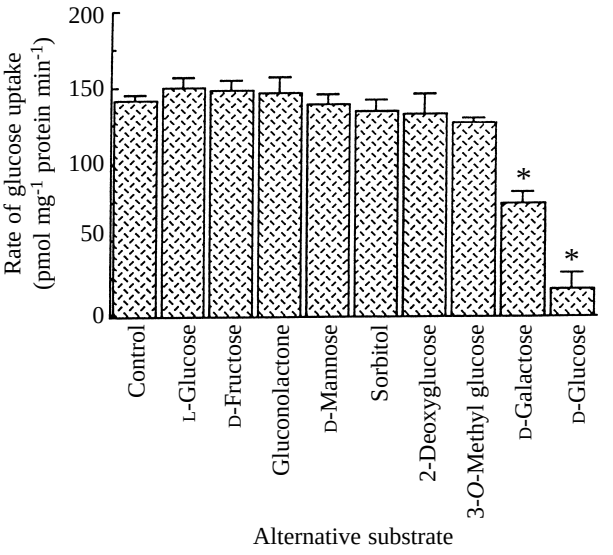

Fig. 5. Inhibition of D-[^{3}H]glucose uptake by alternative substrates. Brush-border membrane vesicles (BBMVs) were preloaded with 500 mmol l^{-1} D-mannitol and 20 mmol l^{-1} Hepes. Samples (5 μl) were then incubated in 95 μl of external medium containing 250 mmol l^{-1} NaCl, 20 mmol l^{-1} Hepes, 0.05 mmol l^{-1} D-[^{3}H]glucose and 5 mmol l^{-1} test substrate. The duration of incubation was 1 min, the time at which maximal D-[^{3}H]glucose accumulation occurs in long-term time courses. Values are presented as total uptake minus non-specific binding, assayed as described in Materials and methods. Transport was stopped with an ice-cold solution containing 250 mmol l^{-1} KCl and 20 mmol l^{-1} Hepes. Values are means + S.E.M., N=4. An asterisk indicates a significant (P<0.05) effect of the test substrate.

To test the relative specificity of the transport system for D-glucose above other potential substrates, the uptake of labelled D-glucose (0.05 mmol l^{-1}) was measured in the presence of a 100-fold excess of unlabelled substrate. After loading with standard mannitol buffer, vesicles were incubated for 1 min in test medium containing NaCl, labelled D-glucose and 5 mmol l^{-1} test sugar. Alternative substrates were chosen for their structural similarity to D-glucose. The data presented in Fig. 5 have had non-specific binding substracted from total uptake. The rate of accumulation of labelled glucose was not significantly affected by any of the related compounds except D-galactose, the C4 epimer of glucose, and D-glucose itself, which would be expected to saturate carrier-mediated transport. These results, in addition to those presented in Figs 1 and 2, show that this carrier is fairly exclusive for Na^+ and D-glucose, with a lower affinity for the closely related D-galactose.

The effect of H^+ concentration on the activity of the glucose carrier is illustrated in Fig. 6. Uptake of D-[^{3}H]glucose was observed in vesicles loaded with solutions ranging in pH from 5.4 to 8.4 and incubated in test medium of the same pH, such that no pH gradients were established. Vesicles were loaded with potassium gluconate/mannitol solution and treated with valinomycin (final $[K^+]$ equal on both vesicle surfaces) then

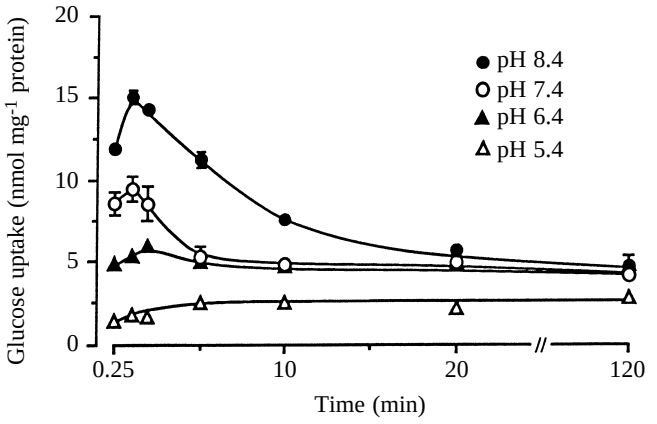

Fig. 6. The effect of bilateral H^+ concentration on the time course of D-[^{3}H]glucose uptake into brush-border membrane vesicles (BBMVs). Four batches of vesicles were prepared, each preloaded with 500 mmol l^{-1} D-mannitol, 50 mmol l^{-1} potassium gluconate, 50 μmol l^{-1} valinomycin and buffered to pH 5.4, pH 6.4, pH 7.4 or pH 8.4. The solution of lowest pH included 40 mmol l^{-1} Mes, the other three included 20 mmol l^{-1} Hepes. All were adjusted to the appropriate pH using Tris or Mes. Samples (20 μl) of the vesicle suspensions were then incubated in 180 μl of external medium of the same pH containing 250 mmol l^{-1} NaCl, 50 mmol l^{-1} potassium gluconate, 0.05 mm D-[^{3}H]glucose. Samples (20 μl) were taken at the time points shown. Transport was stopped with ice-cold external medium lacking glucose. Values are means ± S.E.M., N=4.

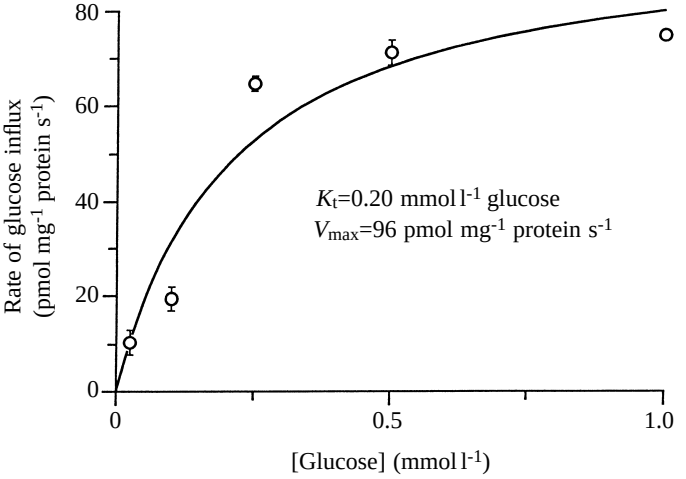

Fig. 7. Kinetics of D-[^{3}H]glucose influx into brush-border membrane vesicles (BBMVs) as a function of external glucose concentration. Vesicles were loaded with 500 mmol l^{-1} D-mannitol, 50 mmol l^{-1} potassium gluconate, 20 mm Hepes and 50 μmol l^{-1} valinomycin. Samples (20 μl) were incubated for 5 s with 95 μl of external solution, with or without phlorizin, containing 250 mmol l^{-1} NaCl, 50 mmol l^{-1} potassum gluconate, 20 mmol l^{-1} Hepes and varying concentrations of D-[^{3}H]glucose. Non-specific binding of D-[^{3}H]glucose to the vesicle exterior and to the Millipore filter on which the vesicles were collected was determined as described in the text and subtracted from total uptake. Transport was stopped with an ice-cold solution containing 500 mmol l^{-1} mannitol, 50 mmol l^{-1} potassium gluconate, 20 mmol l^{-1} Hepes and 1 mmol l^{-1} phlorizin. Values are means ± S.E.M., N=4.

incubated in test solutions of NaCl and potassium gluconate. Fig. 6 shows that, as $[H^+]$ was decreased, the vesicles progressively increased in their ability to sustain concentrative uptake of labelled glucose in the presence of a large inwardly directed Na^+ gradient.

Fig. 7 is a plot of initial rates of glucose uptake by BBMVs *versus* the external concentration of glucose over an incubation period of 5 s in medium in which Na^+ was not limiting. In previous experiments (data not shown), it was confirmed that the glucose influx was a linear function of time for up to 10 s in media containing both 0.05 and 5 mmol l^{-1} glucose. Vesicles were loaded with potassium gluconate/mannitol buffer and treated with valinomycin to prevent the development of a membrane potential. They were incubated in test media containing NaCl, potassium gluconate and D-[^{3}H]glucose at concentrations ranging from 0.025 to 1.0 mmol l^{-1}. Influx was measured in the presence and absence of phlorizin in the external medium. Data are reported as total transport minus non-transport binding. The hyperbolic curve of influx *versus* external glucose concentration (Fig. 7) indicates a saturable Na^+-dependent D-glucose transport pathway with a minimal apparent diffusion component. The Na^+-dependent component of the transport data was further analyzed by fitting to a simple Michaelis–Menten model. The values of kinetic parameters were estimated using an interative non-linear regression curve-fitting program (BPLOT). The maximal rate of glucose influx (J_{max}) was estimated to be 96 pmol mg^{-1} protein s^{-1}. The external glucose concentration necessary to achieve half the maximal transport rate (K_t) was determined to be 0.20 mmol l^{-1} glucose. Aronson and Sacktor (1975) found an apparent K_t of 0.07 mmol l^{-1} glucose in vesicles prepared from rabbit kidney cortex, while Turner and Moran (1982*a*) found an apparent value of 0.35 mmol l^{-1} under zero-*trans* conditions in vesicles prepared from dissected rabbit outer medullary tissue and a low-affinity (K_t=6.0 mmol l^{-1}) high-capacity (J_{max}=10 nmol mg^{-1} protein min^{-1}) system in the rabbit outer cortex.

Analysis of D-glucose influx as a function of external Na^+ concentration was also performed. Vesicles were loaded with potassium gluconate/mannitol solution, treated with valinomycin, then incubated in test medium containing potassium gluconate, labelled D-glucose and NaCl at concentrations ranging from 0 to 300 mmol l^{-1} (TMA-Cl was used to maintain ionic balance). The data are presented as total influx minus Na^+-independent uptake. The sigmoidal activation curve (Fig. 8) suggests that more than one Na^+ is involved in the glucose transport process if a simple Hill model is assumed:

$$J = \frac{J_{max,Na}[Na^+]^n}{(K_{Na})^n + [Na^+]^n} , \quad (1)$$

where $J_{max,Na}$ is the maximal rate of D-glucose influx in the

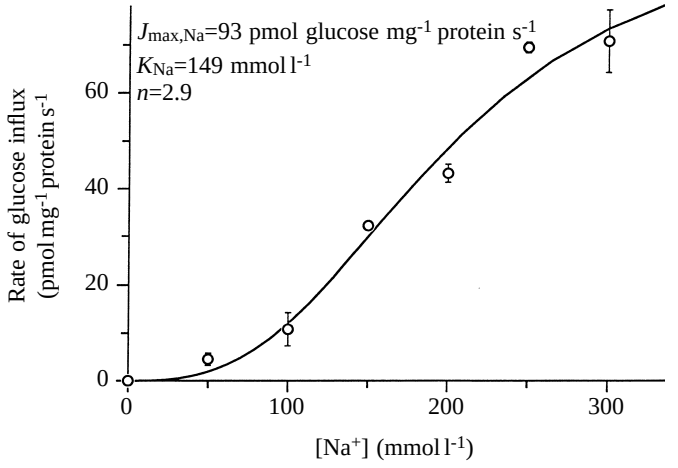

Fig. 8. Kinetics of D-[^{3}H]glucose influx as a function of Na^+ concentration in the external medium. Brush-border membrane vesicles (BBMVs) were loaded with 600 mmol l^{-1} D-mannitol, 50 mmol l^{-1} potassium gluconate, 20 mmol l^{-1} Hepes and 50 μmol l^{-1} valinomycin. Samples (5 μl) of the vesicle suspension were incubated for 8 s in 50 mmol l^{-1} potassium gluconate, 20 mmol l^{-1} Hepes, 0.05 mmol l^{-1} D-[^{3}H]glucose and NaCl ranging in concentration from 0 to 300 mmol l^{-1}. The concentration of Cl^- in the external medium was held at 300 mmol l^{-1} by using compensatory amounts of TMA-Cl. Transport was stopped with an ice-cold solution containing 300 mmol l^{-1} TMA-Cl, 50 mmol l^{-1} potassium gluconate and 20 mmol l^{-1} Hepes. n is the Hill coefficient. Values are means ± S.E.M., N=4.

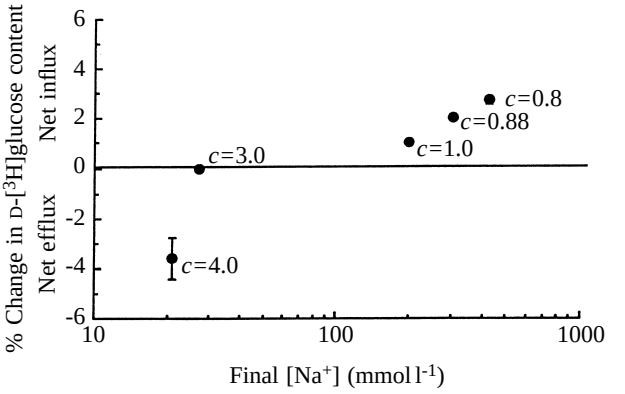

Fig. 9. Static head determination of Na^+:glucose transport stoichiometry. Brush-border membrane vesicles (BBMVs) were loaded with 1 mmol l^{-1} D-[^{3}H]glucose, 10 mmol l^{-1} NaCl, 434.7 mmol l^{-1} TMA-Cl, 100 mmol l^{-1} potassium gluconate, 20 mmol l^{-1} Hepes and 50 μmol l^{-1} valinomycin. Samples (5 μl) were then incubated in 95 μl of external medium. For each separate condition, the medium consisted of 100 mmol l^{-1} potassium gluconate, 20 mmol l^{-1} Hepes and sufficient NaCl balanced with TMA-Cl to yield final Na^+ concentrations of 423 mmol l^{-1} (corresponding to c=0.8, where c is the coupling coefficient), 304.2 mmol l^{-1} (c=0.88), 200 mmol l^{-1} (c=1.0), 27.1 mmol l^{-1} (c=3), 21.1 mmol l^{-1} (c=4) and 0 mmol l^{-1}. Vesicle content after incubation in 0 mmol l^{-1} Na^+ medium was subtracted from total uptake in the other conditions. Transport was stopped with an ice-cold solution containing 444.7 mmol l^{-1} KCl, 100 mmol l^{-1} potassium gluconate, 20 mmol l^{-1} Hepes and 1 mmol l^{-1} phlorizin. Values shown are means ± S.E.M., N=3.

presence of unlimited Na^+, K_{Na} is the concentration of external Na^+ that is needed to reach half the $J_{max,Na}$, and n is the Hill coefficient, a measure of sigmoidicity in the relationship between the concentration of activating substrate (Na^+) and transport rate. The values for the kinetic parameters described above were also estimated using BPLOT. In this way, the apparent $J_{max,Na}$ at 0.05 mmol l^{-1} glucose was found to be 93 pmol glucose mg^{-1} protein s^{-1}. K_{Na} under these conditions was 149 mmol l^{-1} Na^+, and n was 2.9.

The stoichiometry suggested by the activation curve in Fig. 8 was tested by static head analysis in short-circuited vesicles. Briefly, vesicles loaded with labelled D-[^{3}H]glucose and a small amount of NaCl were incubated in media of increasing Na^+ concentration lacking labelled D-glucose. Upon combination of the vesicle suspension and external medium, a 20-fold outwardly directed D-glucose gradient was established. Na^+-dependent influx was measured as the percentage change in D-[^{3}H]glucose content in the vesicles after treatment with the external medium. Non-specific binding and Na^+-independent transport were subtracted from total uptake over the 5 s incubation period. Assuming that each transport event is limited by the binding of the appropriate complement of ligands, depending on the number of sodium ions bound to the carrier, there will be net efflux of D-glucose when there is insufficient Na^+ present to activate transporters and a net influx when there is a surplus of Na^+ to activate D-glucose transport. The concentration of external Na^+ at which the efflux of glucose is balanced by influx through Na^+-dependent cotransport is then used to solve for the coupling coefficient, c, using the following equation:

$$c = \frac{\log_e([\text{glucose}]_{in}/[\text{glucose}]_{out})}{\log_e([Na^+]_{out}/[Na^+]_{in})} . \quad (2)$$

Efflux of labelled D-glucose down the 20-fold concentration gradient was matched by influx mediated by Na^+-dependent transport when a 2.7-fold inwardly directed Na^+-gradient was present (Fig. 9). This corresponds to a coupling coefficient of 3.

Discussion

Previous studies of antennal gland function in decapod crustaceans have focused on the transport properties of the bladder epithelium. This tissue can be easily mounted in an Ussing flux chamber, and the movement of radiolabelled substrate from one side of the chamber to the other is readily quantified. In several of the crustacean crab and crayfish species studied, the bladder epithelium has been shown to be active in transporting solutes such as organic acids (Pritchard and Miller, 1991) and sugars (Gross, 1967; Holliday and Miller, 1984). Structural analysis of the cells of some crab bladders show the appropriate adaptations for transepithelial transport (Miller, 1989).

The role of the labyrinth as an absorptive structure was proposed by Binns (1969), who suggested that the cells of the labyrinth might be important in the active reabsorption of glucose from the urine. Indeed, microscopic studies showing amplified apical and basolateral domains of the epithelial cells of this tissue strongly implicate a secretory and absorptive function (Riegel and Cook, 1975). Although labyrinth tissue also has an epithelium adapted for vectorial solute transport, the study of this component, which makes up the bulk of the antennal gland, has been restricted to the manipulation of tissue slices in which uptake of solutes can be observed (Holliday and Miller, 1984). The ability to access individual cells or membrane domains is limited in these preparations.

The development of techniques to isolate and manipulate plasma membrane vesicles and their subsequent application to crustacean epithelia have enabled us to examine more closely the membrane physiology of the epithelium lining the cavities of the labyrinth (Behnke *et al.* 1990). An earlier study of antennal gland nutrient transport properties showed that the bladder tissue of the lobster had little capacity to mediate Na^+-dependent transport of one amino acid, L-proline, while the labyrinth tissue was rich in Na^+/proline transporters (Behnke *et al.* 1990). Here, the study of labyrinth epithelial function has been extended to an examination of sugar reabsorptive processes.

This study of labyrinth epithelial brush-border membranes indicates that glucose reclamation in lobster antennal glands occurs by a mechanism similar to that present in the ultrastructurally more complex kidneys of vertebrates. Our data show that there are functional membrane-potential-sensitive Na^+-dependent D-glucose transporters present in the BBMVs prepared from lobster renal tissue. The carrier appears to be exclusive of cations other than Na^+, as has been found for Na^+-dependent glucose carriers in rabbit renal cortex (Aronson and Sacktor, 1975) and rat kidney (Kinne *et al.* 1975). Likewise, the transporter shows similar selectivity for D-glucose (Ullrich *et al.* 1976) and sensitivity to the specific inhibitor phlorizin to those found in mammals. The Na^+/glucose transporters in the antennal gland have been found to function within the same range of kinetic parameters as a set of mammalian kidney transporters described previously (Quamme and Freeman, 1987; Turner and Moran, 1982*a*). The crustacean transporters, however, demonstrate a different pH sensitivity from the mammalian forms and have been found to have a higher coupling coefficient between Na^+ and nutrient substrate.

The Na^+/glucose cotransporters studied in the mammalian kidney can be divided into three classes: those of low apparent glucose affinity associated with the more proximal section of the proximal tubule (K_t ranging from 4 to 12 mmol l^{-1} glucose, J_{max} between 9 and 10 nmol min^{-1} mg^{-1}), and those of high affinity and low capacity, thought to predominate in the more distal segment of the proximal tubule (K_t between 0.2 and 0.5 mmol l^{-1} glucose, J_{max} between 1 and 4 nmol min^{-1} mg^{-1}) (Quamme and Freeman, 1987; Turner and Moran, 1982*a*). Examination of the pig kidney and other preparations of the rabbit renal cortex have demonstrated a third class of very high affinity with a K_t of the order of 0.01 mmol l^{-1} glucose (Aronson and Sacktor, 1975; Quamme and Freeman, 1987) and J_{max} of 0.06–1.0 nmol min^{-1} mg^{-1}. The transporter found in the antennal gland BBMVs described in the present study functions within the same kinetic parameters as the intermediate high-affinity class associated with the more distal proximal tubule. This would be consistent with the labyrinth having a role in urine formation analogous to that of the mammalian proximal tubule.

The structural specificity of the Na^+-dependent carrier for D-glucose was established in rat proximal tubule epithelial brush-border membranes by Ullrich *et al.* (1976). Those transporters move only pyranoses in the C1 chair conformation bearing free equatorial hydroxyl groups at position C2 and C3 on the ring. Of several sugars tested in this study, none other than D-galactose affected the accumulation of D-[^{3}H]glucose into antennal gland BBMVs over a 1 min period. Thus, the transporter appears to require that the substrate be a D-pyranose in the C1 chair conformation, that the C2 hydroxyl be equatorial and that C3 be properly hydroxylated. The transporter has the less stringent requirement that the C4 hydroxyl group of the pyranose substrate be equatorial. This is suggested by the fact that D-galactose, which has an axial C4 hydroxyl group, had only a partial inhibitory effect. The profile of inhibitor sensitivity varies slightly among the mammalian transporters studied previously. In some preparations, glucose transport was inhibited by 18–42 % by 10-fold higher concentrations of 3-*O*-methyl glucose (Aronson and Sacktor, 1975; Turner and Moran, 1982*b*), while in lobster BBMVs, glucose transport was unaffected by the presence of this sugar analog. All, however, have been found to have a high preference for glucose and show significant sensitivity to galactose (32–88 % inhibition in mammalian preparations; 25 % in lobster BBMVs). The Na^+-independent system has been found to be stereospecific for the D-isomer of sugars (Kinne *et al.* 1975) but is otherwise relatively non-selective.

In studies on renal tissues from other animals, the glucose derivative phlorizin has been shown to be a potent and specific competitive inhibitor of D-glucose binding to the Na^+-dependent transporter found on epithelial brush-border membranes (Chesney *et al.* 1973). In contrast, it is a relatively poor inhibitor of the Na^+-independent glucose transporters found at the basolateral poles of epithelial cells (Keller, 1968). In whole-animal studies of glucose reabsorption in crustaceans, it was found that injection of phlorizin into the hemolymph of the animal causes glycosuria (Binns, 1969; Burger, 1957; Holliday, 1978; Kirschner, 1967; Riegel and Kirschner, 1960), as it does when it is administered to humans and other mammals. Therefore, those transporters that bind phlorizin in intact tissue, and those that are inhibited by phlorizin in the BBMVs prepared in the present study, are assumed to be responsible for the bulk of glucose reclamation by the renal structure. The kinetics of phlorizin inhibition of the antennal gland Na^+/glucose cotransporter (K_i=3 μmol l^{-1}; Binns, 1969; Holliday, 1978) are similar to those found in the mammalian

kidney, which range between 1 and 7 μmol l^{-1} (Aronson and Sacktor, 1975; Turner and Moran, 1982*b*).

The function of many transporters is sensitive to the pH of the medium in which they are found. Chesney *et al.* (1973) showed that H^+ concentration significantly affected binding of D-glucose to rabbit kidney brush-border membranes. Maximal binding of D-glucose occurred at pH 7.5, which is within the normal range in mammalian urine. A study by Ahearn *et al.* (1985) showed that initial rates of Na^+-dependent D-glucose transport in lobster hepatopancreas, a digestive gland, were stimulated at lower pH and that this higher H^+ concentration lowered the K_t of transport. They proposed that the transporter was adapted to the acidic environment within the tubules of the hepatopancreas diverticula. The luminal fluid of lobster antennal gland labyrinth, however, has been shown to be slightly alkaline (Burger, 1957). In the antennal gland BBMVs prepared in the present study, the pH of the medium in which the vesicles were incubated significantly affected the degree to which D-glucose uptake was stimulated by a transmembrane Na^+ gradient (Fig. 6) over a long-term time course. The greater transport capacity at higher pH may be a reflection of an adaptation of the transporter to the microenvironment within the kidney tubule; the primary structure of the protein may be slightly altered to maintain the tertiary structure under those conditions. H^+ can also function as a substrate under these conditions in some nutrient transport processes, such as that for proline (Roigaard-Petersen *et al.* 1987), although no such mechanism has yet been suggested for glucose transport. The increase in Na^+ stimulation of D-glucose at higher pH could be explained by relief from competitive inhibition by H^+, but the concentration of Na^+ is so high relative to the concentration of H^+ that one would not expect to see such a marked decrease in transport unless the H^+ K_i were extremely low.

The transport stoichiometry of the carrier was determined indirectly by kinetic analysis and more directly by the static head method. Both approaches estimated coupling coefficients of 3. Turner and Moran (1982*a*) found high-affinity (K_t=0.35 mmol l^{-1}), low-capacity (4 nmol min^{-1} mg^{-1} protein) Na^+/glucose transporters associated with the distal segment of the proximal tubule. They also demonstrated a transport stoichiometry of 2 Na^+:1 glucose (Turner and Moran, 1982*b*). The antennal gland carrier described here is comparable with the high-affinity, low-capacity system reported by Turner and Moran (1982*a*), but differs in the number of sodium ions coupled to the movement of each glucose.

It is interesting that all the transporters studied so far in the lobster antennal gland (Ahearn *et al.* 1990) have demonstrated higher activator:solute stoichiometries than their vertebrate counterparts. The lobster antennal gland Na^+/proline transporter (Behnke *et al.* 1990) shows a transport stoichiometry of 2 Na^+: 1 proline compared with the 1:1 relationship seen in the mammalian proximal tubular form (Hammerman and Sacktor, 1977). The Na^+/H^+ exchanger functioning in lobster renal tissue has been found to have a 2 Na^+:1 H^+ rather than a 1:1 relationship (Kinsella and Aronson, 1982).

In many soft-bodied marine invertebrates, a Na^+:nutrient ratio of greater than 2 per transport event has been associated with direct integumental absorption of nutrients, such as free amino acids and sugars, from the surrounding sea water, a process that generally requires the movement of solute against extremely high standing gradients (Gerencser and Stevens, 1989; Pajor *et al.* 1989; Preston and Stevens, 1982; Wright, 1987). The energy available for transport in a transmembrane gradient is a function of the ratio of external to internal activities raised to the power of the number of substrate molecules transported. Therefore, transport of 3 Na^+:1 glucose would be more favorable than a 1:1 transfer for the same inwardly directed Na^+ gradient. The conditions under which these invertebrates function require that slippage be minimal (Gerencser and Stevens, 1989) and that binding of all substances be necessary for translocation no matter what the driving force. Thus, the invertebrate transporters described in the study discussed above show apparent nutrient substrate affinities in the nanomolar range, rather than in the hundred micromolar range demonstrated by glucose transporters previously characterized in vertebrate kidney and the lobster antennal gland carrier described here.

The glucose concentration in the hemolymph of *H. americanus* has been found to range between 1.1 and 1.4 mmol l^{-1} in animals of unknown feeding state (Morgulis, 1923) compared with approximately 5 mmol l^{-1} in animals in which Na^+/glucose transport has been previously described. At these substrate levels, the Na^+-dependent glucose transporter described here with an apparent K_t of 0.20 mmol l^{-1} and J_{max} of 96 pmol mg^{-1} protein s^{-1} (Fig. 7) would be maximally active. As glucose concentration falls to very low levels within the labyrinth as the solute is removed, the transporter would become substrate-limited with respect to glucose, but not with respect to Na^+ (K_{Na}=149 mmol l^{-1}). Urine Na^+ concentration is approximately 470 mmol l^{-1}, nearly isotonic with sea water in this osmoconforming animal, and remains fairly constant throughout the labyrinth (Burger, 1957). A stoichiometry of 3 Na^+:1 glucose would exploit this high concentration of Na^+ and allow the movement of what glucose did bind against a steep concentration gradient into the epithelial cells. It is possible that there may be a very high-affinity glucose transporter in the bladder epithelium which may extract the last traces of glucose from the urine, but the transport properties of the bladder tissue were not studied here. The presence of multiple kinetically distinct transporters could not be detected in our data from the lobster labyrinth BBMVs.

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