Selective Monosaccharide Transport through Lipid Bilayers Using Boronic Acid Carriers

Pamela R. Westmark, Stephen J. Gardiner, and Bradley D. Smith*

Contribution from the Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, Indiana 46556

Received April 17, 1996[⊗]

Abstract: Twenty-one boronic acids were studied for their ability to transport saccharides in and out of liposomes. The rates of liposome efflux were determined using an enzymatic assay, whereas the influx studies used a radiotracer method. All boronic acids examined, except those that were highly hydrophilic, facilitated monosaccharide transport. The order of transport selectivity was sorbitol > fructose > glucose. The disaccharides maltose and sucrose were not transported to any significant degree. Facilitated transport was demonstrated with a variety of liposome types, including multilamellar and unilamellar vesicles with anionic or cationic polar lipid additives. Transport mechanism studies included the accumulation of structure−activity data, as well as systematic investigations of various environmental changes such as pH, added salt, membrane potential, and temperature. Overall, the evidence is strongly in favor of a membrane carrier mechanism. The boronic acid combines reversibly with a diol group on the monosaccharide to produce a tetrahedral, anionic boronate, which is the major complexed structure in bulk, aqueous solution. At the bilayer surface, the tetrahedral boronate is in equilibrium with its neutral, trigonal form, which is the actual transported species. At low carrier concentrations, a first-order dependence on carrier was observed indicating that the transported species was a 1:1 sugar−boronate. At higher carrier concentrations the kinetic order approached 2, suggesting the increased participation of a 1:2 sugar−bisboronate transport pathway. The effect of boronic acids on liposomal bilayer fluidity was probed by fluorescence spectroscopy using appropriate reporter molecules. Adding cholesterol to the liposome membranes reduced translational fluidity by "packing and ordering" the bilayer. Addition of lipophilic arylboronic acids (either free or complexed with monosaccharides) induced a similar but smaller effect.

Introduction

The selective transport of saccharides, such as glucose, across cell membranes is a ubiquitous cellular activity. A typical human red cell, for example, contains around 200 000 glucose transport molecules.[1] It is well recognized that defects in these transport processes can lead to serious medical problems.[2] As a consequence, the mechanisms of biotic sugar transport systems are currently under active investigation.[3] While much structural data has been accumulated, the kinetic picture is still largely unknown.[4] A recent review concluded that, "Virtually nothing is known at present about the molecular mechanism of glucose transport or the transport of any solute across the membrane".[3a]

As part of an interdisciplinary research effort to understand carbohydrate recognition, chemists have started designing artificial sugar receptors. There are two primary goals for this work. The first is to treat the artificial systems as simplified, small molecular weight models whose properties can be probed in a systematic manner. This provides an experimentally accessible way of exploring some of the fundamental questions in sugar recognition. Alternatively, there is the possibility of developing the artificial sugar binders into practical molecular devices.

Artificial sugar receptors can be divided into two groups, those that mimic natural binding systems in the sense that they utilize noncovalent bonding interactions such as hydrogen bonding and those that use non-natural bonding interactions.[5] If the goal is to develop an artificial receptor that operates in water, then an approach based primarily on hydrogen bonding is likely to be very challenging due to competitive bonding with the bulk water. As a consequence, a number of groups have chosen to pursue systems that use non-natural bonding interactions. Boronic acids are of particular interest as they form reversible covalent complexes with sugars in aqueous solution.[6−10] Association constants are typically between 10 and 10^4 M^{-1}. A range of boronic acid receptors have been developed with the capability of molecular functions such as selective binding,[6] allosteric binding,[7] reaction catalysis,[8] chemosensing,[7,9] and membrane transport.[10] This report focuses on membrane

[⊗] Abstract published in *Advance ACS Abstracts,* November 1, 1996.

(1) Widdas, W. F. *Biochim. Biophys. Acta* **1988**, *947*, 385−404.

(2) White, M. K.; McCubrey, J. A. *Int. J. Oncol.* **1995**, *7*, 701−712.

(3) (a) Mueckler, M. *Eur. J. Biochem.* **1994**, *219*, 713−725. (b) Baldwin, S. A. In *Molecular Aspects of Transport Proteins;* De Pont, J., Ed.; Elsevier: New York, 1992; Chapter 6. (c) Quiocho, F. A. *Curr. Opin. Struct. Biol.* **1991**, *1*, 922−933.

(4) (a) Schirmer, T.; Keller, T. A.; Wang, Y.-F.; Rosenbusch, J. P. *Science* **1995**, *267*, 512−514. (b) Vyas, M. N.; Vyas, N. K.; Quiocho, F. A. *Biochemistry* **1994**, *33*, 4762−4768.

(5) Recent reports of artificial sugar receptors that use noncovalent interactions: (a) Coterón, J. M.; Hacket, F.; Schneider, H.-J. *J. Org. Chem.* **1996**, *61*, 1429−1435. (b) Jiménez-Barbero, J.; Junquera, E.; Martín-Pastor, M.; Sharma, S.; Vicent, C.; Penadés, S. *J. Am. Chem. Soc.* **1995**, *117*, 11198−11204. (c) Bonar-Law, R. P.; Sanders, J. K. M. *J. Am. Chem. Soc.* **1995**, *117*, 259−271. (d) Anderson, S.; Neidlein, U.; Gramlich, V.; Diederich, F. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 1596−1600. (e) Inouye, M.; Miyake, T.; Furusyo, M.; Nakazumi, H. *J. Am. Chem. Soc.* **1995**, *117*, 12416−12425.

(6) Wulff, G. *Pure Appl. Chem.* **1982**, *54*, 2093−3000.

(7) James, T. D.; Samankumara Sandanayake, K. R. A.; Shinkai, S. *Supramol. Chem.* **1995**, *6*, 141−157.

(8) Rao, G.; Phillip, M. *J. Org. Chem.* **1991**, *56*, 1505−1512.

(9) Czarnik, A. W. *Acc. Chem. Res.* **1994**, *27*, 302−308.

(10) (a) Smith, B. D. *Supramol. Chem.* **1996**, *7*, 55−60 and references cited therein. (b) Smith, B. D. In *Chemical Separations with Liquid Membranes*; Bartsch, R. A., Way, J. D., Eds.; ACS Symposium Series Number 642; American Chemical Society: Washington, DC, 1996.

S0002-7863(96)01264-4 CCC: $12.00

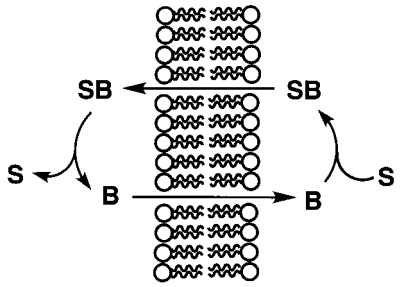


Figure 1. Sugar transport through a lipid bilayer mediated by a boronic acid carrier; S = sugar, B = boronic acid, SB = sugar−boronate complex.

transport, where boronic acids are one of only two classes of artificial compounds that are known to transport sugar derivatives through bilayer membranes.[11] Over the past few years, our group, along with others, has been examining facilitated membrane transport using boronic acid carriers.[10] Initially, the study focused on ways to transport hydrophilic diol-containing compounds through bulk, liquid organic membranes and more recently through polymer-supported liquid membranes.[12] We also examined transport through lipid bilayer membranes and discovered that boronic acids can facilitate the efflux of glucose and ribonucleosides from liposomes.[13,14] In this paper, we describe in full detail our efforts to facilitate the transport of glucose and other saccharides. In short, we have found that boronic acids can selectively transport monosaccarides in and out of liposomes. In addition, we have elucidated the transport mechanism.

Transport Assays

A schematic description of monosaccharide transport through a lipid bilayer mediated by a boronic acid carrier is shown in Figure 1. Both liposome efflux and liposome influx experiments were conducted. Usually a lipid mixture of dipalmitoylphosphatidylcholine (DPPC), cholesterol (C), and egg phosphatidic acid (PA) was used in a ratio of 20:15:2.[15] Compared to unsaturated phospholipids, saturated DPPC forms liposomes that are less permeable to small hydrophilic molecules.[16] In addition, the presence of large amounts of cholesterol within the membrane is also known to decrease permeability.[16] Thus, we expected the liposomes used in this study to be quite stable and relatively impermeable.

Sugar-encapsulated unilamellar vesicles (80 nm diameter) were prepared by the rapid extrusion technique and found to be essentially impermeable to sugar leakage over many hours.[17] Sugar escape from the liposomes was detected spectrometrically using enzymatic assays which produced an NADPH absorption at 340 nm. Generally, the rate of NADPH production was a direct measure of the rate of sugar efflux since the enzymes were unable to penetrate the liposomes, and the rates of sugar efflux were slower than the enzymes turnover rates. This proved to be a straightforward way of monitoring sugar efflux in real time. However, there were a few drawbacks, namely, that some boronic acids inhibited the enzymatic assays making them prohibitively slow, and the low sensitivity required that unnaturally high concentrations of sugar be encapsulated inside the liposomes.

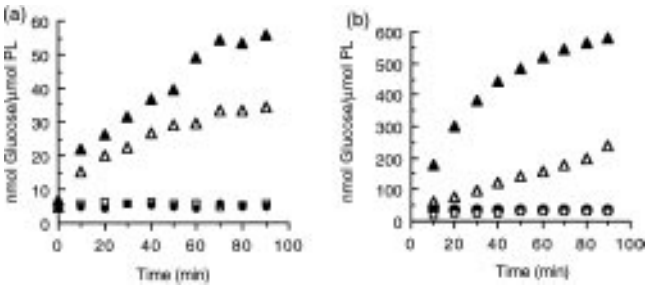


Figure 2. Amount of glucose transported as a function of time. (a) Glucose influx from external solution containing 50 mM glucose into empty liposomes as monitored by radiotracer assay. Transport (at pH 7.5) induced by carriers: ▲ (**3**, 2 mM); △ (**1**, 5 mM); □ (**4**, 5 mM); ● (**8**, 5 mM). Liposome composition DPPC:C:PA, 20:15:2; total lipid concentration 9.4 mM, $T = 25$ °C. (b) Glucose efflux from liposomes containing 300 mM glucose as monitored by enzyme assay. Transport (at pH 7.5) induced by carriers: ▲ (**3**, 1 mM); △ (**1**, 5 mM); □ (**4**, 5 mM); ● (**8**, 5 mM). Liposome composition DPPC:C:PA, 20:15:2; total lipid concentration was 0.75 mM, $T = 25$ °C, PL = phospholipid, reproducibility ±10%.

The enzyme assays were not practical for the influx experiments, therefore a radiotracer method was developed. Empty liposomes with a trace of ^{14}C-labeled DPPC incorporated in the phospholipid membrane were incubated with a sugar solution that contained trace amounts of ^{3}H-labeled sugar and boronic acid carriers. Every 10 min, an aliquot of the mixture was withdrawn and passed through a column of Sephadex gel which separated the liposomal component from the external solution. The ratio of ^{3}H to ^{14}C associated with the liposomal fraction was determined from scintillation counting and considered as a measure of the amount of sugar transported into the liposomes.

A significant difference between these two transport assays is that the efflux experiment results in uphill transport because of the destructive nature of the enzyme assay, whereas the influx experiment simply monitors passive equilibration. Thus, efflux continues until the liposomes are effectively empty of saccharide. Influx, on the other hand, reaches a steady-state position where the electrochemical potential inside the liposome equals that outside (in the absence of a secondary energy source, such as a pH gradient, the final internal and external saccharide concentrations will be equal).

Carrier Effectiveness

The discussion on carrier effectiveness will focus on glucose transport in and out of liposomes; however, it should be noted that similar results were obtained with other monosaccharides (*e.g.*, fructose, sorbitol). The efflux experiments were conducted with a wide range of boronic acids, whereas the influx experiments surveyed a smaller number of carriers. In both cases, the qualitative order for transport enhancement was the same (Figure 2). Examination of the glucose efflux data for carriers **1**−**20**, shown in Table 1, reveals a general pattern that initial transport rates increased as the acidity and lipophilicity

(11) For a remarkable example of artificial channel-mediated glucose transport, see: Granja, J. R.; Ghadiri, M. R. *J. Am. Chem. Soc.* **1994**, *116*, 10785−10786.

(12) Paugam, M.-F.; Bien, J. T.; Smith, B. D.; Chrisstoffels, S.; de Jong, F.; Reinhoudt, D. N. *J. Am. Chem. Soc.* In press.

(13) Westmark, P. R.; Smith, B. D. *J. Am. Chem. Soc.* **1994**, *116*, 9343−9344.

(14) Westmark, P. R.; Smith, B. D. *J. Pharm. Sci.* **1996**, *75*, 266−269.

(15) For discussions on the properties of the bilayer membranes formed by the lipid composition used in this work, see: (a) McMullen, T. P. W.; McElhaney, R. N. *Biochim. Biophys. Acta* **1995**, *1234*, 90−98. (b) Murari, R.; Murari, M.; Baumann, W. J. *Biochemistry* **1986**, *25*, 1062−1067. (c) Mayer, L. D.; Bally, M. B.; Hope, M. J.; Cullis, P. R. *Biochim. Biophys. Acta* **1985**, *816*, 294−302B.

(16) (a) *Liposomes, a Practical Approach*; New, R. R. C., Ed.; IRL Press: Oxford, 1990. (b) Lasic, D. D. *Liposomes; From Physics to Applications*; Elsevier: Amsterdam, 1993.

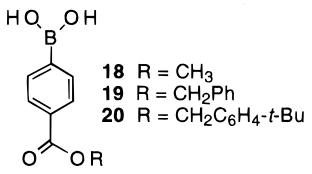
(17) MacDonald, R. C.; MacDonald, R. I.; Menco, B. P. M.; Takeshita, K.; Subbarao, N. K.; Hu, L. *Biochim. Biophys. Acta* **1991**, *1061*, 297−303.

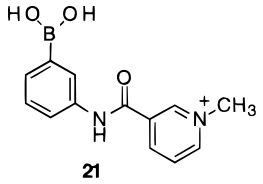
Table 1. Initial Rates of Glucose Efflux at pH 7.5, pK_a Values, and Substituent Hydrophobicity Constants For Various Boronic Acid Derivatives

boronic acid, R−B(OH)$_2$	init rate of efflux[a] (rel rate)	pK_a	sum of π values[b]
phenylboronic acid, **1**	0.4 (1)	8.9[c]	0.0
(3,5-dichlorophenyl)boronic acid, **2**	6 (15)	7.4[d]	1.42
[3,5-bis(trifluoromethyl)phenyl]boronic acid, **3**	12 (30)	7.2[d]	1.76
diphenylborinic acid, **4**	$<10^{-3}$	6.2[d]	2.64
(4-methoxyphenyl)boronic acid, **5**	0.3 (0.8)	9.3[c]	−0.02
(3-methoxyphenyl)boronic acid, **6**	0.3 (0.8)	8.7[d]	−0.02
(4-*tert*-butylphenyl)boronic acid, **7**	12 (30)	9.3[d]	1.98
(4-carboxyphenyl)boronic acid, **8**	$<10^{-3}$	8.4[e]	−4.36
1-butylboronic acid, **9**	1.2 (0.3)	10.4[f]	
boric acid, **10**	$<10^{-3}$	9.0[f]	
(4-bromophenyl)boronic acid, **11**	2.8 (7)	8.6[d]	0.86
(4-fluorophenyl)boronic acid, **12**	0.5 (1.3)	9.1[d]	0.14
(2-methylphenyl)boronic acid, **13**	0.3 (0.8)	9.7[c]	0.56
(3-methylphenyl)boronic acid, **14**	0.5 (1.4)	9.0[c]	0.56
(4-methylphenyl)boronic acid, **15**	0.5 (1.4)	9.3[c]	0.56
(3,5-dimethylphenyl)boronic acid, **16**	1 (2.8)	9.1[d]	1.12
3-pyridylboronic acid, **17**	$<10^{-3}$	4.0[g]	
18	1.5 (3.8)		
19	13 (30)		
20	25 (60)		

[a] Values in nmol of glucose/μmol of PL/min (±15%) induced by 1 mM of boronic acid, PL = phospholipid; total lipid concentration was 0.75 mM. [b] Sum of the hydrophobicity constants for all aryl substituents except the boronic acid (ref 18a). [c] Reference 18b. [d] Determined by the method described in ref 18c. [e] Reference 18d. [f] Reference 18e. [g] Reference 18f.

of the boronic acids increased.[18] The dependence on boronic lipophilicity is most apparent when the fluxes for carriers **18**−**20** are compared. The carriers with more lipophilic ester groups





21

were clearly superior transport agents. The dependence on carrier acidity was harder to demonstrate unambiguously because of the inherent difficulty in delineating the dependence on lipophilicity. Nonetheless, a qualitative survey of Table 1 shows that the more acidic carriers were generally more effective. Perhaps the clearest indication of the importance of carrier Lewis acidity is gained by comparing the transport rates for the equally lipophilic tolyl isomers **13**−**15**. The sterically encumbered ortho derivative **13** had a little over half the transport ability of the meta and para isomers **14** and **15**, respectively. This is consistent with a model where complexation between the sugar and the boronic acid is a prerequisite for transport.

Transport Selectivity

A series of noncompetitive efflux experiments showed a sugar transport enhancement order of sorbitol > fructose > glucose

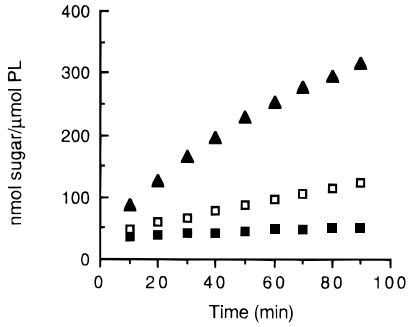


Figure 3. Efflux profiles for liposomes containing 300 mM of ▲ sorbitol, □ fructose, or ■ glucose after treatment with phenylboronic acid (**1**, 1 mM) at pH 7.5. Liposome composition DPPC:C:PA, 20:15:2; total lipid concentration 0.75 mM, T = 25 °C, PL = phospholipid, reproducibility ±10%.

≫ maltose ≥ sucrose (Figure 3).[19] This transport order matches the known order of sugar−boronate complex stabilities (boronic acids favor complexation with compounds containing vicinal *cis*-diols) and is strong evidence in favor of a complexation mediated transport phenomenon.[7,10] To demonstrate the extent of the transport selectivity, competitive influx and efflux experiments were conducted.

The glucose influx experiment described in Figure 2a was repeated except that the "empty" liposomes were incubated with solutions containing equimolar amounts of glucose and sucrose. Each transport experiment involved two concurrent incubations; in one case the glucose included trace ^{3}H-glucose and in the other case the sucrose contained trace ^{3}H-sucrose. When the liposome/glucose/sucrose incubation mixture was treated with boronic acid **1** the amount of glucose associated with the liposomes increased over time in a similar fashion to that shown in Figure 2a. On the other hand, negligible sucrose was incorporated into the liposomes (data not shown). When the nontransporting boronic acid **8** was used, or when boronic acid was omitted, there was no evidence of glucose or sucrose influx. These results are consistent with the selective delivery of glucose (contains a vicinal *cis*-diol) in the presence of weakly complexing sucrose (no vicinal *cis*-diol).

Efflux experiments were conducted with liposomes that encapsulated equal amounts of sorbitol and sucrose. As shown in Figure 4, addition of boronic acid **7** to the liposome mixture resulted in greatly enhanced sorbitol efflux, whereas the sucrose remained untouched (*i.e.*, effectively complete transport selectivity in favor of sorbitol). This efflux result suggests that boronic acids may have hitherto unconsidered therapeutic properties. For example, boronic acids provide a conceptually interesting approach to treating polyol (*e.g.*, sorbitol) accumulation in ocular tissues; a problem that is associated with various types of diabetic complications.[20] Our results suggest that boronic acids may be able to relieve polyol buildup by selectively facilitating cellular efflux.

Transport Mechanism

After successful membrane transport was demonstrated, the first mechanistic question was to prove it was due to a specific sugar complexation event and not to a boronic acid-induced

(18) (a) Chu, K. C. In *Burger's Medicinal Chemistry, Part I. The Basis of Medicinal Chemistry*, 4th ed.; Wolff, M. E., Ed.; Wiley: New York, 1979; p 401. (b) Torsell, K. In *Progress in Boron Chemistry*; Steinberg, H., McCloskey, A. L., Eds.; Pergamon: New York, 1964; p 385. (c) Albert, A.; Serjent, E. P. *The Determination of Ionization Constants*, 3rd ed.; Chapman and Hill: London, 1984. (d) Soundararajan, S.; Badawi, M.; Kohlrust, C. M.; Hageman, J. H. *Anal. Biochem.* **1989**, *178*, 125−134. (e) Babcock, L.; Pizer, R. *Inorg. Chem.* **1980**, *19*, 56−61. (f) Fischer, F. C.; Havinga, E. *Recl. Trav. Chim. Pays.-Bas.* **1974**, *93*, 21−25.

(19) Since boronic acids are also known to form covalent complexes with α-hydroxy acids, we searched for evidence of facilitated transport. Using the UV absorption assay that was developed for nucleoside transport,[14] we found that boronic acids induced large enhancements in the rates of efflux and influx of (*p*-nitrophenyl)glucurinide (data not shown). Therefore, it appears that in addition to monosaccharides and ribonucleosides boronic acids can also be used to transport certain types of α-hydroxy acids.

(20) (a) Burg, M. B.; Kador, P. F. *J. Clin. Invest.* **1988**, *81*, 635−640. (b) Kicic, E.; Palmer, T. N. *Biochim. Biophys. Acta* **1994**, *1226*, 213−218.

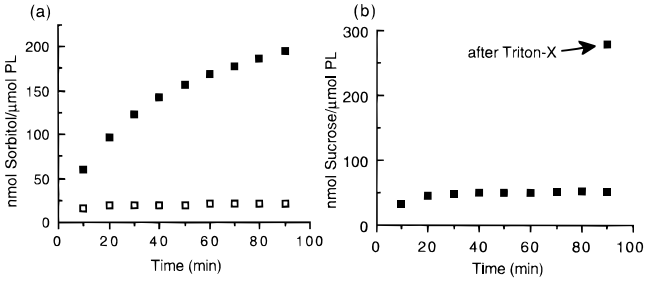


Figure 4. (a) Sorbitol efflux from liposomes filled with 150 mM sorbitol and 150 mM sucrose after treatment with ■ (**7**, 1 mM), or □ (no boronic acid). (b) Sucrose efflux from the same liposome preparation after treatment with ■ (**7**, 1 mM) and then eventually lysing with Triton-X. Liposome composition DPPC:C:PA, 20:15:2; total lipid concentration 0.75 mM, pH = 7.5, T = 25 °C, PL = phospholipid, reproducibility ±10%.

increase in bilayer permeability. Our initial approach was to examine the efflux of various marker compounds that were not expected to be transported under the conditions used to induce monosaccharide transport. In addition to the negligible sucrose transport already discussed, boronic acids **1**−**4** were unable to induce the efflux of anionic monosaccharide derivatives isocitrate, glucose-6-phosphate, and fructose-6-phosphate (as proved by enzymatic assays), as well as the anionic carboxyfluoroscein, calcein (fluorescence assay), and arsenazo III (spectrometric assay).[16a] The structure of the carrier was another variable that was changed in order to gain mechanistic insight. For example, neither excess boric acid nor the extremely hydrophilic boronic acid **8** were capable of inducing monosaccharide influx or efflux. Overall, the data are strongly in favor of a transport carrier mechanism mediated by reversible sugar−boronate complexation.

A more difficult mechanistic issue concerned the molecular details of the transport phenomenon. The problem was addressed by accumulating a range of kinetic and structure−activity evidence. The kinetic order was determined for glucose efflux and influx using carriers **1**, **3**, and **9**. At low carrier concentrations, a first-order dependence was observed, indicating that the transported species had a 1:1 stoichiometry. At higher carrier concentrations the carrier kinetic order approached 2, suggesting the increased participation of a 1:2 glucose−bisboronate pathway.[21] Since transport systems are kinetically related to enzyme reactions it seemed appropriate to gather inhibition data.[22] In this case, efflux and influx rates were determined in the presence of "inhibitors" (highly hydrophilic boronate binding compounds such as anionic sugars that compete for the boronate and lower its effective concentration). These inhibition studies were again in favor of a complexation-mediated transport event. For example, glucose influx rates were determined using carrier **1** in the presence of gluconic acid or sucrose (Figure 5). The sucrose was found to have negligible inhibitory effect on facilitated glucose transport, whereas the gluconic acid was a strong transport inhibitor. This is in agreement with the poor boronate complexation ability of sucrose and the strong boronate complexation ability of gluconic acid (contains several acyclic vicinal diols).[10]

At the molecular level, the bilayer transport mechanism reduces to two limiting possibilities. Is the transported species the neutral trigonal boronate ester **22**, shown in eq 1, or the

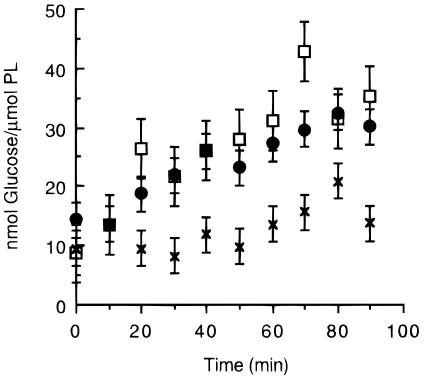
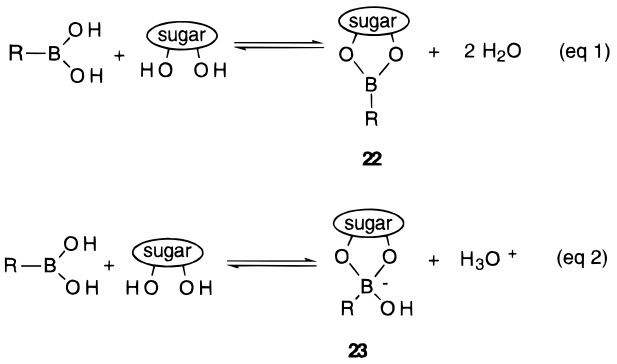


Figure 5. Glucose (50 mM) influx induced by **1** (1 mM) but inhibited by competitive-binding sugars (50 mM): □ (no sugar); ● (sucrose); × (gluconic acid). Radiotracer assay using liposomes of composition DPPC:C:PA, 20:15:2; total lipid concentration 9.4 mM, pH = 7.5, T = 25 °C, PL = phospholipid, error bars reflect the maximum deviation over three experiments.

conjugate base **23**, shown in eq 2 (which is perhaps part of an ion pair)? Indirect evidence was gained by conducting transport experiments under conditions that were expected to facilitate one of the transport pathways. It was felt that this would be an informative approach because one transport mechanism forms a neutral intermediate (**22**), whereas the alternative involves an ionic species (**23**).



First, transport was examined as a function of pH and added counterion. The effect of pH was probed by determining the rates of efflux and influx for samples where the pH of the internal solution was held constant at pH 7.5 and the external solution pH was either 6.5, 7.5, or 8.5 (control fluorescence experiments using encapsulated carboxyfluoroscein showed essentially no decay of the pH gradient over the time frame of the experiment). The resulting pH effect was dependent on the identity of the boronic acid. Rates of efflux and influx were generally a maximum at a pH value just below the pK_a of the boronic acid. This is evidence against **23** as the transported species, because the amount of tetrahedral complex should increase with pH. Transport rates were found to be rather insensitive to cation or anion lipophilicity. Addition of 5 mM sodium perchlorate or tetramethylammonium chloride to the external solution had essentially no effect on transport rates induced by carrier **1**. The more lipophilic tetrabutylammonium chloride had no effect on influx and increased efflux 2- to 4-fold. Various neutral ionophores were examined for their abilities to affect the rates of efflux and influx. Both natural ionophores such valinomycin and gramicidin, as well as artificial ionophores such as 18-crown-6 and [2.2.2]-cryptand, were examined with salts such as sodium or potassium chloride (75 mM) included inside or outside the liposomes. At low ionophore concentrations (1 mM for the artificial ionophores and 1 μM for natural

(21) The association constants for glucose and phenylboronic acid to give 1:1 and 1:2 tetrahedral boronates in aqueous solution are $K_{1:1}$ = 110 M^{-1} and $K_{1:2}$ = 770 M^{-2}. Lorand, J. P.; Edwards, J. O. *J. Org. Chem.* **1959**, *24*, 769−774.

(22) Stein, W. D. *Transport and Diffusion Across Cell Mebranes;* Academic: San Diego, CA, 1986.

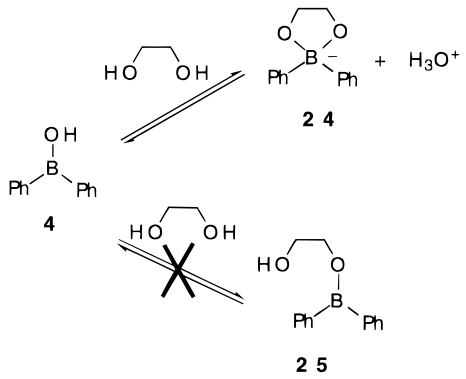


Figure 6. Covalent complexation between a diol and **4** in aqueous solution only producing the chelated tetrahedral borinate anion **24** and not the monodentate trigonal ester **25**.

ionophores), there was little change in rates of influx or efflux. At higher ionophore concentrations (5 mM for the artificial ionophores and 10 μM for natural ionophores), the rates did increase by a factor of 2−4. These results are interpreted as evidence against **23** as a major transport species, because an anionic boronate would be expected to be far more sensitive to changes in cation lipophilicity and/or membrane potential.

Various carriers were examined that had structural features that should have facilitated the formation and transport of anionic boronate **23**. The highly acidic 3-pyridylboronic acid (**17**), which exists as a zwitterion at neutral pH[18f] and the cationic *N*-methylnicotinamide derivative (**21**) which supplies a covalently attached cation to counter a putative anionic boronate, were both unable to induce glucose influx. In addition, diphenylborinic acid (**4**) was also incapable of facilitating glucose influx or efflux. The same negative result was obtained with ribonucleoside transport.[14] Because **4** contains two nonlabile B−C bonds and is relatively acidic (p*K*a = 6.2), it can only form the anionic tetrahedral complex (**24**) when it combines with a diol in aqueous solution (Figure 6). The monodentate borinate ester **25** does not occur to any great extent in water. Previous work with liquid organic membranes has shown that under conditions where an ion pair transport mechanism is operating, the highly lipophilic **24** is readily transported.[23] Taken together, these negative results are strong indirect evidence against **23** as the transported species.

Transport as a Function of Membrane Composition

Most transport experiments were conducted with a lipid mixture of DPPC/C/PA in a ratio of 20:15:2; however, other compositions were examined. Replacing the DPPC with unsaturated lecithin resulted in increased membrane fluidity and a consequent increase in permeability (both background leakage and facilitated transport rates went up). When the amount of cholesterol was systematically varied (DPPC/C/PA, 20:x:2), the transport flux did not show a linear change. Additions up to 10% cholesterol produced slight increases in transport rates, which then dipped when the cholesterol levels reached 20%, only to increase again when the cholesterol level reached 40%.[24] Increasing the temperature from 22 to 50 °C resulted in a fivefold increase in glucose influx (liposomes contained DPPC/C/PA, 20:15:2). This correlates with an increase in membrane fluidity over this temperature range (there is no gel to liquid phase transition for this bilayer composition).[15]

A comparative survey was made of transport with other types of liposomes. Liposomes were prepared by methods known to produce multilamellar vesicles (MLVs), large unilamellar vesicles (LUVs), and small unilamellar vesicles (SUVs).[16] A full characterization of each vesicle system was not possible, so only the glucose efflux per phospholipid content was determined. The observed order of glucose efflux rates from the different liposomes in the presence of **1** was LUV > SUV > MLV, which matches the literature order of relative liposome permeability.[16]

The effect of membrane surface charge was examined. Changing the anionic lipid component (DPPC/C/x, 20:15:2, where x = phosphatidic acid) to a cationic additive such as (*S*)-(2,3-dioleoxypropyl)trimethylammonium chloride (DOTAP), stearyltributylphosphonium bromide, or dodecyltrimethylammonium bromide produced little change in glucose efflux rates, suggesting that the transported species is insensitive to membrane charge (*i.e.*, the transported species is the neutral trigonal boronate ester **22**).

Membrane Fluidity

The final issue concerned the effect of the boronic acids on the bilayer structure. Did the boronic acids alter the bilayer fluidity? Fluorescence spectroscopy using appropriate probe molecules is a versatile means of examining the fluidity of a bilayer membrane.[25] Time-averaged fluorescence experiments were conducted using two well-known fluorescent probes. Rotational fluidity was examined using 1-[(4-trimethylammonium)phenyl]-6-phenyl-1,3,5-hexatriene 4-toluenesulfonate (TMA-DPH), and translational fluidity was investigated using 1-hexadecanoyl-2-(1-pyrenedecanoyl)-*sn*-glycerol-3-phosphatidylcholine (PyDPPC).[25] TMA-DPH intercalates into bilayer membranes in a parallel orientation relative to the other polar lipids with its cationic ammonium group in the polar head group region. The degree in which its rotational freedom is hindered is reflected by the magnitude of its steady-state anisotropy. A decrease in anisotropy reflects an increase in rotational fluidity.[26] With PyDPPC, the ratio of excimer to monomer emission (I'/I) is known to increase as membrane translational fluidity increases.[27]

Fluorescence spectroscopy was used to characterize the membrane fluidity of liposomes that did not contain cholesterol (DPPC/C/PA, 20:0:2). Both probes showed clear evidence for a sharp gel to liquid phase transition around 42 °C. As expected, incorporating >30% cholesterol into the liposome membranes (DPPC/C/PA, 20:15:2) eliminated this phase transition.[27] At 25 °C, the TMA-DPH anisotropy was essentially unchanged upon cholesterol addition, whereas the I'/I ratio for PyDPPC was significantly lower. The cholesterol had reduced translational fluidity by "packing" the membrane.

The following experiments were performed at 25 °C. Liposomes containing either TMA-DHP or PyDPPC were added to solutions of glucose (100 mM in pH 7.5 buffer), gluconic acid (100 mM in pH 7.5 buffer), or pH 7.5 buffer alone. To each solution was then added boronic acid **1** in incremental amounts up to 5 mM. The change in probe response was monitored as

(23) Morin, G. T.; Hughes, M. P.; Paugam, M.-F.; Smith, B. D. *J. Am. Chem. Soc.* **1994**, *116*, 8895−8901.

(24) Cholesterol does not "pack" a bilayer in a uniform manner. At intermediate cholesterol levels, inhomogeneous pockets are formed, which may explain the nonlinear correlation between cholesterol level and bilayer permeability. For a discussion, see: ref 16a, p 19.

(25) (a) Slavik, J. *Fluorescent Probes in Cellular and Molecular Biology*; CRC: Boca Raton, FL, 1994. (b) Haughland, R. P. *Molecular Probes Handbook of Fluorescent Probes and Research Chemicals*; Molecular Probes: Eugene, OR, 1992.

(26) Prendergast, F. G.; Haugland, R. P.; Callahan, P. J. *Biochemistry* **1981**, *20*, 7333−7337.

(27) (a) Galla, H.-G.; Hartman, W. *Chem. Phys. Lipids* **1980**, *27*, 199−219. (b) Mulders, F.; Van Langen, H.; Van Ginkel, G.; Levine, Y. K. *Biochim. Biophys. Acta* **1986**, *859*, 209−218.

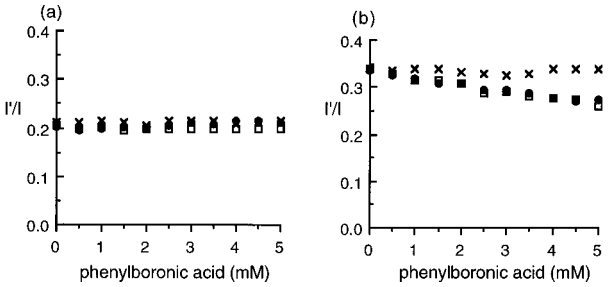
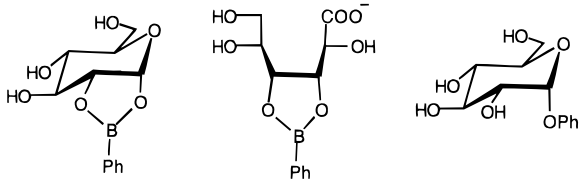


Figure 7. Change in excimer/monomer ratio (I'/I) upon addition of phenylboronic acid (**1**) to PyDPPC-containing liposomes incubating in solutions of × (gluconic acid, 100 mM); ● (glucose, 100 mM); □ (buffer control). All samples were buffered at pH 7.5; $T = 25$ °C. (a) Liposome composition DPPC:C:PA, 20:15:2; (b) liposome composition DPPC:C:PA, 20:0:2.

a function of boronic acid addition. Regardless of sample composition, the TMA-DPH anisotropy did not change upon boronic acid addition. The PyDPPC response, however, depended on both the composition of the liposomes as well as the external solution. When the liposomes contained cholesterol (DPPC/C/PA, 20:15:2), the I'/I ratio remained low and invariant upon boronic acid addition (Figure 7a). However, in certain cases, the liposomes that did not contain cholesterol (DPPC/C/PA, 20:0:2) showed a dose dependent response. When the liposomes were in buffer or glucose solution, the I'/I ratio decreased as boronic acid was added. When the liposomes were in gluconic acid solution, the I'/I ratio did not change (Figure 7b). These fluorescent reponses did not vary with time.

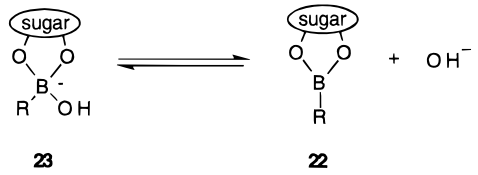
These results can be explained in the following way. *Adding cholesterol to liposome membranes reduces translation fluidity by "packing and ordering" the bilayer. Addition of lipophilic arylboronic acids induces a similar but smaller effect.* If the membrane already incorporates large amounts of cholesterol, then these "packing" effects have already occurred and the addition of boronic acid induces no observable change in fluidity. In the presence of glucose, the boronic acid forms a trigonal phenylboronate ester, such as **26**,[28] which can also intercalate into the membrane (the first step toward membrane transport) and decrease translational fluidity. In the presence of gluconic acid, however, the analogous gluconic acid phenylboronate ester (**27**)[29] is still anionic and does not penetrate the negatively charged membrane. Thus, there is no change in membrane fluidity.



26 **27** **28**

Conclusion

Lipophilic boronic acids are capable of greatly facilitating the transport of monosaccharides through lipid bilayers. The order of transport enhancement for the monosaccharides examined was sorbitol > fructose > glucose, which matches the known order of sugar−boronate complexation stabilities. The disaccharides maltose and sucrose were not transported to any significant degree. The evidence is strongly in favor of a carrier mechanism. The boronic acid combines reversibly with a diol group on the monosaccharide to produce the tetrahedral boronate **23** which is the major complexed structure in bulk, aqueous solution. At the bilayer surface, the tetrahedral boronate is in equilibrium with its neutral, conjugate acid (**22**), which is the actual transported species. Transport mediated by reversible



23 **22**

formation of a trigonal boronate ester has been reported before with bulk, liquid organic membranes.[10] Precedence for this mechanism occurring in bilayer membranes is the fact that weak organic acids and bases can permeate bilayers via their chemically neutral forms, even under conditions where the neutral forms are present in minor amounts.[30]

At low carrier concentrations, a first-order dependence on carrier was observed indicating that the transported species was a 1:1 sugar−boronate such as **26**.[28] Liposome leakage experiments with aryl glycosides such as **28** (whose lipophilicity is similar to **26**) found that these compounds diffuse unassisted through a lipid bilayer at a rate that is close to the facilitated rates recorded in Table 1. This suggests that attachment of one lipophilic boronate group to a monosaccharide provides enough transient lipophilicity to induce partitioning into a bilayer. At higher carrier concentrations, the carrier kinetic order approached 2, indicating the increased participation of a 1:2 sugar−bisboronate transport pathway.[21]

It is possible that boronic acids may be useful as selective bilayer transport agents. In addition to the application already discussed concerning polyol efflux, boronic acids should be capable of delivering a wide range of hydrophilic diol-containing compounds, such as monosaccharides, ribonucleosides, and α-hydroxy acids, into cells. The area of drug delivery is a long term goal and many obstacles need to be overcome. An interesting short-term application is the possibility of using boronic acids to load cells or vesicles with hydrophilic drugs or biochemical reagents such as fluorescent probes.

Experimental Section

Materials. Reagents were obtained from the following sources: all lipids (Avanti Polar Lipids) except phosphatidic acid (Sigma); all enzymes (Sigma); all enzyme substrates (Sigma); (*R*)-1,2-di[1-^{14}C]-palmitoyl-phosphatidylcholine (^{14}C-DPPC, Amersham); ^{3}H-sucrose (fructose-1-^{3}H(N)) and ^{3}H-glucose (Sigma); Triton-X-100 (Aldrich); Sephadex G-50 (Pharmacia); dialysis tubing (Spectrum); Fluorodyne scintillation fluid (Life Sciences); all fluorescent probes (Molecular Probes), (*p*-nitrophenyl)glucurinide and phenyl α-D-glucoyranoside (Sigma). UV spectra were obtained on a Perkin Elmer Lambda 2 instrument. Fluorescence data was obtained on a Perkin Elmer LS 50B luminescence spectrometer.

Boronic Acids. Boronic acids **1**−**6** and **8**−**16** were obtained commercially (Aldrich or Lancaster). Compounds **7**[13] and **17**[18f] were

(28) For simplicity, boronate ester **26** is drawn as the pyranose isomer; however, it is well-known that boronic acids usually form complexes with monosaccharides in their furanose configuration. Norrild, J. C.; Eggert, H. *J. Am. Chem. Soc.* **1995**, *117*, 1479−1485.

(29) Structure **27** is postulated on the basis of the following work. (a) Ferrier, R. J. *Adv. Carbohydr. Chem.* **1978**, *35*, 31−80. (b) van Duin, M.; Peters, J. A.; van Bekkum, H. *Tetrahedron* **1985**, *41*, 3411−3421. van Duin, M.; Peters, J. A.; Kieboom, A. P. G.; van Bekkum, H. *Carbohydr. Res.* **1987**, *162*, 65−78.

(30) (a) Eastman, S. J.; Hope, M. J.; Cullis, P. R. *Biochemistry* **1991**, *30*, 1740−1745. (b) Madden, T. D.; Harrigan, P. R.; Tai, L.; Bally, L. O.; Mayer, T. E.; Redelmeier, H. C.; Loughrey, H. C.; Tilcock, C. P. S.; Reinisch, L. W.; Cullis, P. R. *Chem. Phys. Lipids* **1990**, *53*, 37−46. (c) Rottenberg, H. In *Methods in Enzymology*; Fleischer, S., Fleischer, B., Eds.; Academic Press: London, 1989; Vol. 172, p 62−85.

prepared by standard literature procedures where the appropriate bromo precursor was treated with butyllithium followed by trimethyl borate.

(A) Ester Boronic Acids 18−20. These compounds were prepared by alkylating (4-carboxyphenyl)boronic acid (**8**) using the following general procedure. Potassium hydrogen carbonate (2 mmol) was added to a solution of **8** (0.6 mmol) in DMF (30 mL). The mixture was stirred at reflux for 2 h. The appropriate alkylating agent (dimethyl sulfate, benzyl bromide, or 4-*tert*-butylbenzyl bromide, 0.7 mmol) was added, and the mixture was heated overnight at 60 °C. After standard workup, the crude product was purified by column chromatography. For **18**: ^{1}H NMR (CD_3COCD_3 plus 1 drop of D_2O) 3.87 (s, 3H), 7.96 (s, 4H) ppm; ^{13}C NMR (CD_3COCD_3 plus 1 drop of D_2O) 52.3, 116.0, 129.0, 132.3, 135.0, 167.5 ppm; HRMS (positive FAB with glycerol matrix) $[M + 57]^+ = 237.0934$, calcd for M = $C_8H_9BO_4$ 237.0936. For **19**: ^{1}H NMR (CD_3COCD_3) 5.37 (s, 2H), 7.32−7.51 (m, 5H), 7.97 (d, 2H), 8.02 (d, 2H) ppm; HRMS (positive FAB with glycerol matrix) $[M + 57]^+ = 313.1284$, calcd for M = $C_{14}H_{13}O_4B$ 313.1262. For **20**: ^{1}H NMR (CD_3COCD_3) 1.29 (s, 9H), 5.32 (s, 2H), 7.42 (s 4H), 7.97 (s, 4H). Due to extensive fragmentation, HRMS could not be performed.

(B) 3-(*N*-Methylnicotinamidophenyl)boronic Acid (21). Nicotinic acid (1.82 mmol) and (3-aminophenyl)boronic acid (1.82 mmol) were coupled together using *N*,*N*′-carbodiimidazole (1.82 mmol) in THF. A standard workup produced 3-(nicotinamidophenyl)boronic acid as a white solid (yield 49%): ^{1}H NMR (CD_3SOCD_3 plus 1 drop of D_2O) 7.33 (t, 1H), 7.54 (m, 2H), 7.79 (d, 1H), 8.00 (s, 1H), 8.27 (d, 1H), 8.72 (d, 1H), 9.06 (s, 1H) ppm; ^{13}C NMR (CD_3COCD_3 plus 1 drop of D_2O) 123.4, 124.5, 126.9, 128.6, 129.3, 130.9, 131.6, 136.4, 138.4, 148.9, 152.4, 165.1 ppm; HRMS (positive FAB with glycerol matrix) $[M + 57]^+ = 299.1213$, calcd for M = $C_{15}H_{16}N_2BO_4$ 299.1206. A solution of 3-(nicotinamidophenyl)boronic acid (0.33 mmol) in acetone was treated with iodomethane (1.0 mmol). After 3 h a precipitate had formed, which was filtered to give **21** as its iodide salt (yield 25%): ^{1}H NMR (CD_3COCD_3) 4.43 (s, 3H), 7.39 (t, 1H), 7.63 (d, 1H), 7.88 (d, 1H), 8.01 (s, 1H), 8.12 (s, 1H), 8.29 (t, 1H), 9.02 (d, 1H), 9.13 (d, 1H), 9.51 (s, 1H) ppm; ^{13}C NMR (CD_3COCD_3 plus 1 drop of D_2O) 49.4, 133.5, 136.9, 137.9, 139.1, 141.6, 147.7, 154.3, 154.5, 155.9, 158.0, 171.7 ppm; HRMS (positive FAB with glycerol matrix) $[M + 56]^+ = 313.183$, calcd for M = $C_{16}H_{19}N_2BO_4$ 313.1363.

Liposome Preparation.[16] A chloroform solution of dipalmitoylphosphatidylcholine (DPPC, 2 μmol), cholesterol (C, 1.5 μmol), and egg phosphatidic acid (PA, 0.2 μmol) was evaporated using a rotary evaporator (<30 °C), and the lipid film was dried under vacuum for 1 h (longer drying times had no apparent effect on observed transport results). The liposomes were dispersed in 200 μL of marker solution (depending on the purpose of the experiment, the solution was either water or 35 mM sodium phosphate buffer with 75 mM NaCl at pH 7.5) with the aid of a Vortex mixer. Pyrex beads were added before vortexing to facilitate removal of the lipid film from the sides of the flask. The resulting opaque dispersion was frozen in an ethanol/dry-ice bath and then allowed to thaw in a water-bath at 37 °C. This freeze−thaw cycle was repeated 10 times. The resulting mixture was extruded, at room temperature, 29 times through a 19 mm polycarbonate filter (Nucleopore) with 100 nm diameter pores using a hand-held Basic LiposoFast device purchased from Avestin, Inc., Ottawa, Canada.[17] The extrusion device consisted of a machined housing that secured the polycarbonate filter between two 0.5 mL syringes. The liposomal mixture was forced back and forth from one syringe to the other. An odd number of passages ensured that the mixture always ended up in the receiving syringe. This method has been shown to produce LUVs with a mean diameter of 80 nm.[17] To separate untrapped marker compound, the mixture was dialyzed against NaCl solution (0.15 M) for at least 2 h using dialysis tubing of 15 000 MW cut-off. Ocassionally, Sephadex G-50 minispun columns were used to rapidly separate untrapped marker from the liposomes.[16] The amount of phospholipid in the liposomal fraction after separation was determined using the Stewart assay and the amount of encapsulated glucose by the enzymatic assay decribed below.[16] The encapsulation volume was typically around 1.0 μL/μmol of lipid.

Liposome Transport Assays. (A) Glucose Efflux. The rate of glucose efflux was determined by the method of Kinsky.[31] Aliquots of the above liposome preparation (usually 40 μL, final concentration of phospholipid plus cholesterol was 0.75 mM) encapsulating 300 mM of glucose were added to two 1 mL cuvettes. One cuvette contained the complete assay solution of 35 mM sodium phosphate, pH 7.5, 75 mM NaCl, 2 mM $MgCl_2$, 2.5 units of enzyme (hexokinase/glucose-6-phosphate dehydrogenase), 1 mM ATP, and 0.5 mM NADP. The other cuvette contained the same reagents except for the NADP and was a control solution used to correct for background absorption at 340 nm. The boronic acids were added as small aliquots of concentrated aqueous or DMSO solutions (the final concentration of DMSO was almost always less than 5%), and the absorbance at 340 nm was monitored over time. The initial efflux rates were determined from the amount of glucose transported after the first 10 min. There was no glucose consumption if the glucose was inside the liposomes and boronic acids were absent, proving that the enzymes were unable to penetrate the liposomes. Facilitated efflux generally occurred over a period of hours, whereas enzyme turnover rates were much faster.[31] If the glucose was not encapsulated within the liposome, then it was totally consumed by the coupled enyzme system within 1 min. The total amount of glucose encapsulated inside the liposomes was determined by lysing the liposomes with 100 μL of 10% Triton-X-100 solution at the end of each assay.

(B) Glucose-6-phosphate Efflux. The procedure was the same as that for glucose efflux except the liposomes were prepared with 300 mM glucose-6-phosphate as the encapsulated marker compound, and ATP and hexokinase were deleted from the assay.

(C) Fructose Efflux. The procedure was the same as that for glucose efflux except the liposomes were prepared with 300 mM fructose as the encapsulated marker compound and the enzyme phosphoglucoisomerase (5.0 units/mL) was added to the mixed enzyme system.[32]

(D) Fructose-6-phosphate Efflux. The procedure was the same as that for glucose efflux, except the liposomes were prepared with 300 mM fructose-6-phosphate as the encapsulated marker compound and the enzyme phosphoglucoisomerase (5.0 units/mL) was added to the mixed enzyme system, while ATP and hexokinase were deleted.

(E) Sorbitol Efflux. The procedure was similar to that for glucose efflux, except the liposomes were prepared with 300 mM sorbitol as the encapsulated marker compound and the enzyme was sorbitol dehydrogenase (2 units/mL).

(F) Sucrose Efflux. The procedure was the same as that for fructose efflux except the liposomes were prepared with 300 M sucrose as the encapsulated marker compound and invertase (1.0 units/mL) was added. Note that 2 equiv of NADPH are produced for each equivalent of sucrose consumed.

(G) Isocitrate Efflux. The procedure was the same as that for glucose efflux except the liposomes were prepared with 300 mM isocitrate as the encapsulated marker compound, the assay enzyme was isocitrate dehydrogenase (1.5 units/mL), and the ATP was deleted.[33]

(H) Arsenazo III Efflux. Liposomes were prepared with 3 mM arsenazo III as the encapsulated marker compound. The assay utilizes the 560 nm to 606/660 nm spectral shift of arsenazo III that occurs when the dye binds calcium ions. Both the normal assay with arsenazo inside and calcium outside the liposomes, as well as the reverse assay with arsenazo and calcium inside and EDTA outside, were performed. The literature method was followed, which monitored the change in absorbance at 660 nm.[16]

(I) Carboxyfluoroscein/Calcein Efflux.[16] Liposomes were prepared with carboxyfluoroscein (CF, 100 mM in 10 mM sodium phosphate buffer, pH 7.7) and glucose (300 mM) as the encapsulated marker compounds. CF fluorescence is linearly proportional to concentration between 3 and 30 μM. Above 30 μM self-quenching becomes dominant. To monitor CF efflux, an aliquot of the liposome preparation (40 μL) was added to a glass fluorescence cuvette containing 1 mL of

(31) Kinsky, S. C. In *Methods in Enzymology*; Fleischer, S., Packer, L., Eds.; Academic Press: London, 1974; Vol. 32, p 501−512.

(32) Beutler, H.-O. In *Methods of Enzymatic Analysis*, 3rd ed.; Bergmeyer, H. U., Ed.; Verlag: Weinheim, 1983; Vol. 6, pp 321−327.

(33) Crowe, L. M.; Womersley, C.; Crowe, J. H.; Reid, D.; Appel, L.; Rudolph, A. *Biochim. Biophys. Acta* **1986**, *861*, 131−140.

the complete glucose assay solution as well as the appropriate boronic acid. The change in CF fluorescence at 520 nm (excitation at 470 nm) was monitored for 90 min before the liposomes were lysed by addition of Triton-X-100 (300 μL of a 10% solution). The calcein assay followed the same procedure except the calcein was encapsulated at pH 7.3 and the fluorometer settings were slightly different (excitation at 491 nm and emission at 511 nm).

(J) Radiotracer Assay for Glucose Influx. A stock solution containing sucrose (50 mM) and glucose (50 mM, doped with 8 μCi/mL of 15.2 Ci/mmol ^{3}H-glucose) was prepared. Empty liposomes containing 70 mM sodium phosphate at pH 7.5 were prepared as described above, except the phospholipid lipid layer was doped with ^{14}C-DPPC (112 mCi/mmol) at a ratio of 0.25 μCi/10 μmol of DPPC. The glucose influx experiment was initiated when 475 μL of liposome solution, 475 μL stock solution, and 50 μL of boronic acid solution (aqueous or DMSO) were combined at 25 °C. Every 10 min, a 100 μL sample was withdrawn and spun-down through a 1 mL Sephadex G-50 column.[16] The liposome fraction was added to 10 mL of scintillation fluid, and the ^{3}H and ^{14}C activity levels were counted using a scintillation counter. Each influx experiment was repeated with independent solutions. Sucrose influx was determined using the same method except the sucrose was doped with tracer and the glucose was not.

Membrane Fluidity Studies. (A) Fluorescence Assay for Rotational Fluidity Using TMA−DPH.[26] A liposome mixture containing only buffer solution was prepared using the procedure described above and diluted to a concentration of 345 μM phospholipid. TMA-DPH (0.5 mol % probe to phospholipid) was added, and the mixture was immediately vortexed and then allowed to incubate for 18 h. The fluorescence assay used a 3 mL cuvette that was stirred magnetically at 25 °C. The cuvette was filled with probe-containing liposomes (58 μM phospholipid) in buffer solution (35 mM sodium phosphate, 75 mM NaCl, pH 7.5). The *G* value (correction for instrument polarization response which causes the ratio of I_V/I_H to deviate from unity) was calculated. After each addition of boronic acid (10 μL of concentrated boronic acid solution), the polarization and anisotropy values were determined. The excitation wavelength was 360 nm, and the emission wavelength was 427 nm, with slit widths of 4 and 3.5, respectively. Perkin Elmer StdPol. Software was used to obtain $I_{V(V)}$ (emission intensity of vertically polarized light parallel to the plane of excitation), $I_{V(H)}$ (emission intensity of horizontally polarized light perpendicular to the plane of excitation), polarization, and anisotropy values.

(B) Fluorescence Assay for Translational Fluidity.[27] The liposomes were prepared as described above, except that PyDPPC (2 mol % for liposomes without cholesterol and 5 mol % for liposomes with cholesterol) was added to the starting phospholipid solution in chloroform. The final concentration of phospholipid in the 3 mL fluorescence cell was 3.5 μM. The excitation wavelength was 320 nm; emission wavelengths were 397 and 479 nm.

Acknowledgment. We thank Professor R. Bretthauer for allowing us to use his radiochemical facilities. We are grateful to Jeffrey Bien, Jennifer Riggs, Linbee Valencia, and Carol Monahan for assistance. We thank two referees for their careful and thoughtful reviews. This work was supported by the National Science Foundation (CHE96-27099). B.D.S. acknowledges a Cottrell Scholar award of Research Corporation.

Supporting Information Available: Representative raw data for transport kinetic order, pH effects, counterion effects, and membrane composition (9 pages). See any current masthead page for ordering and Internet access instructions.

JA961264H