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D-Glucose Transport Across the Apical Membrane of the Surface Epithelium in *Nereis diversicolor*

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Summary. Epidermal D-glucose transport was investigated *in vivo* in the brackishwater polychaete worm *Nereis diversicolor*. Transfer across the apical membrane is rate-limiting to D-glucose uptake, but the cuticle and/or mucus presents some resistance to D-glucose diffusion between bulk solution and transporting membrane. Maximal D-glucose influx is about 10^{-12} mol sec^{-1} per cm^2 of apical plasmalemma. Under natural conditions (~ 1 μM D-glucose in the medium), backflux from the epidermal transport pool is negligible, but a significant paracellular outflux may occur. D-glucose influx across the apical membrane is Na^+-dependent and completely inhibitable by phlorizin and harmaline; phloretin is less effective, and cytochalasin B has no effect. Influx is moderately depressed by KCN and iodoacetate. α-methyl-D-glucopyranoside is an effective substitute of D-glucose in transport. Animals acclimated to a low salinity, in which epidermal salt transport takes place, show a marked decrease of D-glucose transport capacity. On transfer of animals from a high to a low salinity, or vice versa, the corresponding change of influx occurs after a time-lag of at least an hour. Permeability of the epidermis to simple diffusion of D-glucose is 8×10^{-8} cm sec^{-1} (on basis of gross epidermal area).

Key words: *Nereis diversicolor* (Annelida, Polychaeta); epithelial glucose transport, monosaccharides, mannitol; D-glucose, L-glucose, D-mannose, D-galactose, 3-O-methyl-D-glucose, 2-deoxy-D-glucose, α-methyl-D-glucopyranoside; inhibition of transport; phlorizin, phloretin, harmaline, cytochalasin B, KCN, iodacetic acid; epidermis, apical membrane; cuticle, unstirred layer; Na-dependence; salinity-dependence of transport

Some animals acquire their food energy by absorbing low-molecular organic material through the body surface. Tapeworms (Cestoda) and spiny-headed worms (Acanthocephala), both intestinal parasites without alimentary tract, take up amino acids and monosaccharides through specific transport systems in the integumentary cell membranes, much like those present in the intestinal mucosa of their vertebrate hosts. Also the parasitic flatworms (Trematoda) – although possessing a gut – have the capability of absorbing organic substances through tegumentary transport systems (Read, Rothman & Simmons, 1963; Pappas & Read, 1975). Similar mechanisms undoubtedly are involved in the nutrition of the Pogonophores, free-living marine animals lacking a gut (Little & Gupta, 1969; Southward & Southward, 1970). A nutritional role of integumentary uptake of organic compounds even has been proposed for marine invertebrates in general; in fact, this latter idea has puzzled biologists through almost a century, and the theme still is much debated (Jørgensen, 1976; Stephens, 1967, 1972; Sepers, 1977). While it has been known for a long time that monosaccharides and amino acids are present in all marine habitats (Wangersky, 1978), concentrations always are orders of magnitude lower than those prevailing in the body fluids of animals. Diffusional loss of nutrients across the epidermis therefore is a potential burden, and transepidermal net uptake from the exterior must occur against considerable concentration gradients.

The functional integrity of epithelia with respect to solute transport results from an orderly asymmetry of the epithelial cells, the apical and basolateral plasma membranes differing from each other in their complement of transport systems and permeability characteristics (Ussing & Leaf, 1978). Also the 'tightness' or 'leakiness' of the intercellular junctions plays an important role in defining the overall characteristics of a transporting epithelium (Erlij & Martinez-Palomo, 1978). Our current picture of epithelial transport of organic nutrients is based mainly on those verte-

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brate organs engaged in mass transcellular transport, i.e. small intestine and kidney (Curran, 1972; Kinne, 1976; Munck, 1976; Silverman, 1976; Schultz, 1977; Ullrich, 1979). Although epidermal solute exchange in marine invertebrates may differ in many respects from that in the vertebrate epithelia, it seems likely that a deeper understanding of the former likewise demands an examination at the cell membrane level. This approach is used in our laboratory in studies of epidermal monosaccharide and amino acid transport across the invertebrate integument.

This paper focuses on the validation of measurements *in vivo* of the unidirectional influx of D-glucose across the apical epidermal cell membrane of *Nereis diversicolor* (Annelida, Polychaeta), a worm inhabiting the sediment of shallow brackishwater areas. An account of the characteristics of D-glucose transport by this membrane is provided. Although the study of transport phenomena is often simplified by the use of nonmetabolizable substrates, and by suitable *in vitro* preparations, the present work deliberately utilizes the naturally most abundant hexose under environmentally realistic conditions to form a reference for further investigation. A preliminary report has been published (Gomme, 1979).

Materials and Methods

Animals, Media

Animals (*Nereis diversicolor* Müller) were collected in batches of 100–300 throughout the year from mud flats in Vellerup Vig, Zealand, Denmark. In the laboratory, the worms were kept unfed and acclimated for at least 5 days at 15 °C to dilutions of aerated artificial seawater (ASW), prepared according to Hale (1958). 100% ASW (34.4% salinity) contains (in mM): 470 Na^+, 53.6 Mg^{2+}, 10.2 Ca^{2+}, 9.7 K^+, 0.07 Sr^{2+}; 548 Cl^-, 28.2 SO_4^{2-}, 2.4 HCO_3^- (nominal), 0.8 Br^-, 0.4 H_3BO_3. The pH is about 7.2. Glass tubes were provided as artificial burrows for the animals during storage. Worms of both sexes were used indiscriminately within the first two weeks after capture, but injured or ripe specimens were discarded. Animals were exposed individually to the test solutions, the weight of each worm being recorded beforehand. The temperature of 15 °C was maintained in all experiments.

Labeled Compounds Chemicals

Radioactively labeled substrates were purchased from The Radiochemical Centre, Amersham, England. Precautions were taken to minimize radiolysis on storage (Evans, 1976), and the radiochemical purity was checked regularly by thin-layer chromatography. When necessary, chromatographic purification was performed. All other reagents used were of analytical grade.

Uptake and Influx Experiments

Unless otherwise noted, animals were preincubated for 15–60 min in D-glucose free ASW and subsequently incubated in 10 ml ASW of the same salinity, containing radioactive substrate; unlabeled material was added as required, and the substrate concentration (1–1800 μM) remained constant throughout the experiment. After incubation, the animals were rinsed 5 sec in unlabeled ASW: A complete removal of adhering radioactivity was found to require 20–40 sec; a 5-sec rinsing was chosen as a compromise, since 90–95% of adhering medium is then removed while diffusional loss of metabolically formed ^3HHO is kept below 2% (*unpublished observations*). After the rinse, the animals were extracted for 48 hr in 10 ml 70% ethanol at room temperature. Approximately 90% of the radioactivity reached equilibrium with the ethanol within this period. For liquid scintillation counting, 4 ml ethanolic extract was added to 10 ml scintillation fluid containing 3.3 g PPO and 0.13 g DMPOPOP per liter of toluene:Triton X-100 (2:1 vol/vol). The resulting counting system was stable and was shown by a double-ratio test (Bush, 1968) to be functionally homogenous in spite of the heterogenous character normally ascribed to Triton scintillants (Fox, 1976). Thus, counting efficiency could be evaluated by the external standard method, using individual standard curves for each experiment. Counting was performed at +12 °C.

In this paper, the term *uptake* signifies the amount of radiolabeled substrate or its metabolic products (dpm mg^{-1} or fmol mg^{-1}) extractable from animals after a specified incubation period, including substrate adhering to the animal surface after rinsing. The term *influx* will be reserved for the estimated rate of uptake (dpm mg^{-1} min^{-1} or fmol mg^{-1} min^{-1}), as controlled by some rate-limiting barrier in the epidermis, later to be identified as the apical membrane. D-glucose influx was determined by incubating 3–4 groups of 8 animals in the radioactive medium for periods of 1–4 min. The slope of the resulting progress curve will be shown to represent influx across the apical membrane. Influx of D-mannitol was evaluated by a modified procedure: After incubation for either 30 or 60 min, rinsing was extended to 2 min to remove all loosely adhering material before extraction. Influx was determined from the mean uptake, assuming a linear time-course.

Removal of D-Glucose from Medium

After preincubation in D-glucose free ASW as above, animals were incubated for 100–160 min in 5 ml ASW with an initial D-[6-^{3}H]glucose concentration of 5 μM. The medium was stirred gently by a stream of moist atmospheric air. Samples of 10 μl were taken from the medium at intervals and evaporated on a strip of lens paper to remove volatile material (^3HHO). After transfer to a scintillation vial, the paper was soaked in 25 μl distilled water, and 10 ml of the following scintillant added: 1000 ml toluene, 150 g naphthalene, 300 ml 2-ethoxyethanol, 7 g PPO and 0.6 g DMPOPOP. The samples were homogenous, and spot-checks with addition of internal standard verified that all counted with the same efficiency.

Washout Experiments

Animals were incubated for 30 min in ASW with D-[6-^{3}H]glucose, or with a mixture of D-[6-^{3}H]glucose and D-[1-^{14}C]mannitol. After a 5-sec rinse they were transferred to 10 or 25 ml ASW with a known concentration of unlabeled substrate. This washout medium was stirred by air bubbling. Samples of 100 μl were taken at intervals, evaporated, redissolved and subjected to liquid scintillation counting as described. After washout, extractable radioactivity in the animals was determined. In double-label experiments, counting efficiency for each isotope was evaluated by means of toluene internal standards.

Net Uptake of D-Glucose

Each animal was incubated for 120 min in 2.5 ml ASW with an initial concentration of unlabeled D-glucose of about 1 μM. Initial and final D-glucose concentrations were determined by the enzy-

matic-fluorimetric method of Hicks and Carey (1968), which is absolutely specific for D-glucose. The difference between concentrations was used to estimate the average rate of net D-glucose uptake during the exposure.

Calculations

Uptake (Q^*W) as a function of animal weight (W) in the range 10–1000 mg was found to obey the relation (Gomme & Ahearn, *unpublished*):

$$Q^*W = kW^{0.662}. \quad (1)$$

The exponent (0.662) is not significantly different from 0.667, the theoretical value derived from the surface law[1]. The experimental animals in the present study were in the range 150–600 mg (mean 310 mg), and to compensate for variation in animal weight, all uptake values were standardized according to the equation:

$$Q^*_{st} = Q^* \frac{W W_{st}^{0.667}}{W_{st} W^{0.667}}. \quad (2)$$

W_{st} is the weight of an arbitrarily chosen reference animal, W_{st}= 300 mg. The 'standard activity uptake', Q^*_{st}, clearly has the connotation of 'the amount of substrate transported through or bound to unit surface area,' the latter being defined as 1/300 of the area of a 300 mg reference worm. The exact surface area, expressed in conventional units, is unknown; whenever needed for comparative purposes, the epidermis of a 300 mg worm will be assumed to have a gross surface area of 10 cm^2.

D-glucose influx was determined by least-squares linear regression analysis of standard uptake *vs.* time, using the reciprocal experimental variance of uptake values for each time period as a weighting factor. Uptake values for a given time period followed the normal distribution and a check for linearity was performed. The slope of regression lines were compared in the manner of Hald (1952).

Results

The ultrastructure of the polychaete integument has been described by several authors (for a review, *see* Richards, 1978). Figure 1 is a semi-diagrammatic representation of the surface epithelium of *Nereis* with its adjacent cuticle.

The *Nereis* integument was found by Ahearn and Gomme (1975) to constitute the avenue of D-glucose uptake from the medium, since the animals never drink under the experimental conditions used. The surface microflora was found to accumulate D-glucose to a negligible extent, a conclusion later corroborated (Gomme, *unpublished*). After incubation in an ASW-medium with micromolar radioactive D-glucose, most of the labeled material in the animal was seen to be located in the peripheral tissues, mainly the epidermis; a considerable part of the substrate was metabolically degraded. Evidence was presented that D-glucose uptake by the integument occurs via substrate-specific processes, and it was tempting to provisionally designate the apical membrane of the epidermal cells as the rate-limiting barrier.

[1] Least-squares linear regression analysis on basis of the relation $\log(Q^*W) = a + b \log W$ gave the slope estimate: $b = 0.655 \pm 0.030$ (N = 100).

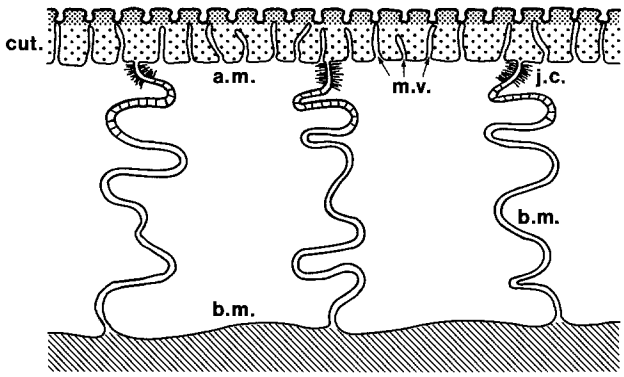

Fig. 1. Schematic representation of the *Nereis* integument. Gland cells not shown. The junctional complex (*j.c.*), composed of zonula adhaerens and septate junction, is believed to form an apical collar around each epidermal cell, partitioning the cell membrane into two distinct regions: The apical membrane (*a.m.*) facing the cuticle, and the baso-lateral membrane (*b.m.*). The cuticle (*cut.*) is composed of (1) a basal stratum (about 2 µm) with a network of 0.1 µm collagen fibers embedded in a homogenous polysaccharide matrix; (2) a more electron-dense epicuticle (0.2–0.4 µm), and (3) a thin, dense outer layer (0.02 µm). Substances pass the cuticle by simple diffusion, and two routes apparently are open for transepithelial passage: Diffusion through the intercellular spaces, and transport into or through the cell. The tips of the microvilli (*m.v.*) may be in direct contact with the surrounding medium. The presence of microvilli increases the surface area of the apical membrane by a factor of 3–8 (estimated from EM-micrographs)

Time-Course of D-*glucose Uptake. Effect of Inhibitors*

When measuring epidermal D-[6-^{3}H]glucose influx into *Nereis*, incubation of animals in the radioactive medium should not exceed 5–10 min; after that period, backflux from the animal of labeled metabolites, especially ^{3}HHO, may affect the results (Ahearn & Gomme, 1975), and ethanol extraction no longer reveals the total amount of substrate accumulated. The rate of D-glucose uptake may be estimated from a time-course curve as shown in Fig. 2(*A*). For brief incubations, such curves are always linear with positive intercepts on the vertical axis. The magnitude of the intercepts, expressed on basis of an animal of standard size (300 mg), usually corresponds to the amount of labeled substrate present in 0.5–5 µl incubation medium.

The question arises whether the slope of a line as in Fig. 2(*A*) actually reflects influx across the apical epidermal membrane, as tacitly assumed by Ahearn and Gomme (1975), or alternatively the rate of metabolic conversion of exogenous D-glucose in the cells (metabolic trapping) or even some other process. In the latter cases, kinetic information on the transfer process at the apical membrane should be revealed

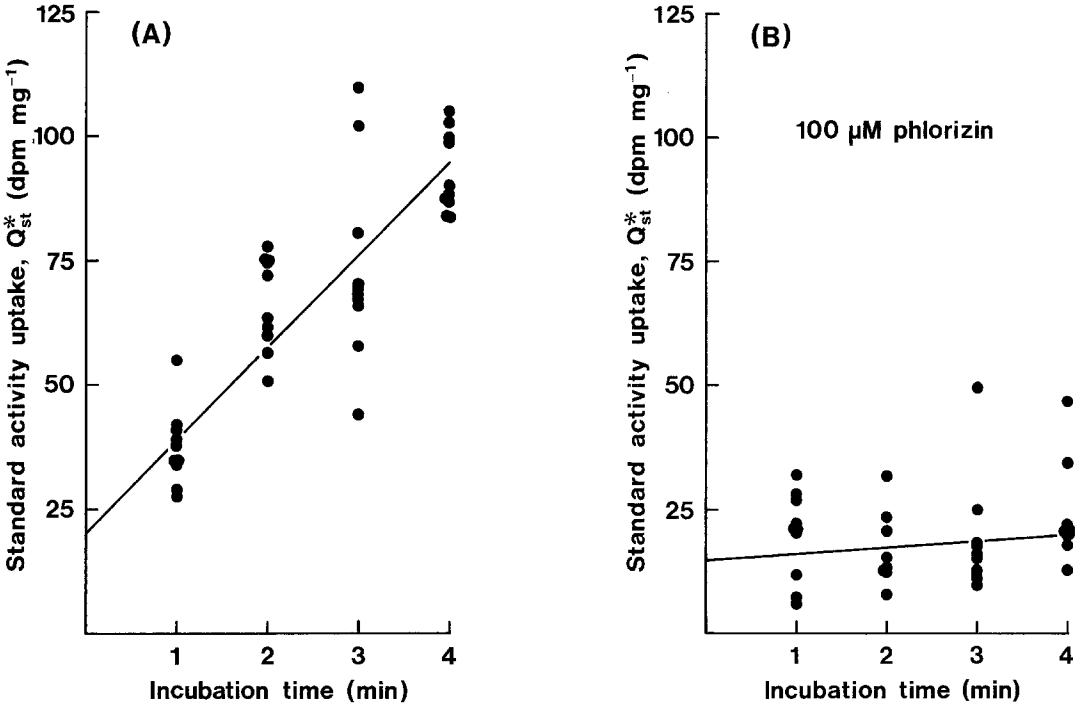

Fig. 2. (*A*) Time-course of standard activity uptake (Q^*_{st}) on incubation in 1.0 µM D-[6-^{3}H]glucose (50% ASW). Standard D-glucose uptake was calculated as described in Materials and Methods to compensate for variation in animal size. The line was fitted by a weighted, linear least-squares regression analysis. The slope of the line is 17.8 dpm mg^{-1} min^{-1} or 12.3 fmol mg^{-1} min^{-1}, and the intercept on the vertical axis is 23.0 dpm mg^{-1} or 15.8 fmol mg^{-1} (N=40). (*B*) Time-course of standard activity uptake under similar conditions, but in the presence of 100 µM phlorizin in the incubation medium. The slope of the line is 1.32 dpm mg^{-1} min^{-1} or 1.01 fmol mg^{-1} min^{-1}, and the intercept is 16.7 fmol mg^{-1} (N=38). (*See also* the first two lines of Table 1.) Addition of phlorizin reduces the slope to 7% of the control value, but does not significantly affect the intercept. In this and similar experiments, the variance of uptake values for a given incubation time is approximately proportional to the mean uptake

only during ultra-short incubations, which are technically unfeasible; using the time-scale of Fig. 2, the first part of the uptake curve would be 'hidden' in the positive intercept. Conversely, if the slope is in fact representative of apical influx, the intercept demonstrably should be due to radioactive material outside the apical membrane.

Figure 2(*B*) shows that phlorizin, known to be a competitive inhibitor of D-glucose transport in some animal cells (Lotspeich, 1961), also inhibits D-glucose uptake by *Nereis* epidermis; this is seen from the markedly reduced slope of the time-course curve in the presence of the drug. The intercept, however, is unchanged (*see also* Table 1). The concentrations of phlorizin used are so low as to rule out a direct metabolic action (Lotspeich & Keller, 1956; Parsons, Smyth & Taylor, 1958; Newey, Parsons & Smyth, 1959), and the compound must exert its effect on the level of the plasma membrane. Phlorizin penetrates biological membranes rather slowly (Stirling, 1967), the only exception known to the author being the secretion of phlorizin by mammalian renal tubule (Silverman, 1976). Diffusion of phlorizin through the intercellular junctions of the epidermis is not likely, due to the limited permeability of the latter to even smaller molecules such as D-glucose (Ahearn & Gomme, 1975). Thus, the immediate and exclusive effect of the drug on the slope of the uptake curve (but not on the intercept) apparently results from an inhibitory action on the uptake mechanism in the apical cell membrane. Consequently, the intercept is assumed to represent labeled material connected with structures external to the outer membrane of the epidermal cells.

Table 1 also shows the effect of phloretin, the aglucone of phlorizin, on D-glucose transport. Again, inhibition is only manifest in the slope of the curve. Phloretin appears to be a less efficient inhibitor than phlorizin, when similar concentrations of the two substances are compared.

Also the alkaloid, harmaline, reduces the slope of the progress curve, but leaves the intercept unchanged (Table 1). At the concentration used, harmaline is known to inhibit Na^+-dependent sugar uptake across the apical membrane of the mammalian intestinal epithelium, but it has no effect when added to the serosal side (Sepulveda & Robinson, 1974, 1975). Thus, the above interpretation of Fig. 2 is further strengthened.

In contrast, cytochalasin B, which inhibits D-glu-

Table 1. Effect of inhibitors on D-glucose transport

Inhibitor	C_i (μM)	C (μM)	J_{in} (fmol mg^{-1} min^{-1})	$Q^*_{t=0}$ (fmol mg^{-1})	$Q^*_{t=0}$ equivalent volume (μl/300 mg)
(Control)	0	1.0	12.3 ± 0.9	15.8 ± 0.1	4.8
Phlorizin	100	1.0	1.0 ± 1.0*	12.7 ± 0.1	3.8
Phlorizin	25	1.0	−0.3 ± 1.2*	15.3 ± 0.1	4.6
Phloretin	25	1.0	8.2 ± 1.5	20.4 ± 0.1	6.1
(Control)	0	1.0	15.7 ± 2.0	9.4 ± 0.1	2.8
Phloretin	100	1.0	3.7 ± 0.8*	10.1 ± 0.1	3.0
(Control)	0	700	1825 ± 719	5233 ± 176	2.2
Phlorizin	100	700	−1098 ± 1332*	11240 ± 221	4.8
(Control)	0	5.0	185 ± 12	91.6 ± 0.6	5.3
Harmaline	4000	5.0	31 ± 13*	88.1 ± 0.6	5.3
(Control)	0	5.0	122 ± 14	130 ± 8	7.8
Cytochalasin B	40	5.0	106 ± 9	16 ± 7	1.0

C is the concentration of D-[6-^{3}H]glucose in the incubation medium, C_i the concentration of unlabeled inhibitor. J_{in} denotes the transport rate (influx), as determined from the slope of a line as in Fig. 2, and $(Q^*)_{t=0}$ is the intercept; both are given as the least-squares estimate ± SE. Significant deviation of fluxes from the control value ($p<0.05$), as evaluated by the t-test, is indicated by *. Note that since uptake values are composite (*see* text), only part of the variation along the ordinate is due to membrane transport. Hence, the statistical comparison underestimates the actual differences between fluxes. In the right-hand column, the intercept is expressed as the equivalent volume of adhering incubation medium (μl per standard animal of 300 mg). Each group of data represents experiments performed on a single batch of animals, and are therefore directly comparable.

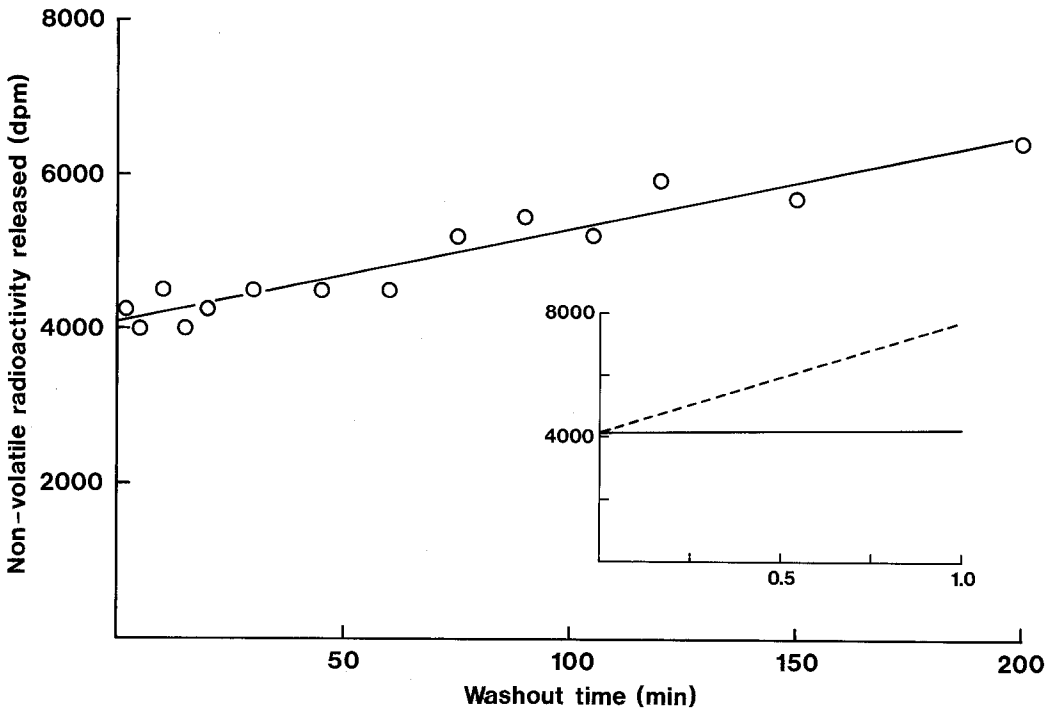

Fig. 3. Washout of nonvolatile radioactivity (medium samples evaporated before counting to remove metabolic ^{3}HHO) from a single animal (277 mg) after 30 min incubation in 5.0 μM D-[6-^{3}H]glucose (50% ASW). Specific activity in the incubation medium: 549 dpm pmol^{-1}. Washout medium: 25 ml 50% ASW containing 5 μM unlabeled D-glucose; under these conditions, less than 2% of D-glucose in the washout medium will be removed by the animal during the experiment. The intercept on the vertical axis is equivalent to 7.5 pmol D-[6-^{3}H]glucose, or to 1.5 μl incubation medium adhering to animal surface. The slope of the line (dQ^*/dt) is 0.072 fmol mg^{-1} min^{-1}, when using the same specific activity as in the incubation medium. Simple linear regression analysis was used to determine these parameters. *Insert:* Washout of volatile (^{3}HHO) and nonvolatile radioactivity during the first minute of washout. The full line is a repetition of the main frame. The broken line represents the initial washout of total radioactivity, using the results of Ahearn and Gomme (1975), Table 1, to estimate the washout of volatile radioactivity (^{3}HHO)

Table 2. Washout of nonvolatile radioactivity

Salinity (% ASW)	Washout medium (ml; μM D-glucose)	dQ^*/dt (dpm mg^{-1} min^{-1} $\times 10^4$)	Expected J^*_{in} (dpm mg^{-1} min^{-1} $\times 10^4$)	$Q^*_{t=0}$ (dpm mg^{-1})	$Q^*_{t=0}$ mean equiv. volume (μl)
		−62		23	
		−64		21	
20	25; 0	104*	(0)	26	5.4
		−258*		46	
		112		51	
		58		20	
		−256*		28	
50	25; 0	437*	(0)	15	2.3
		905*		7	
		255*		10	
		248*		3	
		11		7	
65	10; 25	38*	−50	32	1.1
		282*		13	
		434*		4	
		−425*		59	
		5		22	
65	10; 5	−77*	−220	28	5.2
		183*		8	
		−320		79	
		3985*		0	
		7130*		0	
80	25; 0	128*	(0)	11	1.6
		1075*		17	

Animals incubated with 5.0 μM D-[6-^{3}H]glucose (specific activity: 549 dpm $pmol^{-1}$). The rate of release of nonvolatile radioactivity, dQ^*/dt, was estimated as in Fig. 3. An asterisk (*) denotes a rate significantly different from zero ($p<0.05$). The expected rate of uptake of labeled D-glucose (J^*_{in}) is based on influx data, some of which are presented in Table 5, on the initial concentration in the washout medium (col. 2 of this Table), and on the magnitude of the washout intercept (col. 5); the latter two quantities give the initial specific activity of labeled D-glucose in the washout medium. A negative rate corresponds to uptake into the animal. When the initial D-glucose concentration is zero (rows 1, 2, and 5), the expected J^*_{in} is also zero, but binding of the very small amount of radioactive material to surfaces in the washout chamber is anticipated.

cose transport in some animal cells (Czech, Lynn & Lynn, 1973; Vinten, 1978), is shown in Table 1 to be ineffective in the present case.

Washout of Nonvolatile labeled Material: The 'Surface Compartment'

The problem of adhering substrate was further investigated in a series of washout experiments. Figure 3 shows the result of a typical one, and a summary of 24 experiments is compiled in Table 2.

It is evident from Fig. 3 that the washout of nonvolatile radioactivity from preloaded animals is a biphasic process, as was the uptake. The initial rapid release represented by the vertical intercept is found in all experiments (Table 2, columns 5 and 6). Expressed as the equivalent volume of incubation medium, the intercepts are in good agreement with the amount of adhering medium estimated from uptake curves. Thus, both types of experiment demonstrate that a 'surface compartment' of labeled D-glucose is created during incubation, and not completely eliminated during the 5-sec rinse in unlabeled medium. The slope of the washout curve therefore is a measure of the net release of nonvolatile labeled compounds from structures below the level of the apical epidermal membrane.

In uptake studies, the adherence of labeled substrate outside the apical membrane may be avoided by increasing the rinsing time, but only at the risk of losing labeled material from the tissue. The insert in Fig. 3 illustrates this point. The release of activity (nonvolatile + volatile) during the first minute of washout is estimated from previously published data and shown by the broken line (*see* figure legend for details).

Washout of Nonvolatile Labeled Material: Leakage from the Epidermis

The slope of the curve in Fig. 3 is significantly positive, indicating a net release of radioactive D-glucose and/or nonvolatile (organic) D-glucose metabolites throughout the experiment. The washout medium in this case contained unlabeled D-glucose (initially 5 μM), and at the start of the washout period it received a sudden supply of labeled D-glucose from the 'surface compartment' mentioned above. Since the D-glucose uptake system(s) must be operative also under washout conditions, removal from the medium of some nonvolatile radioactivity as D-[6-^{3}H]glucose is to be expected. The slope of the curve therefore results from two opposite processes: The release of nonvolatile labeled material from the animal, and the uptake of labeled D-glucose from the washout medium. In the experiment of Fig. 3, the former process must have been dominating, as seen from the positive slope.

The average rate of change of nonvolatile radioactivity in the medium (dQ^*/dt) was determined by linear regression analysis for each of the 24 washout experiments, representing a variety of experimental conditions (Table 2); all curves conformed with the assumption of a linear time-course. In 13 of the 24 cases, dQ^*/dt was significantly greater than zero ($p < 0.05$), and at least these animals must have released labeled organic material to the surroundings. This conclusion was substantiated by comparing the individual dQ^*/dt's, not to the zero level, but to the expected rate of D-glucose uptake during the washout period (Table 2, column 4). However, as clearly seen in column 3, there is a considerable variation among animals, also those subjected to identical experimental conditions; 5 animals even show an uptake of nonvolatile material during the washout period.

Nonvolatile labeled material released is only a minute fraction of what remains in the animal. Thus, in the experiment of Fig. 3, 3×10^5 dpm could be extracted after the washout period. The relevance of searching for a significant leakage stems from the fact, that the specific activity in the particular animal compartment exchanging with the exterior may be orders of magnitude lower than that in the incubation medium. Therefore, even a small release of nonvolatile radioactivity may signal an energetically important outflux. To the extent that leakage of labeled metabolites does occur, the compounds may be discharged from the epidermal cells via the apical cell membrane, or come from the extracellular fluid, passing the integument by diffusion in the intercellular spaces. Alternatively, the material may originate from mucus production, or even excretion, since a small amount of exogenous D-glucose enters the coelomic fluid, from which urine is formed (Ahearn & Gomme, 1975).

Table 3. Effect of phlorizin on D-[1-^{14}C]mannitol and D-[6-^{3}H]glucose uptake, corrected for adhering medium

	Without phlorizin	With 100 μM phlorizin
D-[1-^{14}C]mannitol	35.5 ± 14.7 (N = 20)	27.2 ± 22.8 (N = 19)
D-[6-^{3}H]glucose	2481 ± 753 (N = 20)	69.5 ± 34.5 (N = 19)

Uptake values were standardized according to Eq. (2) to compensate for differences in animal weight. All values in fmol mg^{-1}, mean ± SD.

Diffusion of D-Glucose to the Apical Membrane

The suitability of D-[1-^{14}C]mannitol as an indicator of adhering D-[6-^{3}H]glucose (extracellular marker) was investigated. Two groups of animals, a total of 39, were incubated for 30 min in 65% ASW with an equimolar mixture of D-[6-^{3}H]glucose and D-[1-^{14}C]mannitol (5 μM); 100 μM phlorizin was added to the incubation medium of one group. After a 5-sec rinse in unlabeled ASW, each animal was transferred to 10 ml 65% ASW with 5 μM unlabeled D-glucose and D-mannitol. The washout of nonvolatile radioactivity from each animal was followed for 10 min, enough to estimate the intercept of the washout curves for ^{3}H and ^{14}C. At the end of the washout period, the animals were extracted in 70% ethanol, and the accumulated ^{3}H- and ^{14}C-activity in the extracts determined. Since adhering radioactive material from the incubation medium has been removed during the washout period, the radioactivity of the extract represents material transported through the apical membrane during incubation. The results are summarized in Tables 3 and 4, and in Fig. 4.

The uptake of D-mannitol through the epidermis is very slow, as indeed expected for an extracellular marker (Table 3). On this background, phlorizin is seen as an extremely effective inhibitor of D-glucose transport across the outward-facing membrane. Both in the presence and absence of phlorizin, the intercepts of the washout curves for nonvolatile ^{3}H- and ^{14}C-activity were in the expected range: Expressed as the equivalent volume of incubation medium, 90% of the values fell within the interval 0.5–5 μl (Table 4). When phlorizin was present to inhibit the influx of D-glucose, there was a close correspondence between the D-[6-^{3}H]glucose and D-[1-^{14}C]mannitol intercepts (Fig. 4*A*). This finding strongly confirms the interpre-

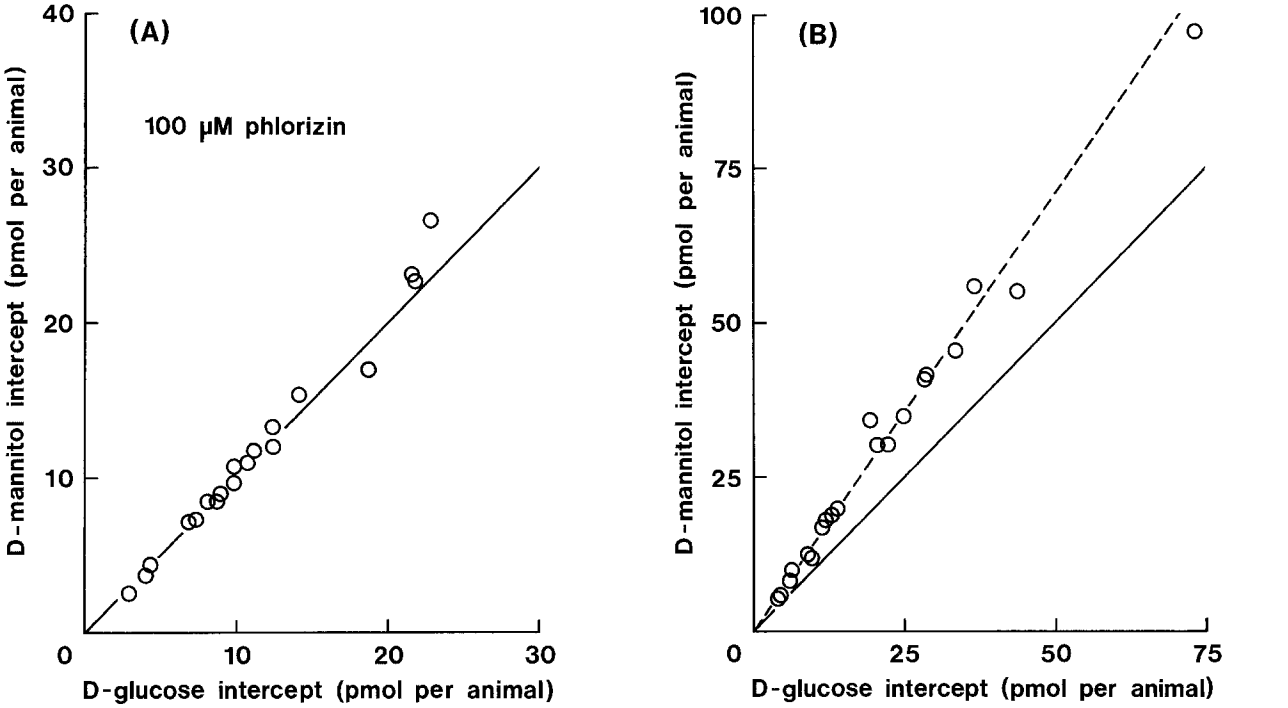

Fig. 4. Relation between the intercepts of concomitant washout curves for D-[1-^{14}C]mannitol and D-[6-^{3}H]glucose. Each point represents the two recorded intercepts for a single animal. (*A*) 100 μM phlorizin added to both incubation and washout medium to block D-glucose transport. The line of identity is shown. (*B*) No phlorizin in the media. The line of identity no longer describes the relation between the variables. The slope of the broken line is 1.43

Table 4. Effect of phlorizin on D-[1-^{14}C]mannitol and D-[6-^{3}H]glucose washout intercepts

	Without phlorizin	With 100 μM phlorizin
(*a*) Expressed as fmol mg^{-1} (*cf.* Table 3)		
D-[1-^{14}C]mannitol	81.9 ±55.8 (N=20)	42.0 ±26.5 (N=19)
D-[6-^{3}H]glucose	58.1 ±42.6 (N=20)	40.3 ±23.4 (N=19)
(*b*) Expressed as equivalent volume of adhering medium (μl) per standard animal (300 mg)		
D-[1-^{14}C]mannitol	4.91 ± 3.35 (N=20)	2.52 ± 1.59 (N=19)
D-[6-^{3}H]glucose	3.49 ± 2.56 (N=20)	2.42 ± 1.40 (N=19)

Same experiment as in Table 3 and Fig. 4. Values standardized to compensate for differences in animal weight, using Eq. (2). Mean ± SD.

tation of uptake curves given above. However, in the absence of phlorizin, the D-[6-^{3}H]glucose intercepts were consistently lower than the D-[1-^{14}C]mannitol intercepts from the same animals (Fig. 4*B*). The average ratio between the two was found to be 0.70 ± 0.05 (N=20). Thus, when D-glucose transport across the apical membrane is functional, the 'surface compartment' of this compound is partially emptied.

These results may be explained by assuming the presence of an unstirred layer apposing the epidermis, through which D-[6-^{3}H]glucose moves by diffusion. The mobility of the compound in this layer must be sufficiently low to produce a significant concentration gradient, the concentration being lowest at the apical membrane, where D-glucose is taken into the cell[2]. As a consequence, the total amount of D-[6-^{3}H]glucose in this layer is less than the amount of D-[1-^{14}C]mannitol, provided D-glucose transport into the epidermis takes place. D-[1-^{14}C]mannitol, although it has the characteristics to qualify as an extracellular marker, in this case will lead to an overestimate of D-[6-^{3}H]glucose outside the transport barrier.

The use of D-mannitol as an extracellular marker was discontinued. However, the data provides information on the diffusion of D-glucose between the bulk medium and the transporting apical membrane (*see* Discussion, and Gomme, 1981).

Epidermal Permeability to D-Mannitol

Determination of nonadhering D-mannitol uptake after long incubations, as in Table 3, provides a basis

[2] It was attempted to confirm this conclusion by repeating the experiment of Fig. 4(*B*), using chilled (0 °C), phlorizin-containing rinsing medium, which would presumably inhibit uptake. However, results were confounded by provoked leakage of minute amounts of D-[6-^{3}H]glucose accumulated in the cells during incubation.

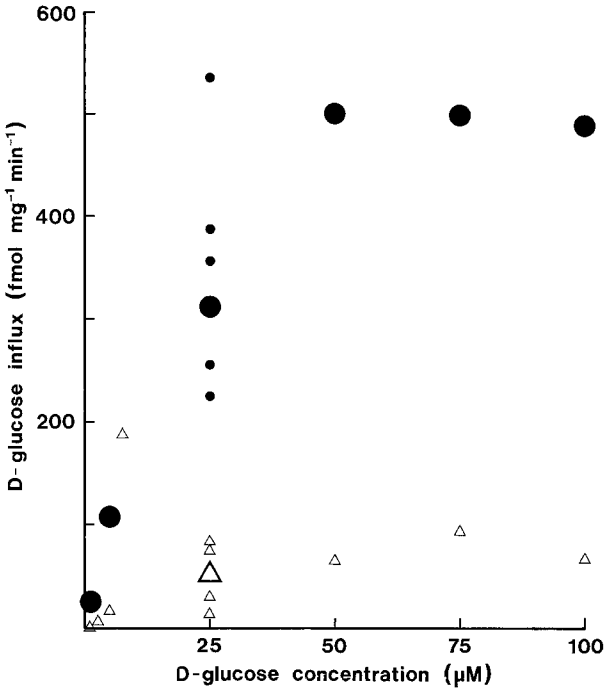

Fig. 5. Concentration dependence of D-glucose influx in 35% ASW (triangles) and 65% ASW (circles). Animals acclimated to the respective salinity for at least 5 days before the experiment. Small symbols show the results of individual experiments, large symbols the weighted average of several experiments performed on different batches of animals

for assessing the epidermal permeability to this substance. Animals were incubated for 30 or 60 min in 5 μM (5×10^{-9} mol cm^{-3}) D-[1-^{14}C]mannitol, left to washout for 2 min, and extracted in ethanol. The rate of uptake follows an upward-skewed distribution with a mean of 0.80 fmol mg^{-1} min^{-1}, and a median of 0.63 fmol mg^{-1} min^{-1} (N = 108). Assigning an epidermal surface area of 10 cm^2 to an animal of standard size (300 mg) leads to the following estimate of epidermal D-mannitol permeability: $p = (0.80 \times 10^{-15} \times 300)/(10 \times 5 \times 10^{-9} \times 60) = 8.0 \times 10^{-8}$ cm · sec^{-1}.

Salinity- and Concentration Dependence of D-*Glucose Influx*

It was demonstrated above that the intercept of uptake curves as in Fig. 2 is due to labeled substrate in a 'surface compartment' outside the apical epidermal membrane. In the Discussion, it will be shown that the slope of uptake curves indeed represents D-glucose influx across the apical membrane. Epidermal D-glucose influx was investigated at various substrate concentrations, and in animals acclimated to a low (35% ASW) and a high salinity (65% ASW). Results are displayed in Fig. 5. It should be noted that, due to the diffusion-restraining properties of peripheral epidermal structures, D-glucose concentration at the apical membrane is lower than the bulk concentration shown in the figure, and concentrations probably vary along the length of the microvilli.

When 35% ASW was used for acclimation and incubation, D-glucose influx never exceeded 100 fmol mg^{-1} min^{-1}. In the higher salinity, 10-fold larger fluxes were encountered in the same concentration interval. Also, influx is positively correlated to the D-glucose concentration of the medium, and there are signs of saturation. However, a substantial variability among replicate flux estimates is a prominent feature, which tends to obscure any functional relationship between influx and concentration. A number of additional experiments at 65% ASW were performed at high D-glucose concentrations in an attempt to achieve an unequivocal saturation of the transport system(s). The results suggest that saturation occurs at 600–800 μM. Although the intercept values were in each case within the previously established range, transport into the epidermis appeared erratic at concentrations above 100 μM, as seen from a large relative standard error of the flux estimates. A kinetic characterization of D-glucose influx was relinquished.

To provide a comparison to the influx data just presented, some animals were incubated individually in 5 ml 35 or 65% ASW with an initial concentration of 5 μM D-[6-^{3}H]glucose, and the decrease in the medium of labeled nonvolatile material was monitored throughout 120 min. It has been shown above that only a small fraction of the accumulated radioactivity is again released to the surroundings as nonvolatile material. Therefore, nonvolatile radioactivity in the medium may be used as a measure of D-glucose concentration. During the 120 min incubation, D-glucose concentration never reached below 3.5 μM, and it was a linear function of time throughout the experiment. The average rate of D-glucose removal was calculated from the slope of the line, and the results are summarized in Table 5. Although a completely different technique, the data confirm that animals acclimated to and tested in 35% ASW remove D-glucose significantly slower than those in 65% ASW. The rate of removal corresponds fairly well with the range of influx values found with the method of Fig. 2.

Effect of Acclimation and Incubation Salinity

In the experiments of Fig. 5 and Table 5, animals were acclimated to the experimental salinity for at least 5 days before measurement. The results therefore do not reveal whether the salinity dependence of D-glucose influx is due to the animals' state of salinity acclimation, or is a function of the acute salinity of the incubation medium.

Table 5. Rate of D-glucose removal from medium

Salinity (% ASW)	Rate of uptake	Standard rate of uptake[a]	Average exp. error[b]
	(fmol mg^{-1} min^{-1})		
35	46.9 ± 18.1	56.7 ± 19.4	7.5
65	132 ± 39	143 ± 34	8.8

Rate of D-glucose removal estimated from the rate of decrease of nonvolatile radioactivity in the incubation medium. Animals from a single batch were acclimated to the test salinity indicated. Initial D-glucose concentration of medium: 5.0 µM. Each line represents the average uptake rate (± SD) for N = 7 experiments (animals).

[a] Uptake values standardized according to Eq. (2).

[b] The experimental error was determined in each experiment as the standard error of estimate for linear regression analysis.

Table 6. D-glucose influx as function of acclimation and incubation salinity

Acclimation salinity (% ASW)	Incubation salinity (% ASW)	D-glucose concentration (µM)	D-glucose influx (fmol mg^{-1} min^{-1})
35	35	25	84 ± 26
65	35	25	217 ± 25
65	65	25	358 ± 35
35	35	25	63 ± 36
65	35	25	382 ± 41
65	65	25	226 ± 27
35	35	25	13 ± 27
35	65	25	48 ± 25
65	65	25	538 ± 56
35	35	25	31 ± 29
35	65	25	39 ± 27
65	65	25	446 ± 46

Each group of 3 lines are from experiments on a single batch of animals, and are therefore directly comparable. Standard error of estimate for flux values is shown.

Table 7. Na^+-dependence of D-glucose influx

Salinity (% ASW)	Na^+ concentration of incubation medium (µM)	D-glucose concentration (µM)	D-glucose influx (fmol mg^{-1} min^{-1})
65	0[a]	1.0	4.1 ± 1.5
65	37[a]	1.0	16.8 ± 2.5
65	306	1.0	35.9 ± 3.1
65	0[a]	1.0	1.2 ± 1.5
65	306	1.0	33.3 ± 2.9
100	56[a]	1.0	38.7 ± 5.2
100	470	1.0	40.7 ± 3.2

Each group of data are from experiments on a single batch of animals.

[a] Na^+ partially or fully replaced by choline.

Table 6 shows that animals acclimated to 65% ASW, but incubated in 35% ASW medium, retain an elevated D-glucose influx characteristic of the higher salinity, at least for the duration of the experiment (4 min). Conversely, animals transferred from 35 to 65% ASW do not instantaneously adjust to the higher influx level of 65% ASW. Therefore, D-glucose influx takes some time to adjust to the salinity of the incubation medium. To check this conclusion, supplementary experiments were performed by following the disappearance of nonvolatile labeled material in the medium. Each animal was tested at the two salinities in consecutive experimental periods. Throughout the 160-min incubation in the ultimate salinity, the uptake rate did not change noticeably, as the time-course remained linear.

Na^+-Dependence of Influx

Table 7 demonstrates that a complete removal of Na^+ from the incubation medium (and replacement with $choline^+$) completely eliminates D-glucose influx, whereas a partial substitution is less effective. Na^+-dependence of D-glucose transport is characteristic of a variety of epithelia (Schultz & Curran, 1970; Schultz, 1977). In mammalian tissues such as the small intestinal epithelium and proximal kidney tubule, the electrochemical potential gradient for Na^+ across the apical membrane is widely believed to constitute the driving force of active D-glucose transport. Although unequivocal Na^+ dependence of epidermal D-glucose influx is shown in Table 7, there is as yet not evidence that the same mechanism operates in the apical membrane of *Nereis* epidermis as in the well-studied mammalian epithelia.

Effect of Metabolic Inhibitors

Preincubation of animals for 30 min in 65% ASW with 5 mM iodoacetate and 5 mM KCN, followed by incubation with 5 µM D-[6-^{3}H]glucose in 65% ASW containing the same inhibitors, was performed to test the dependence of D-glucose transport on cellular energy metabolism. The substances are known inhibitors of glycolysis and oxidative phosphorylation. D-glucose influx was reduced from a control value of 144 ± 8 fmol mg^{-1} min^{-1} to 71 ± 11 fmol mg^{-1} min^{-1} (all animals taken from same batch), but it remained significantly above zero. The partial inhibition may be due to an incomplete blocking, or to D-glucose influx being partially independent of cellular metabolism. As a further complication, *Nereis* is known to shift easily to anaerobiosis (Schöttler, 1978).

Table 8. Net D-glucose uptake

Salinity (% ASW)	Phlorizin concentration (μM)	Initial D-glucose conc. (μM)	Final D-glucose conc. (μM)	Average net flux (fmol mg^{-1} min^{-1})	No. of expts.
35	0	0.71 ± 0.09	0.72 ± 0.06	–	20
65	0	0.72 ± 0.12	0.22 ± 0.12	34.7	20
65	0	0.95 ± 0.10	0.43 ± 0.11	34.9	6
65	0	0.86 ± 0.17	0.47 ± 0.22	27.1	6
65	0	1.13 ± 0.11	0.67 ± 0.14	33.2	6
65	100	0.90 ± 0.05	1.08 ± 0.21	–	7
65	100	1.05 ± 0.09	1.05 ± 0.43	–	7

Animals incubated for 120 min in 2.5 ml ASW. The rate of net D-glucose uptake determined from the difference between initial and final concentrations (given as mean ± SD). No control was included to check for possible hydrolysis of phlorizin during the extended incubation.

Table 9. Influx other monosaccharides than D-glucose

Substrate	Substrate concentration (μM)	Salinity (% ASW)	Influx (fmol mg^{-1} min^{-1})	Reference level (fmol mg^{-1} min^{-1})
D-mannose	5.0	50	−2.9 ± 4.4	16[a]
D-galactose	5.0	50	7.8 ± 4.5	16[a]
3-O-methyl-D-glucose	5.0	35	2.6 ± 3.5	19[b]
3-O-methyl-D-glucose	5.0	65	8.5 ± 4.7	108[b]
L-glucose	5.0	65	−1.6 ± 6.3	108[b]
2-deoxy-D-glucose	5.0	65	4.5 ± 4.6	108[b]
α-methyl-D-glucose	5.0	65	56.1 ± 19.9	108[b]

[a] Expected D-glucose influx, based on a single experiment, not a simultaneous control.
[b] Expected D-glucose influx based on several experiments under similar conditions (weighted average of data in Table 5).

Net Uptake of D-*Glucose*

None of the data presented provide evidence on the net transport of D-glucose by the epidermis, since D-glucose outflux is not accounted for. Although D-glucose outflux from the epidermal transport pool appears to be minimal (*see* washout experiments), there still is a possibility of significant outflux from the extracellular fluid along a paracellular route. Table 8 shows that D-glucose net flux in 65% ASW is of the same order of magnitude as influx under comparable conditions. Therefore, outflux of D-glucose along all possible routes is modest. No significant net uptake was found in 35% ASW, or in 65% ASW when 100 μM phlorizin was added to the incubation medium. Both results are in agreement with the tracer experiments presented above.

Epidermal Influx of Various Monosaccharides

Table 9 shows the results of influx studies performed on various hexoses. Only one of the sugars included in the survey, α-methyl-D-glucopyranoside, is transported by *Nereis* epidermis at the concentration tested; influx is seen to be close to that normally experienced by D-glucose, taking into account the normal variability of D-glucose influx.

α-methyl-D-glucopyranoside also is an able inhibitor of D-glucose influx, as shown by the results of a sample experiment: When tested at 1 μM D-glucose in 65% ASW, 100 μM of the compound reduced D-glucose influx from the control value 54 ± 4 to 16 ± 2 fmol mg^{-1} min^{-1}. α-methyl-D-glucopyranoside is not a substrate for hexokinase (Dixon & Webb, 1979), and the compound may therefore be used as a nonmetabolizable analog of D-glucose in further studies.

Table 9 also shows that D-glucose transport into *Nereis* epidermis is highly stereo-specific, since L-glucose influx is insignificant.

Discussion

The realization that dissolved organic matter plays an important role in the trophic structure of the marine environment forms the background of the present work. There is now increasing evidence, that integumentary absorption of dissolved organic material by

'soft-bodied' marine invertebrates cannot be neglected as a source of nutriment, and efforts in many laboratories are directed towards quantifying parenteral feeding under environmentally relevant conditions (Jørgensen, 1976). A number of papers deal with the integumentary exchange of D-glucose in various groups of nonparasitic metazoan invertebrates (*Coelenterata:* Stephens, 1962; Yerokhin, 1971; Schlichter, 1975. *Annelida:* Ernst & Goerke, 1969; Ahearn & Gomme, 1975. *Mollusca:* Pequignat, 1973; Schulte, Clark & Lawrence, 1973; Bamford & Gingles, 1974; Swift, Conger, Exler & Lakshmanan, 1975. *Echinodermata:* Ferguson, 1967; Fontaine & Chia, 1968. *Pogonophora:* Southward & Southward, 1970). Little is known, however, about the physiological mechanisms, and it is not clear to which extent epidermal D-glucose uptake in marine invertebrates relies on functional principles already known from other animal tissues. Any such investigation of mechanisms should depart from a thorough understanding of the *in vivo* situation.

Uptake of exogenous D-glucose by intact *Nereis diversicolor* was studied by three complementary techniques: (1) Brief incubation in media with radioactive D-glucose allowed for determination of initial fluxes. Due to the low rate of membrane transport, and to the persistence of a 'surface compartment' under conditions not compromising initial-rate measurements, the precision of flux estimates cannot be regarded high. Adding to this problem is a pronounced batch-to-batch variability, and even variation within the same group of animals kept together under standardized conditions. Initial fluxes were not determined in a way likely to represent a steady state of the transporting epithelium; this is partly due to the animals being preincubated in a D-glucose free medium, so that the intraepidermal D-glucose concentration is probably rising throughout incubation. (2) Experiments on removal of nonvolatile labeled material from a medium with radioactive D-glucose may give a better estimate of the steady-state rate of D-glucose uptake, due to the longer time-scale in this kind of experiment. Initial fluxes are not *a priori* obtainable this way, but the slow washout of nonvolatile radioactive material suggests that the 'removal' and 'influx' procedures within experimental error should give fairly identical results, provided membrane transport is the rate-limiting event. (3) Net D-glucose uptake was determined by following the disappearance of D-glucose from the medium, using a specific and sensitive chemical assay. Methods (2) and (3) only could be applied to low D-glucose concentrations (< 5 µM), since the change in medium concentration would otherwise be too slow. All three methods are subject to the limitation of the D-glucose concentration at the transporting membrane not being accurately known due to the presence of 'unstirred' layers.

Identity of the Rate-Limiting Barrier

The study of D-glucose uptake by animal cells, using tracer methods, often requires extremely brief incubations, if the characteristics of the membrane barrier are to be revealed. Often, the distinction between the rate of membrane transfer and the rate of metabolic conversion of the translocated substrate is not carried out successfully (e.g., *see* Graff, Wohlhueter & Plagemann, 1978). There is no *a priori* reason why the intercept of uptake curves (Fig. 2) or washout curves (Fig. 3) could not result from rapid accumulation of D-glucose in, or elimination from, an epidermal compartment. The present paper, however, presents compelling evidence in favor of the intercepts corresponding to material peripheral to the outermost epidermal cell membrane: (1) Intercepts are unchanged after application of inhibitors (phlorizin, harmaline), which in other epithelia selectively block D-glucose transport at the apical membrane. The same is true for a less specific inhibitor (phloretin), which also depresses epidermal D-glucose uptake. (2) In double-label experiments with D-[6-^{3}H]glucose and D-[1-^{14}C]mannitol, intercepts are identical, except for a slight systematic difference probably due to establishing a concentration gradient of D-glucose in structures outside the apical membrane. (3) Manipulating the rate of uptake by varying D-glucose concentration and salinity of the medium, reveals no correlation between either of these variables and the magnitude of the intercept. (4) Although the figures are not included in this paper, substitution of Na^+ in the incubation medium did not change the intercepts of uptake curves used for flux estimation (Table 7), and uptake studies with other hexoses (Table 9) all gave intercepts equivalent to those obtained for D-glucose. Thus, the data fulfill the criteria set up by Heichal, Ish-Shalom, Koren and Stein (1979) for a true extrapolation of the uptake curve to zero time.

Rinsing of animals after incubation removes radioactive material equivalent to 50–100 µl of incubation medium with a half-time of about 1 sec (data not shown). Part of the 0.5–5 µl remaining after the 5-sec rinse may also be due to the cuticle or the overlying mucus layer entrapping some labeled substrate. The duration of the rinse was balanced so as to limit the fraction of extracted radioactivity originating from outside the transport pool, and at the same time minimizing the loss of labeled metabolic products (^{3}HHO) from the epidermal cells.

The slope of uptake curves evidently must represent processes in the epidermis or deeper tissues. Ex-

ogenous D-glucose is taken up through the apical membrane, part of it is metabolized, and the sequestered material (including metabolic products) subsequently may pass the basolateral membrane and appear in the extracellular spaces or in the various body tissues (Ahearn & Gomme, 1975). On this basis, how can the slope of the uptake curve be interpreted?

Biber and Curran (1970) discuss a similar problem pertaining to Na^+ transport in frog skin epithelium. (With this substrate, metabolic alteration obviously is out of the question.) In their experiments, the intercepts were zero because the authors introduced an accurate correction by means of an extracellular marker. In a mathematical analysis of the relevant kinetic model, they came to the general conclusion that, provided the time-course curve is linear in the investigated interval, the slope always represents unidirectional influx across the outermost epithelial membrane.

In the present experiments on *Nereis,* a nonzero intercept is the rule. However, there is strong evidence that the intercept is not due to material accumulated within or below the epidermis. Therefore, it is tempting to extend the conclusion reached by Biber and Curran (1970) to D-glucose uptake by *Nereis* integument: If the linearity of the uptake curve is assured, and if the epidermis is the site of substrate accumulation, the slope is an accurate measure of unidirectional influx across the apical epidermal membrane.

That uptake is in fact linear up to about 10 min was already shown by Ahearn and Gomme (1975), and linearity was also assured in every experiment of the present paper. In addition, Ahearn and Gomme (1975) provided evidence that exogenous D-glucose amasses in the epidermis, since phosphorylation of the compound proceeds without the delay attributable to mixing within an extracellular pool, and since labeled material recovered from the extracellular fluid was only a small fraction of the total amount extractable. Consequently, the apical epidermal membrane emerges as a strong candidate for the rate-limiting barrier.

Unfortunately, one reservation must be made with respect to this inference: The rapid metabolization of D-glucose upon entry into the epidermis opens the possibility of a biochemical transformation, rather than membrane transport, being rate-limiting. However, Biber and Curran's (1970) argument, based on a formal compartmental analysis, applies equally well for membrane transport and biochemical reactions. If a substrate transformation is in fact rate-limiting, it must be intimately associated with uptake of D-glucose into the cell, so that only a negligible pool is present 'between' membrane translocation and the decisive metabolic reaction. This seems a remote possibility, since the nonmetabolizable D-glucose analog, α-methyl-D-glucopyranoside, is transported almost as fast as D-glucose, showing membrane transport to be functionally dissociated from cellular metabolism. That the analog in fact uses the D-glucose transport system(s) as a transmembrane vehicle is indicated by its ability to inhibit D-glucose influx. Thus, the present paper strongly suggests that the slope of uptake curves as in Fig. 2 represents the unidirectional influx of D-glucose across the apical epidermal membrane, not the rate of metabolic trapping of transported substrate.

The intra-epidermal D-glucose concentration is unknown. It is certainly not very high, as exogenous D-glucose is metabolized rapidly after entering the epidermal pool (Ahearn & Gomme, 1975). Assigning – for the purpose of illustration – an arbitrary value of 1 μM, and assuming D-glucose to be uniformly distributed within 10 cm^2 epidermis of 5 μm depth, leads to an estimated pool size of about 5 000 fmol, a fair amount as compared to the influxes reported (Fig. 5).

Unstirred Layers. Role of the Cuticle

A layer of unstirred medium in contact with the tissue often is a problem in studies of epithelial transport, since the supply of substrate to the transport sites is restricted (Dietschy, Sallee & Wilson, 1971). As a consequence, the substrate concentration 'seen' by the membrane is lower than that prevailing in the bulk medium. In the influx experiments, the incubation medium was never stirred mechanically, although the animals by moving about in the vessel produce some convection. Figure 4 suggests that, during transport, a concentration gradient of substrate is established in a region just outside the epidermis; this region probably includes the cuticle and mucus layer, as well as an unstirred layer of medium.

As an alternative interpretation of the experiment, the deviation of the points of Fig. 4(*B*) from the line of identity may be due to cellular accumulation of D-glucose during the brief rinsing period. Estimates based on the data in Tables 4 and 5 suggest that cellular uptake is insufficient to explain the observed effect: The concentration of D-glucose just outside the apical membrane then should be virtually unchanged throughout the 5-sec rinse, which contrasts with previously mentioned observations. If, however, D-glucose should not be rapidly removed from a thin layer outside the apical membrane, this only can be due to a low D-glucose mobility in this region, i.e. a potential for creating concentration gradients during net transport.

The assumption of a concentration gradient of D-[6-^{3}H]glucose being established in the vicinity of

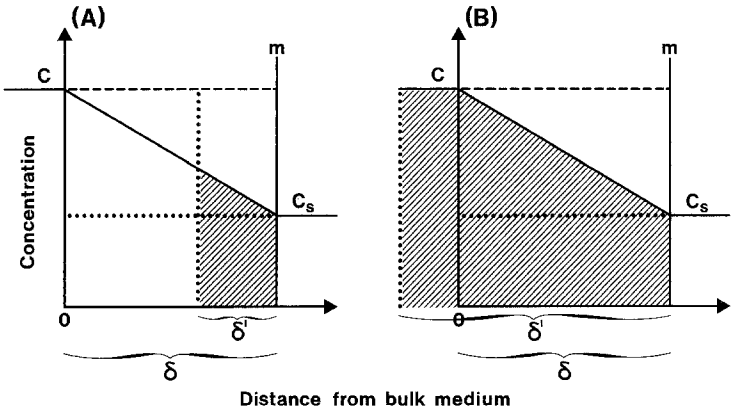

Fig. 6. Simple geometrical model (with reference to Fig. 4) to estimate the D-glucose concentration (C_s) just outside the apical epidermal membrane (m) under steady-state conditions, the presence of microvilli and cuticular material being neglected. C is the D-glucose concentration in the bulk incubation medium; δ is the operational thickness of the unstirred layer, which is assumed to be homogenous, so that the same diffusion coefficient applies throughout; δ' is the thickness of the layer left on the animal surface after rinsing. The hatched area represents the amount of D-[6-^{3}H]glucose (Q_{glu}) in the unstirred layer when transport across m is operative. The area of the large rectangle under the broken line and to the right of the vertical dotted line represents the amount of D-[6-^{3}H]glucose when transport is blocked by phlorizin, equivalent to the amount of D-[1-^{14}C]mannitol (Q_{man}). All material to the left of the vertical dotted line is removed during rinsing. Note, that δ is an operational quantity, as defined by Dainty and House (1966); the formalism is useful, although actual concentration profiles are not linear between a solid surface and the bulk medium. (*A*) The concentration gradient extends into the material removed by rinsing, and simple geometry shows C_s to be:

$$C_s=\frac{Q_{glu}/Q_{man}-f/2}{1-f/2};\ f=\delta'/\delta.$$

(*B*) The rinsing procedure does not remove material from the region of the concentration profile, and

$$C_s=(1-2f(1-Q_{glu}/Q_{man}))C.$$

The two equations define C_s as a smooth function of C in the unknown parameter f

the transporting apical membrane further is supported by evidence presented in the following paper (Gomme, 1981).

Fick's law describes the diffusional flux of a substance in a homogenous medium as a function of the concentration gradient. Assume that the superficial diffusion-restraining layer is a slab of constant thickness throughout the plane surface of the epidermis, and that this layer is homogenous with respect to the diffusion coefficient of D-glucose; obviously, this is an oversimplication, justified only for illustrative purposes. If D-[6-^{3}H]glucose is neither produced nor consumed in this region, the concentration gradient in the stationary state will be constant at all depths (Sten-Knudsen, 1978), resulting in a linear concentration profile.

As shown in Fig. 6, a range of possible D-glucose concentrations at the apical membrane (C_s) may be determined from this simple model, given the results of Fig. 4. Using the values $C=5.0$ μM and $Q_{glu}/Q_{man}=0.70$, and considering all possibilities with respect to the part of the diffusion-restraining layer actually removed by rinsing, we obtain for the permissible f-values: $0<C_s<3.5$ μM. Thus, the D-[6-^{3}H]glucose concentration at the transporting membrane under these conditions is at least 1.5 μM, or 30%, lower than the bulk phase concentration.

By neglecting for the moment the effect of the cuticle and the mucus, an estimate of the operational thickness, δ, of the unstirred water layer may be obtained from Fick's law:

$$\delta=(D/J)(C-C_s). \tag{3}$$

D is the diffusion coefficient for D-glucose in dilute aqueous solution, 0.67×10^{-5} cm sec^{-1} (Weast, 1969). D-glucose influx, J, is taken as 100 fmol mg^{-1} min^{-1} (*cf.* Fig. 5), or 5.0×10^{-14} mol cm^{-2} sec^{-1} for a 300 mg animal with 10 cm^2 surface. For the above values of C and C_s we obtain: $0.20<\delta<0.67$ cm. Thus, assuming a negligible diffusion-restraining effect of the cuticle and mucus, the unstirred water layer at the integumentary surface should be at least 0.20 cm thick.

The half-time, $t_{\frac{1}{2}}$, for diffusional equilibration in a layer of thickness δ is (Dainty & House, 1966):

$$t_{\frac{1}{2}}=0.38\ \delta^2/D. \tag{4}$$

Using $\delta>0.20$ cm, we obtain $t_{\frac{1}{2}}>2\,200$ sec. This value clearly is unrealistic, since the equilibration of the 'surface compartment' with the environment is complete well within the first minute (*see also* Fig. 3). Consequently, the actual D of the 'unstirred layer' must be smaller than that applicable to diffusion in water, since this would be the only way to achieve

a high peripheral diffusion resistance (δ/D) at the same time as a low $t_{\frac{1}{2}}(\delta^2/D)$. A numerical example will illustrate this point: Assuming $t_{\frac{1}{2}}=5$ sec, $\delta=2$ μm (the cuticle thickness), and a partition coefficient of unity, we obtain: $D=3\times10^{-9}$ cm sec^{-1}. Probably, the cuticle is responsible for a large part of the peripheral diffusion resistance (*see also* Gomme, 1981).

Characteristics of D-*Glucose Transport across the Apical Membrane*

Comparable results were obtained whether D-glucose influx was measured directly as in Fig. 2, or estimated from the rate of D-glucose removal from the medium. This agreement is understandable in the light of outflux from the epidermal transport pool being minute, as illustrated by the washout experiments. Net uptake of D-glucose, at least from concentrations close to 1 μM, is of the same order of magnitude as influx, and thus the sum of D-glucose outflux to the medium from epidermal and extracellular pools must be small. Investigations in this laboratory suggest that D-glucose concentration in the natural habitat of *Nereis* diversicolor is also of the order of magnitude of 1 μM (Minck & Olesen, *personal communication*), and the animals therefore are likely to experience a net integumentary D-glucose uptake in nature, supplementing food intake via the alimentary tract. In addition, the capacity of D-glucose absorption, taken together with the high peripheral diffusion resistance, may be seen as a way of reducing D-glucose leakage from the animal surface (Gomme, 1981).

The D-glucose transport system(s) responsible for this uptake is highly substrate-specific. Of six hexoses tested as alternative substrates only one, α-ethyl-D-glucopyranoside, showed a significant uptake. D-glucose transport systems in animal cell membranes tend to fall into two categories: (1) The accumulating systems such as in the apical membrane of vertebrate small intestinal and proximal tubular epithelia, and (2) the systems in epithelial basolateral membrane and in apolar cells. The test substrates were so chosen that all, except L-glucose, would be transported to some extent by at least one of the type systems. The failure to demonstrate transport of some of these substances by *Nereis* epidermis does not, however, necessarily imply a lack of reactivity with the transport site(s) in the apical membrane. It may be due to the substrate concentration being too low to effect an uptake that may be visible at higher, environmentally unrealistic, concentrations. The substrate-specificity of D-glucose transport in *Nereis* epidermis is presently the subject of further investigation in this laboratory.

D-glucose influx across the apical membrane clearly is Na^+-dependent, which also is a well-known feature of apical D-glucose transport in mammalian small intestinal and proximal tubular epithelia, but distinct from transport across the basolateral membrane and in nonepithelial cells (Kinne, 1976; Schultz, 1977; Wilson, 1978; Ullrich, 1979). Interestingly, phlorizin is a more effective inhibitor of D-glucose influx than the aglucone, phloretin. This pattern is also found in apical plasmalemma of the mammalian epithelia, but in the basolateral one and in apolar cells, the relative potency of the two inhibitors is about reversed (Stein, 1967; Bihler & Cybulsky, 1973; Murer, Hopfer, Kinne-Saffran & Kinne, 1974; Kimmich & Randles, 1975; Kleinzeller, Dubyak & Mullin, 1976). Harmaline, which is believed to interact with the Na^+-site on the D-glucose carrier in the small intestine (Sepulveda & Robinson, 1974, 1975), also blocks D-glucose transport into *Nereis* epidermis, whereas cytochalasin B, a distinct inhibitor of D-glucose transport in apolar cell types (Czech et al., 1973) does not act on *Nereis* apical membrane. The sum of this evidence strongly points towards a molecular similarity between the D-glucose transporter in the apical membrane of *Nereis* epidermis and in the epithelia mentioned. This also would imply, that the Na^+-dependency of the *Nereis* system is due to a Na^+ electrochemical potential gradient propelling D-glucose across the apical membrane, although the energetics of transport has not yet been explored in this species. The ability of α-methyl-D-glucopyranoside to replace D-glucose is additional evidence in favor of the assumed molecular resemblance, since this analog is the most effective replacement of D-glucose in Na^+-dependent transport (Silverman, 1976).

Concentrative D-glucose transport into the Cestodan (tapeworm) tegument similarly is Na^+-dependent and phlorizin-sensitive (Pappas & Read, 1975), and *Nereis* epidermis possibly explore the same molecular machinery as these parasitic forms and mammalian nutrient-absorbing epithelia. Remarkably, another group of gutless intestinal parasites, the Acanthocephalans, utillizes a nonconcentrative tegumentary D-glucose transport that is neither Na^+-dependent nor phlorizin-sensitive (Starling & Fisher, 1975). For the Trematodes, there is conflicting evidence: D-glucose transport through the apical epidermal membrane in the liver fluke *Fasciola hepatica* is Na^+-and phlorizin-insensitive (Isseroff & Read, 1974), whereas the opposite is true for 2-deoxy-D-glucose transport in *Schistosoma mansoni*, the blood fluke (Uglem & Read, cited by Pappas & Read, 1975).

In spite of the qualitative similarity between D-glucose transport in *Nereis* epidermis and in the concentrative systems mentioned, area-specific fluxes in *Nereis* epidermis might be expected to be smaller than in the classical nutrient-absorbing epithelia. The max-

imal D-glucose influx observed in *Nereis* acclimated to 65% ASW was about 5000 fmol mg^{-1} min^{-1}, corresponding to about 3 pmol cm^{-2} sec^{-1}, or 0.5 pmol cm^{-2} sec^{-1} when expressed on the basis of the true area of folded apical membrane (*cf.* Fig. 1). Surprisingly, this value is compatible with that cited by Hopfer (1978) for maximal D-glucose influx across intestinal brush-border membrane (1.3 pmol cm^{-2} sec^{-1}). The possibility appears that the larger overall transport capacity of the small intestine as compared to *Nereis* epidermis may result from the more extensive folding of the apical membrane in the former tissue.

Epidermal D-glucose influx is significantly smaller in *Nereis* acclimated to a low salinity (35% ASW), than in the higher salinity (65% ASW). A similar salinity-dependence has been described for the uptake of glycine by *Nereis* (Stephens, 1964). The present paper shows the salinity dependence of D-glucose influx to result from a regulatory action of the epidermis on part of the D-glucose transport system, rather than from the Na^+-dependence of transport. Whereas the effect is acutely manifest when removing Na^+ from the incubation medium, a change in medium salinity takes some time to effect a corresponding adjustment of D-glucose influx. The time-course is not clear from the present data, but it extends over hours rather than minutes. Furthermore, the Na^+ concentration in the lower salinity (35% ASW) is 165 mM, much too high to reduce influx by itself. The salinity range corresponding to a depressed D-glucose influx (<45% ASW, data not shown) is coextensive with that of osmoregulatory net NaCl uptake by the epidermis (Potts & Parry, 1964). The conclusions by Stephens (1964), that osmoregulation and epidermal amino acid uptake in *Nereis* are mutually exclusive thus is extended to D-glucose, but the physiological basis of the phenomenon remains obscure.

The data on concentration dependence of D-glucose influx at various salinities do not form a sufficient basis for characterizing the D-glucose transport system(s) kinetically. This is due in part to the complex morphology of the apical aspect of the epidermis, taken together with the unstirred layer characteristics of the cuticle: The D-glucose concentration at the transporting membrane is not well defined. Also the poor reproducibility of influx measurements (biological variability), and the cellular nonsteady state undoubtedly created during the influx experiments precludes a reasonable kinetic analysis. Consequently, there is no basis for guessing about the number of distinct transport agencies, or their substrate affinity. The preliminary conclusion by Ahearn and Gomme (1975) on this matter apparently rested on undue simplification.

Permeability of the Epidermis to Simple Diffusion

Determination of the rate of D-mannitol uptake provided an estimate of the epidermal permeability to this compound: 8×10^{-8} cm sec^{-1}. This estimate is inaccurate to the extent that the assigned surface area (10 cm^2 for a 300 mg worm) is in error; no measurements of the surface area of *Nereis* are available. The derived permeability, however, is not likely to deviate by more than a factor of 2–3 from the true value. The D-mannitol permeability, therefore, is of the same order of magnitude as that known for 'tight' epithelia such as frog skin (2.5×10^{-7} cm sec^{-1}; Mandel & Curran, 1972) and toad urinary bladder (1.9×10^{-7} cm sec^{-1}; Wright & Pietras, 1974).

D-mannitol does not react with transport proteins in animal cell membranes, and it therefore must cross the epidermis by simple diffusion. Due to its physical similarity to D-glucose, it may be used as an analog of simple D-glucose diffusion, which may occur along either of two pathways: Across the epidermal cells via the apical membrane, or along the intercellular junctions. Bimolecular lipid membranes tend to have D-glucose permeabilities around 4×10^{-8} cm sec^{-1} (Jain, 1972), although values down to 10^{-10} cm sec^{-1} have been reported (Wood, Wirth & Morgan, 1968). The presence of microvilli in the apical membrane increases the surface area of *Nereis* epidermis by a factor of about 5 (*cf.* Fig. 1), and it may be possible to ascribe most or all of the observed epidermal D-mannitol permeability to the plasma membrane. On the other hand, the data set no limits to the part of the diffusional permeability that must be referred to the intercellular junctions.

If the assumption of a low intraepidermal D-glucose concentration is correct (*see above*), diffusional D-glucose loss through the lipid part of the apical membrane can only be modest. To this must be added, however, the outflux through the D-glucose carrier(s), which must be thermodynamically reversible. The rapid phosphorylation of D-glucose upon entering the cell (Ahearn & Gomme, 1975) may be seen as a way of reducing loss of the compound to the outside. A saving of D-glucose probably would result from the cells investing metabolic energy on the transport system. If the Na^+-dependency of D-glucose transport is taken to imply the validity of the Na^+-gradient hypothesis, the ratio between D-glucose influx and outflux can be derived from the D-glucose concentration ratio across the apical membrane, from the electrochemical potential gradient of Na^+, and the coupling ratio between D-glucose and Na^+. No data are presently available for this analysis, but there is no basis for fully neglecting outflux through the transport sites. In fact, if the Na^+-gradient hypothesis

applies, and the coupling coefficient is close to unity (Goldner, Schultz & Curran, 1969), this would set a limit to the intraepidermal D-glucose concentration, if D-glucose losses through the apical membrane are not to be unacceptably high.

An even greater latitude exists in evaluating the diffusional leakage across the intercellular junctions. The junctional complexes of *Nereis* epidermis consists of zonula adhaerens and septate desmosomes, of which the latter are most likely to serve as an occluding girdle. There has been some ambiguity about the ability of the septate junctions to prevent bypass diffusion in the intercellular cleft (McNutt & Weinstein, 1973). The data on D-mannitol influx in the present paper demonstrates that the junctional complex of *Nereis* epidermis is at least as effective as the zonula occludens (tight junction) of vertebrates in maintaining a low paracellular permeability to small nonelectrolytes.

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