ELSEVIER

Biochimica et Biophysica Acta 1515 (2001) 177–188

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

www.bba-direct.com

Detection of two distinct transporter systems for 2-deoxyglucose uptake by the opportunistic pathogen *Pneumocystis carinii*

Mireille Basselin-Eiweida, Edna S. Kaneshiro *

Department of Biological Sciences, University of Cincinnati, ML 0006, Cincinnati, OH 45221, USA

Received 27 March 2001; received in revised form 5 July 2001; accepted 30 August 2001

Abstract

Since the opportunistic pathogen *Pneumocystis carinii* grows only slowly in vitro, the mechanism of glucose uptake was investigated to better understand how the organism transports nutrients. Using the non-metabolizable analogue 2-deoxyglucose, two uptake systems were detected with Q_{10} values of 2.12 and 2.09, respectively. One had a high affinity (K_m = 67.5 μM) and the other a low affinity (K_m = 5.99 mM) for 2-deoxyglucose uptake. Glucose or deoxyglucose phosphate products from transported radiolabeled substrates were not detected during the incubation times used in this study. Both systems were inhibited by mannose, galactose, fructose, galactosamine, glucosamine, and glucose but not by allose, 5-thioglucose, xylose, glucose 6-phosphate and glucuronic acid. Salicylhydroxamate, KCN, iodoacetate, and 2,4-dinitrophenol inhibited the high-affinity transporter, suggesting it required ATP. Ouabain, monensin, carbonyl cyanide *m*-chlorophenylhydrazone, and *N*,*N*′-dicyclohexylcarbodiimide also inhibited deoxyglucose uptake, as did the replacement of Na^+ in the incubation medium with choline, indicating requirements for Na^+ and H^+. The high-affinity system was also inhibited by the protein synthesis inhibitors cycloheximide and chloramphenicol. In contrast, the low-affinity system transported deoxyglucose by facilitated diffusion mechanisms. Unlike the human erythrocyte glucose transporter GLUT1, the *P. carinii* transporters recognized fructose and galactose and were relatively insensitive to cytochalasin B, suggesting that the *P. carinii* glucose transporters may be good drug targets.

Keywords: Glucose transport; Active transport; Facilitated diffusion; *Pneumocystis*

1. Introduction

Pneumocystis carinii is an opportunistic eukaryotic pathogen of the lungs that can be found in mammals lacking disease symptoms, but it also can be responsible for major morbidity and mortality in those with defective immune systems. In immunodeficient hosts, the organism can grow rapidly in the lung alveolus where it causes inflammation, major damage to the alveolar epithelium and blocks exchange of gases between the alveolar lumen and blood capillaries. Effective chemotherapeutic treatment available to prevent or clear *P. carinii* pneumonia (PcP) includes trimethoprim/sulfamethoxazole (TMP/SMX), trimetrexate, pentamidine and atovaquone. However, these can cause severe side effects in some patients [1]. Membrane transport processes represent potential targets for drug development, since it is by these

Abbreviations: 2-DOG, 2-deoxy-D-glucose; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; KCN, potassium cyanide; 2,4-DNP, 2,4-dinitrophenol; Q_{10}, temperature coefficient; SHAM, salicylhydroxamic acid; NKA, (Na^+-K^+)-ATPase

* Corresponding author. Fax: 513-556-5280.

E-mail address: edna.kaneshiro@uc.edu (E.S. Kaneshiro).

0005-2736/01/$ – see front matter
PII: S0005-2736(01)00412-6

mechanisms that the organism imports vital nutrients. Blocking normal nutrient uptake may result in clearing the infection caused by these organisms.

The organism proliferates extracellularly bathed by fluid rich in lung surfactant secreted by alveolar type II epithelial cells. Approx. 90% of lung surfactant is lipids with lower amounts of proteins and carbohydrates. In contrast to growth in the lung, organisms placed in axenic culture [2,3] or co-cultivated with mammalian cells [4,5] multiply only very slowly, and most *P. carinii* culture protocols do not sustain long-term passage and proliferation in vitro [6]. Because of slow growth in vitro, much of the organism's basic biochemistry has yet to be examined. Performing biochemical experiments on *P. carinii* are not trivial but they can be done. Most studies have been done using purified organisms isolated from immunosuppressed animal models.

Glucose is a nutrient that serves as an important carbon source in prokaryotic and eukaryotic organisms. Organisms satisfy their requirement for this sugar by synthesizing it, hydrolyzing glucose-containing complex molecules such as glycogen, or by acquiring it from exogenous sources. That glucose is a major structural precursor in the organism is suggested by the identification of this sugar as the dominant monosaccharide component of the *P. carinii* cyst wall [7], and the high 1,3-β-glucan synthetase activity measured by the incorporation of [^{14}C]-UDP-glucose into cell wall β-glucans [8]. [^{14}C]-Glucose utilization via the classic aerobic glycolytic pathway to produce $^{14}CO_2$ was reported [9], but the purity of the organism preparations used for that earlier study was not defined. Recently, it was found that *P. carinii* requires glucose for axenic growth in vitro; 1–5.5 mM glucose sustained culture growth over 3 weeks, whereas cell proliferation was not observed without glucose (Clarkson, A.B., Jr., Merali, S., pers. commun.).

Glucose transport systems have been studied in many prokaryotic and eukaryotic cells [10–29]. In most mammalian cells, the rate of glucose metabolism is limited by the transport of exogenous glucose across the plasma membrane. The mechanism of glucose uptake most commonly encountered is by facilitated transport via glucose-specific translocation systems described as carriers, transporters, or permeases. Both facilitated diffusion [15–19] and active transport for glucose uptake have been identified. Co-transport (linked transport, coupled transport) requiring specific ions such as Na^+ [20–22] or H^+ [11,23–29] for symport translocation of glucose has been characterized. Although glucose transporter systems are among the most studied among different cell types, there is only little information on the mechanism of glucose transport in *P. carinii*. In the present study, we analyzed the uptake by *P. carinii* of radiolabeled glucose and the non-metabolizable analogue 2-deoxy-D-glucose (2-DOG).

2. Materials and methods

2.1. Chemicals

D-[U-^{14}C]Glucose (10 mCi/mmol, >99% purity) was obtained from ARC (St. Louis, MO, USA). 2-Deoxy-D-[2,6-^{3}H]glucose (44 Ci/mmol, 1 mCi/ml) was purchased from Amersham Pharmacia Biotech (Piscataway, NJ, USA). The radiochemical purity determined by HPLC was 96.8% and the distribution of the label was 51.8% and 48.2% for the H-2 and H-6 position, respectively. All other compounds including unlabeled D-hexose sugars, glucose analogues, inhibitors and bovine serum albumin were of the highest quality available from Sigma-Aldrich (St. Louis, MO, USA). D-Galactose (>99%) was from Sigma-Aldrich or Acros Organics (Pittsburgh, PA, USA). 5-Thio-D-glucose was from ICN (Costa Mesa, CA, USA), and sodium azide and potassium cyanide (KCN) were from Fisher Scientific (Pittsburgh, PA, USA).

2.2. Organisms

P. carinii f. sp. *carinii* was isolated from the lungs of corticosteroid-immunosuppressed rats with PcP [30,31]. Viral antibody-negative female Lewis rats (Harlan Sprague–Dawley, Indianapolis, IN, USA) were immunosuppressed with methylprednisolone acetate (Depo-Medrol; Upjohn, Kalamazoo, MI, USA) and were twice inoculated intratracheally with 10^6–10^7 mixed life cycle stages of cryopreserved organisms [31]. Lung homogenates containing *P. carinii* that were used for inoculation into the rats were prepared under aseptic conditions in the absence of

antibiotics [31]. Aliquots of these preparations were cryopreserved and only those that tested negative for common bacteria (Mueller Hinton agar, Difco, Detroit, MI, USA) and fungi (Sabouraud dextrose agar, Difco) were used for infecting rats. After 8–10 weeks of immunosuppression, moribund rats were killed and their lungs were perfused, excised, and cut into small pieces.

The organisms were isolated and purified by procedures previously described [31]. Briefly, lung pieces were homogenized (Stomacher; Tekmar, Cincinnati, OH, USA) with the mucolytic sulfhydryl agent glutathione (reduced form), which caused the detachment of organisms from host cells and other *P. carinii* organisms. Purification involved sieving and a series of centrifugation steps at different speeds, followed by membrane microfiltration. The purity of the organism preparations was documented by microscopic, biochemical, immunochemical, and microbiological analyses, all of which indicated >95–100% purity [31,32].

These preparations contained 10–30% cystic forms. The percentage of viable organisms in the preparations was conservatively estimated between 80% and 95% by the fluorescence dual staining assay [31]. Process control pellets resulting from subjecting the lungs of a single normal, untreated rat or an immunosuppressed, uninfected rat to the same isolation and purification protocol did not produce visible pellets after the final centrifugation step. The highest (not average) total protein remaining in the centrifuge tube was <300 μg ($n=10$) [31]. In contrast, the volume of *P. carinii* packed pellets purified from a single infected rat (10^8–10^9 organisms) was 0.3–0.4 ml, containing approx. 5 mg protein [31].

2.3. Uptake of glucose and 2-DOG

Organisms (10^8–10^9) were centrifuged at $926\times g$ for 10 min at 4°C and then the pellet was resuspended at a density of approx. 5×10^7 cells/ml of a HEPES-buffered solution (100 mM HEPES, 1.8 mM $CaCl_2$, 145 mM NaCl) at pH 7.4. Uptake of D-[U-^{14}C]-glucose was performed by incubating organisms with 0.5 μCi of the radiocompound plus various concentrations of non-radioactive glucose in a final volume of 0.8 ml incubation solution. Uptake of deoxyglucose (1 μCi of 2-[2,6-^{3}H]DOG) and analyses of the effects of various sugars or inhibitors were performed by adding various concentrations of the compounds to the organism suspensions. After 1 min incubation at 37°C, uptake was terminated by the addition of 2 ml of ice-cold HEPES-buffered solution supplemented with 100 mM glucose or 2-DOG, and high-speed centrifugation ($3000\times g$) at 4°C for 2 min. The supernatant was aspirated, and then 5 ml of ice-cold HEPES-buffered solution were carefully layered over the pellet without disrupting the pellet or resuspending the cells. The sample was then subjected to centrifugation for 1 min, the supernatant removed, and then the radioactivity in the resultant pellet was measured by liquid scintillation counting (efficiencies were 45% and 94% for ^{3}H and ^{14}C, respectively). Alternatively, the organisms in pellets were resuspended and washed with the ice-cold HEPES-buffered solution, and then centrifuged. The two protocols for removing excess extracellular radioactive compounds gave the same results. The equivalent results were attributed to the small size of the cell pellets, i.e. insignificant amounts of radioactive incubation medium were trapped in between organisms in the final pelleted material.

To analyze the effects of various inhibitor compounds and ionophores, the organisms were preincubated at 37°C with the compound for 10 min prior to the initiation of 2-DOG uptake analysis. The protein concentration of cells was measured by the method of Lowry et al. [33] with bovine serum albumin as standard. 2-DOG uptake rates were expressed as nmol or pmol of 2-DOG/mg of *P. carinii* total proteins/min. Estimates of cell volume (cysts, 32.6 fl; trophozoites, 94.7 fl), an average of 20% cysts in the preparation, and an estimate of 1 mg protein of the preparation represents 2.6×10^8 organisms, were taken from previously reported analyses [31].

2.4. Phosphorylated glucose

After intact cells had been incubated with radiolabeled glucose or 2-DOG for 1 min, incorporation into hexose phosphates was examined. Protein and nucleic acid macromolecules were precipitated by adding 0.2 ml of 5.6% (w/v) perchloric acid to the cell pellet, and after centrifugation, the phosphorylated compounds in the soluble fraction were precipitated with $ZnSO_4$ and $Ba(OH)_2$ according to the

procedure described by Somogyi [34]. Briefly, after neutralization and centrifugation, 0.25 ml 0.15 M $ZnSO_4$, and then 0.25 ml 0.15 M $Ba(OH)_2$ were added to the supernatant fraction. After 5 min at room temperature, the suspension was centrifuged, and then the resultant pellet and supernatant fractions were analyzed for radioactivity. Samples not treated by $ZnSO_4$ and $Ba(OH)_2$ precipitation to which 0.5 ml of H_2O was added, served as controls.

2.5. Data analyses

Uptake was analyzed using the software program Grafit (version 4.0.12, Erithacus Software, Sigma). Statistical analyses ($n=3$) were performed using Microsoft Excel 2000. The K_m and V_{max} values were determined by linear transformation of the Michaelis–Menten equation using the Eadie–Hofstee plot, and the method of Spears et al. [35] for demonstrating the presence of two independent transporter systems. The temperature coefficient (Q_{10}), which represents the increase in velocity (k) observed when the temperature is raised by 10°C, was calculated by the van 't Hoff equation: $Q_{10}=(k_2/k_1)^{10/t2-t1}$.

3. Results

3.1. Uptake of glucose

The initial uptake rate of the natural substrate glucose was measured over a 1 min period using different external glucose concentrations from 1 mM to 50 mM. Uptake of radiolabeled glucose appeared to reflect first order kinetics (Fig. 1A,B), indicating that glucose uptake by *P. carinii* was carrier-mediated. It is assumed that glucose acquired exogenously by the organism is metabolized; nonetheless, glucose uptake appeared linear up to 5 mM, and saturation was observed at approx. 20 mM. The apparent K_m was estimated at 5.2 ± 0.5 mM and the apparent V_{max} was 3560 ± 106 pmol/mg of total *P. carinii* protein/min.

3.2. Kinetics of 2-DOG uptake

The glucose analogue 2-DOG was then employed to investigate the mechanism of glucose uptake by *P. carinii* in greater detail. The time course of 100 μM and 10 mM 2-DOG accumulation in *P. carinii* was studied using incubation times from 1 min to 30 min (Fig. 1C). The initial rate was approximately linear up to 10 min for both substrate concentrations. After 30 min incubation, the levels of 2-DOG in the cells reached a plateau between 13 and 15 nmol/mg protein when extracellular 2-DOG concentration was 100 μM, whereas a plateau was seen at a higher intracellular 2-DOG content of 63–65 nmol/mg protein when extracellular 2-DOG concentration was 10 mM. It was estimated that intracellular 2-DOG concentrations under the two incubation conditions after 30 min were 1.1–1.3 mM and 5.4–5.5 mM, respectively (calculated using the average percentage of cysts in the preparations and the known volumes and protein content of the cells). Uptake against a concentration gradient was evident only when low (100 μM) extracellular 2-DOG was used. The observations suggest that glucose is translocated across the cell surface membrane by active transport (evident at low extracellular 2-DOG concentration) and that additional amounts can enter the cells (at high extracellular 2-DOG concentration) via a separate mechanism, e.g. facilitative diffusion.

Initial uptake rates of 2-DOG were determined for 1 min at various external 2-DOG concentrations from 0.029 μM to 50 mM. The process exhibited first order kinetics; saturation occurred at approx. 20 mM 2-DOG (Fig. 1D). The kinetics of 2-DOG uptake was comparable to that of the natural substrate glucose (Fig. 1A,B), indicating that 2-DOG was an appropriate analogue to study the mechanism of glucose uptake by *P. carinii*. The Eadie–Hofstee plot of the data showed two straight lines, which suggests that the organism had two independent transport systems for glucose uptake (Fig. 1C). The K_m values were 67.5 ± 2.5 μM and 5.99 ± 0.21 mM for the high- and low-affinity system, respectively. The V_{max} values were 237 ± 22 pmol/mg protein/min and 3035 ± 70 pmol/mg protein/min, respectively. The kinetic parameters of the low-affinity, high-capacity 2-DOG transporter system were similar to the values obtained using 1–50 mM glucose in the initial experiments that were performed testing whether 2-DOG and glucose were equivalent as substrates taken up by *P. carinii*.

The effect of temperature on 2-DOG uptake was

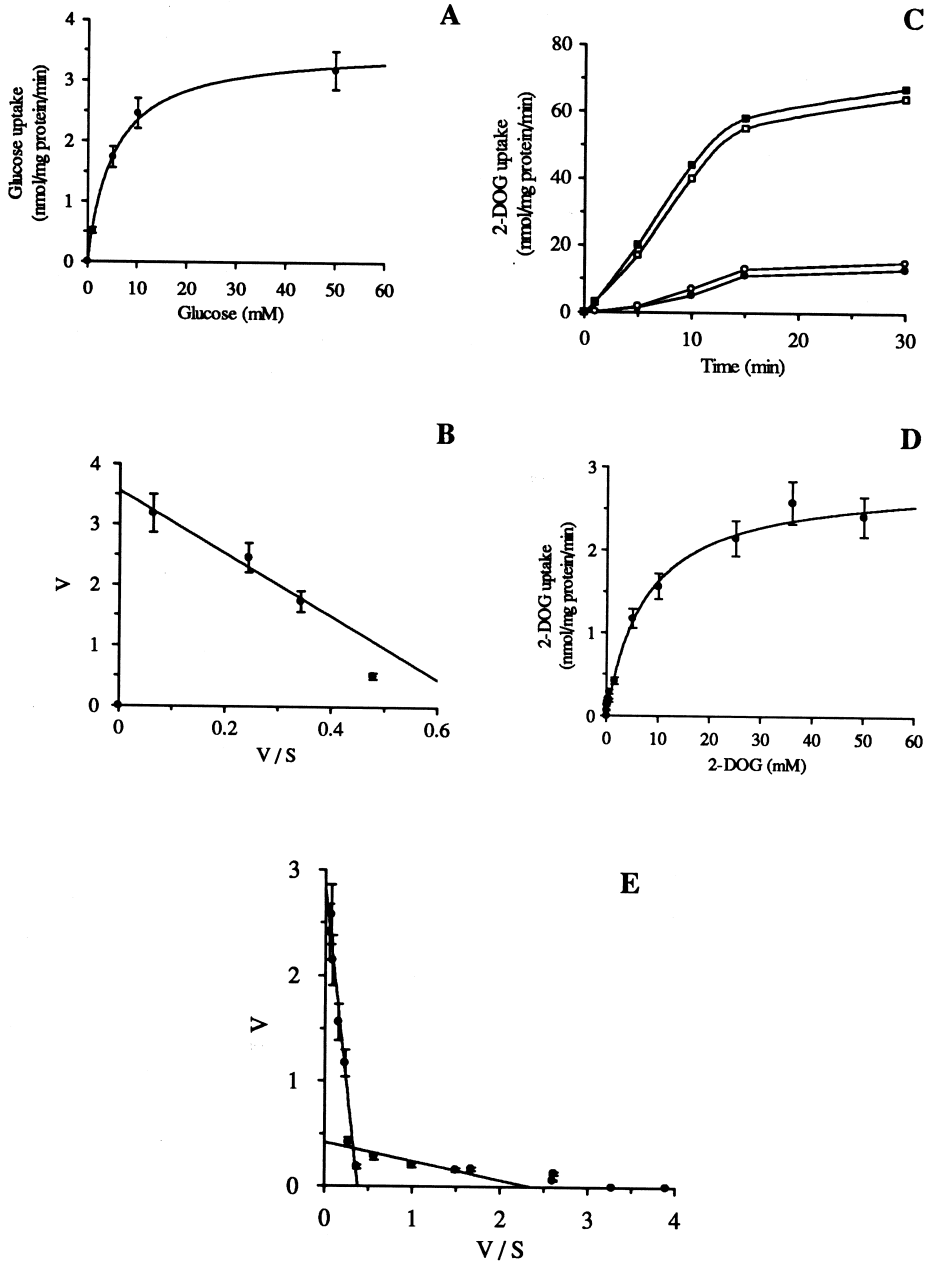

Fig. 1. Kinetics of glucose and 2-DOG initial uptake rates in *P. carinii*. (A) Uptake of glucose at various substrate concentrations. Points are means ± S.E.M.; $n = 3$. (B) Double reciprocal plot of the glucose uptake. (C) Uptake of 2-DOG measured as a function of time using substrate concentrations of 100 μM (circles) and 10 mM (squares). Closed and open symbols represent two independent experiments. (D) Rate of 2-DOG uptake at various substrate concentrations measured at 37°C for 1 min. Saturation was observed at approx. 20 mM 2-DOG, similar to that of glucose uptake shown in A. Thus, this analogue is an appropriate compound for studying the glucose transporter systems in *P. carinii*. Each point represents the means ± S.E.M. of three separate experiments. (E) Eadie–Hofstee plots of 2-DOG uptake data shown in D demonstrating the presence of two transporter systems (two different slopes).

examined by placing *P. carinii* suspensions at 4°C and comparing the rates measured with those obtained at 37°C (controls). Uptake was reduced at 4°C; 2-DOG uptake by the high-affinity transporter was only 6.1 ± 2.8% of control, and uptake by the low-affinity was 6.9 ± 2.5% of control. The Q_{10} values calculated were 2.12 and 2.09, respectively, both of which are typical for carrier-mediated transport.

3.3. Substrate specificity of the glucose transport systems

Uptake by the high-affinity and the low-affinity systems was measured using a substrate concentration of 100 μM and 10 mM 2-DOG, respectively, and the effects of various hexose sugars and analogues were tested. In the presence of a 10-fold excess of various sugars 2-DOG uptake was strongly inhibited by glucose (Table 1), indicating that glucose and 2-DOG are both taken up by *P. carinii* via the same transporters. Strong inhibition occurred with mannose (92–96% inhibition of the high-affinity system; 56–75% inhibition of the low-affinity system). This suggests that the hydroxyl group at the C-2 position of the sugar is not essential for recognition by the transporters, but it may have an influence on the function of the low-affinity system. Lower levels of inhibition (approx. 50%) of 2-DOG uptake by galactose, the 4-epimer of glucose, was observed. By increasing the ratio of galactose/2-DOG by 100, the high- and low-affinity systems were inhibited by $70 \pm 2\%$ and $65 \pm 3\%$, respectively. These results may indicate that the hydroxyl group at the C-4 position is involved in the recognition of sugars by the transporter. Fructose also inhibited both systems by half when this sugar was present in a fructose/2-DOG ratio of 10 (Table 1). With an increased ratio of 100, the high- and low-affinity systems were inhibited by $66 \pm 4\%$ and $71 \pm 3\%$, respectively. The IC_{50} for fructose inhibition of 2-DOG uptake was estimated at approx. 1 mM. Glucosamine and galactosamine were less effective than glucose and galactose, respectively, indicating that the transporters preferred substrate analogues lacking charged amino groups, and that the oxygen in the C-2 position, like that in glucose, was an important structural feature recognized by the transporter systems.

The analogue 5-thioglucose did not inhibit either transport system (Table 1). Using a ratio of 100 times, an only 25% and 23% inhibition of the high- and low-affinity transport system, respectively, was observed. These data indicate that the ring oxygen is an essential requirement for binding to the carrier, probably involving hydrogen bonds. Allose, glucuronic acid, glucose 6-phosphate, and xylose did not inhibit either of the 2-DOG uptake systems (Table 1). The same results were obtained by increasing the ratio of analogue/2-DOG by 100 (data not shown). The lack of interaction between transporters and these compounds suggests that the C-3 and C-6 hydroxyl group configurations as found in glucose are

Table 1
Effect of D-glucose analogues and disaccharides on the two 2-DOG uptake systems in *P. carinii*

Oxygen function	Hexose substrate/analogue	2-DOG transport (% of control)	
		High-affinity system	Low-affinity system
Monosaccharides			
C-2	Glucose	3.8 ± 1.4	25 ± 4
	Glucosamine	42 ± 7	46 ± 5
	Mannose	6.2 ± 1.1	34 ± 9
C-3	Allose	81 ± 2	91 ± 4
C-4	Galactose	49 ± 2	54 ± 4
	Galactosamine	68 ± 7	71 ± 2
C-5	5-Thioglucose	95 ± 2	97 ± 3
C-6	Fructose	57 ± 2	54 ± 3
	Glucuronic acid	90 ± 6	92 ± 3
	Glucose 6-phosphate	84 ± 1	87 ± 3
	Xylose	87 ± 1	88 ± 6
Disaccharides			
	Lactose	82 ± 1	159 ± 5
	Maltose	77 ± 1	83 ± 5
	Sucrose	94 ± 10	93 ± 1

Transport of 100 μM (high-affinity system) and 10 mM (low-affinity system) 2-DOG was determined at pH 7.4 for 1 min in the presence of competitors (10-fold excess). Control uptake rates of 2-DOG by the two systems were 245 ± 5 and 3015 ± 21 pmol/mg protein/min, respectively. Percent of control values are expressed as means ± S.E.M. of three independent experiments.

essential structural features. Also, since glucose 6-phosphate and glucuronic acid are both charged at pH 7.4, the absence of effect of these compounds on 2-DOG uptake by *P. carinii* indicates that a negative charge at the C-6 region of the substrate prevents binding with the carriers.

The disaccharides lactose (galactose β (1,4), glucose), maltose (glucose α (1,4), glucose) and sucrose (glucose α (1,2), β fructose) did not inhibit either of the 2-DOG uptake systems (Table 1). The same results were obtained by increasing the ratio of disaccharide/2-DOG for both systems to 100 (data not shown). These results showed that the transporter is specific for monosaccharides.

It was previously shown that uptake of the amino acid serine by *P. carinii* required the presence of glucose in the medium [36]. To examine whether this was due to co-transport of the amino acid with glucose, the effects of 1 mM and 100 mM serine on 2-DOG uptake were tested. Serine did not stimulate or inhibit 2-DOG uptake by the organism; uptake by the high-affinity system was 89 ± 6% of control without serine, and uptake by the low-affinity system was 91 ± 3% of control. The same results were obtained when serine was increased to 100-fold excess over that of 2-DOG using 10 mM and 1 M serine for the high- and low-affinity system, respectively (data not shown). The effect of serine on glucose uptake was examined to determine whether the lack of effect of this amino acid on 2-DOG uptake was related to differences in structure of the natural substrate and the non-metabolizable analogue. Uptake of 5 mM radiolabeled glucose was not altered by the presence of 5 mM, 50 mM or 100 mM exogenous serine. Compared to controls lacking exogenous serine, uptakes were 97 ± 4%, 116 ± 4% and 95 ± 5%, respectively.

3.4. Analysis of phosphorylated substrates

In some cells, glucose uptake involves a cell surface membrane hexokinase activity that phosphorylates the substrate as part of the translocation process. Using 5 mM radiolabeled glucose (1 μCi), or 10 μM or 100 μM radiolabeled 2-DOG (1 μCi) at pH 7.4 for 1 min the soluble fraction of *P. carinii* cells was analyzed for phosphorylated products that were isolated by precipitation. Radioactivity in the phosphorylated glucose or 2-DOG pelleted material was not detected. Increasing the radioactivity of the substrate to 5 μCi did not result in detectable radiolabeled hexose phosphates. In two separate experiments using 5 μCi 2-DOG, 99.9% and 91.6% of the radioactivity were in the supernatant. Likewise, using 5 μCi glucose, 98.7% and 96.3% of the radioactivity were in the supernatant. These results indicated that putative glucose or 2-DOG phosphates produced from exogenous radiolabeled hexoses were undetectable under the experimental conditions used in this study, and suggest that the glucose transport process in *P. carinii* does not involve phosphorylation.

3.5. Effect of ATP synthesis inhibitors on 2-DOG transport

The mechanisms and properties of the two 2-DOG transport systems were investigated by probing them with different types of inhibitors (Table 2). The oxidase inhibitor salicylhydroxamic acid (SHAM) was highly effective in reducing 2-DOG uptake, which is consistent with reports of an active alternative oxidase system in *P. carinii* [37,39]. Sodium azide, which was previously shown to significantly reduce intracellular ATP levels [37] and cytoplasmic pH in *P. carinii* [38], did not inhibit 2-DOG uptake. The mitochondrial oxidative phosphorylation inhibitors dinitrophenol (DNP) and KCN were previously shown to reduce ATP levels in *P. carinii* [37], and KCN was reported to also reduce oxygen consumption [40]. These compounds inhibited the high-affinity 2-DOG uptake system, and like azide and SHAM, this inhibitor had no effect on the low-affinity system. To test the effects of inhibiting ATP production via glycolysis, iodoacetate was tested for inhibitory activity on 2-DOG uptake. It was previously reported that iodoacetate strongly inhibited the conversion of glucose to CO_2 by *P. carinii* [9], but those studies were performed on organism preparations for which adequate proof of purity was lacking. Our results indicated that iodoacetate had only a slight inhibitory effect on 2-DOG uptake; glucose metabolism was not measured. Thus, glycolysis apparently is not a major source of ATP used by the organism for glucose transport. When the combination of iodoacetate, KCN, azide, and SHAM was tested, additive or synergistic effects were not detected. These analy-

ses on the effects of ATP synthesis inhibitors indicate that the high-affinity 2-DOG uptake system required energy from ATP, and that inhibition of the alternative oxidase pathway was more effective than blockage of the classical cytochrome-containing electron transport chain.

3.6. Effects of other inhibitors

Cycloheximide and chloramphenicol, classic inhibitors of cytosolic and mitochondrial protein synthesis, respectively, were effective in inhibiting protein synthesis in *P. carinii* [9]. Organisms pretreated for 10 min with either compound exhibited diminished 2-DOG uptake by the high-affinity system only (Table 2). When both inhibitors were tested together, an additive (or perhaps a slightly synergistic) effect was observed.

Cytochalasin B, an inhibitor of actin polymerization, is also a potent inhibitor of the human erythrocyte glucose transporter with a K_i of approx. 0.5 μM [41]. In *P. carinii*, 2-DOG uptake by the high-affinity system was more sensitive to cytochalasin B than was the low-affinity carrier system. However, the cytochalasin B concentration required to inhibit 2-DOG uptake by *P. carinii* was more than approximately a thousand times higher than that reported for mammalian transporters [41] (Table 2). Thus, the 2-DOG transporter in *P. carinii* is relatively insensitive to this inhibitor compound compared to that reported for mammalian systems.

It was earlier reported that 8.4 μM of the anti-*P. carinii* drug pentamidine inhibited glucose utilization by 59%, and that the drug also inhibited protein synthesis by 50% at a concentration of 17 μM [9]. In the present study, 100 μM pentamidine was found to have no inhibitory effect on 2-DOG uptake by *P. carinii* (Table 2). The possibility that pentamidine inhibits glucose metabolism was not ruled out.

Since hexose and H^+ translocation have been

Table 2
Effect of inhibitors and ionophores on 2-DOG uptake by *P. carinii*

Inhibitors	Inhibitor concentration	2-DOG uptake (% of controls)	
		High-affinity system	Low-affinity system
Azide	2 mM	122 ± 21	98 ± 8
SHAM	1 mM	33 ± 4	98 ± 2
Azide+SHAM	2 mM+1 mM	38 ± 1	104 ± 4
2,4-DNP	2 mM	48 ± 1	98 ± 1
KCN	2 mM	69 ± 3	107 ± 2
Iodoacetate	1 mM	72 ± 1	112 ± 1
Iodoacetate+KCN+azide+SHAM	1 mM+2 mM+2 mM+1 mM	36 ± 2	104 ± 2
Cycloheximide	1 mM	52 ± 4	94 ± 5
Chloramphenicol	1 mM	53 ± 2	90 ± 2
Cycloheximide+chloramphenicol	1 mM+1 mM	19 ± 2	97 ± 3
Cytochalasin B	150 μM	58 ± 3	90 ± 4
	200 μM	40 ± 2	ND
	300 μM	ND	62 ± 4
Pentamidine	100 μM	112 ± 13	94 ± 2
CCCP	1 μM	37.1 ± 1.2	ND
	10 μM	7.6 ± 0.4	ND
	50 μM	3.3 ± 0.3	87 ± 5
DCCD	100 μM	28 ± 2	105 ± 3
Monensin	100 μM	88 ± 10	81 ± 2
	150 μM	72 ± 2	ND
	300 μM	55 ± 3	83 ± 2
Ouabain	1 mM	75 ± 1	96 ± 11
	5 mM	46 ± 2	75 ± 2
	10 mM	ND	77 ± 3

Organisms were preincubated with compounds then tested for the uptake of 2-DOG. The initial rates of 100 μM and 10 mM 2-DOG were measured over a 1 min period. Percent of control values are expressed as the means ± S.E.M.; $n = 3$.

found to be coupled in several cell types, the proton ionophore carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was tested for its effect on 2-DOG uptake by *P. carinii*. CCCP has been previously shown to cause a drop of cytoplasmic pH [38] in this organism, but the ionophore had no effect on the uptake of tyrosine, arginine [32], or serine [37]. CCCP was the most potent inhibitor tested in the present study. At a concentration of only 1 μM, uptake via the high-affinity 2-DOG transporter was reduced by more than 50%. Like CCCP, the plasma membrane H^+-ATPase inhibitor *N*,*N*′-dicyclohexylcarbodiimide (DCCD) was previously shown to decrease *P. carinii* intracellular pH [38], and DCCD also caused dissipation of the cell membrane potential [42]. This inhibitor also reduced 2-DOG uptake by the high-affinity system, suggesting that a transmembrane H^+ gradient was needed for glucose uptake by *P. carinii*. The effects of DCCD and CCCP on glucose transport may be the result of disruption of the proton gradient across the inner mitochondrial membrane that led to decreased ATP and/or the proton gradient across the cell surface membrane.

Since symport co-transport of glucose with Na^+ is common in biological systems, the role of Na^+ on 2-DOG uptake in *P. carinii* was investigated. The high-affinity 2-DOG uptake system was sensitive to both the (Na^+-K^+)-ATPase (NKA) inhibitor, ouabain, and the Na^+/H^+ antiport ionophore monensin (Table 2). Furthermore, replacement of NaCl in the buffered incubation solution with equimolar choline chloride resulted in a marked decrease in 2-DOG uptake by the high-affinity transporter. After inhibition in Na^+-free medium, recovery of the 2-DOG uptake was Na^+ concentration-dependent (Fig. 2). The stoichiometry of extracellular Na^+ and 2-DOG uptake, according to the Hill plot, was 1:1. The results on testing Na^++glucose co-transport by *P. carinii* are consistent with the finding of a gene, by expressed sequence tag (EST) techniques, in the ongoing *Pneumocystis* Genome Project, which is homologous to a gene coding for a low-affinity Na^+/glucose transporter (SGLT2) of human kidney cells. The low-affinity system was not inhibited by removal of extracellular Na^+, but may have functioned more efficiently under those conditions. The uptake of 2-DOG by the low-affinity high-capacity system in the Na^+-free medium was 140 ± 4% of control.

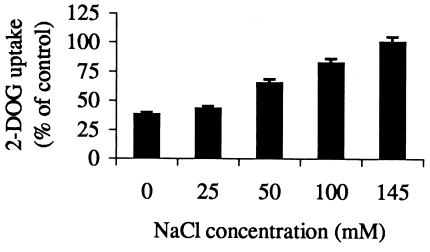

Fig. 2. Effects of Na^+ concentration on 2-DOG uptake by *P. carinii*. 2-DOG high-affinity transporter (100 μM) was measured at various sodium concentrations in the incubation medium, which was altered by isosmotic substitution of NaCl with choline chloride. Values are expressed as means ± S.E.M. of percent of control (250 ± 22 pmol/mg protein/min); $n = 3$.

4. Discussion

4.1. Glucose nutritional requirement and uptake mechanisms in P. carinii

In this report we demonstrate that *P. carinii* can take up glucose via two distinct transporter mechanisms. The high-affinity transporter system appears to require energy from ATP, extracellular Na^+ and a H^+ gradient across the cell surface membrane. The reason for inhibition of 2-DOG uptake by ouabain is not obvious since it binds to the external K^+-binding site of the NKA complex within the cell surface membrane. It appears that unlike cells whose cell surface membrane potentials are primarily maintained by these pumps, the membrane potential across the cell surface of *P. carinii* involves a different mechanism, i.e. a proton gradient. It is not known whether there is an NKA in the cell surface membrane of *P. carinii*, but these results suggest that the organism has an ouabain-binding NKA, and that it inhibits 2-DOG uptake as a consequence of reduced Na^+ being pumped to the external surface of the cell. It is not known if the requirement for H^+ is for maintenance of a proton motive force at the cell membrane to drive glucose uptake, or if the *P. carinii* carrier molecule requires binding of both glucose and H^+ to function.

By having two glucose transporter systems, the organism can obtain glucose at optimal rates under

a broad range of nutrient concentrations in the environment. When glucose is available in high concentrations, both the active transport and facilitated diffusion systems would provide high levels of this nutrient to the organism. Facilitated diffusion of glucose is commonly found among diverse organisms, having been described in human erythrocytes [16], *Trypanosoma cruzi* [17], *Trypanosoma brucei brucei* bloodstream form [18], *Crithidia luciliae* [19], *Plasmodium falciparum* [15] and *Saccharomyces cerevisiae* [27]. When the exogenous glucose level is low and the facilitated diffusion mechanism is not operational, the high-affinity uphill active transport mechanism would continue providing glucose uptake by the cells. In *P. carinii*, this system is linked for symport active transport with $Na^{+,}$ which may function in a similar manner as the glucose+Na^+ systems described in intestinal brush border, renal epithelial cells [20,21], and the bacterium *Vibrio parahaemolyticus* [22]. Other inhibitors of Na^+ transport, amiloride, benzamil, and dimethyl amiloride, inhibited proliferation of *P. carinii* short-term culture [43]. It is not known if the inhibition of glucose transport in the absence of extracellular Na^+ contributes to the inhibition of growth of the organism.

H^+/glucose symport systems have been identified in bacteria [10–12,23], *Leishmania* [29], *T. brucei brucei* procyclic forms [28], *Schizosaccharomyces pombe* [25] and the water fungus *Achlya* [24]. In *Escherichia coli*, it has been shown that melibiose transport requires both Na^+ and H^+ translocation [23]. The results demonstrating the requirement of the high-affinity 2-DOG transporter for a H^+ gradient suggest that the system is directly driven by a proton motive force at the cell surface, which in turn requires ATP for the development and maintenance of the transmembrane proton gradient.

The glucose requirement for *P. carinii* growth in an axenic culture medium is an important finding for understanding the nature of the organism's nutrition, and this also provides insight into the mechanism of uptake of other nutrients. *P. carinii* does not take up serine in the absence of glucose in the medium, although there was no obvious energy requirement found for serine uptake. An alternative explanation was that glucose and serine uptake were linked and transported by a common carrier system. In the present study, the finding that serine had no effect on 2-DOG uptake eliminated the idea of co-transport, thus the influence of glucose on amino acid uptake by *P. carinii* remains unexplained.

4.2. Comparison of *P. carinii* and mammalian GLUT glucose transporters

Analysis of the substrate specificity of *P. carinii* transport systems showed that hydroxyl groups located at positions C-3, C-5 and C-6 are vital for recognition by the transporters whereas positions C-2 and C-4 are less important. The *P. carinii* 2-DOG uptake systems recognized fructose, which differs from the mammalian erythrocyte GLUT1 glucose transporter [44]. The mammalian liver glucose transporter GLUT2 is able to transport fructose but has only a low affinity for this hexose sugar [45], and thus differs from the high-affinity 2-DOG uptake system of *P. carinii*. Similar to the Na^+/glucose co-transporter of *V. parahaemolyticus* [22], both *P. carinii* 2-DOG uptake systems were inhibited by galactose. In contrast, the mammalian GLUT1 transporter exhibited only very low affinity for galactose [44]. It was reported that the renal Na^+/glucose co-transporter system SGLT2 [46] also has only low affinity for this sugar. The ability of the *P. carinii* 2-DOG uptake systems to interact with fructose and galactose indicates that there are important differences between glucose carriers in mammals and *P. carinii*. Also, the *P. carinii* 2-DOG uptake systems were far less sensitive to cytochalasin B compared to GLUT1 [41]. These differences may be targeted for the development of new chemotherapeutic approaches for *P. carinii* infection.

Acknowledgements

The authors thank Mike A. Wyder for technical assistance. This work was supported in part by a US Public Health Service Grant RO1 AI29316.

References

[1] P.D. Walzer, *Pneumocystis carinii*, in: G.L. Mandell, J.E. Bennett, P. Dollin (Eds.), Principles and Practice of Infectious Diseases, Churchill Livingstone, New York, 2000, pp. 2781–2795.

[2] M.T. Cushion, D. Ebbets, Growth and metabolism of *Pneumocystis carinii*, J. Clin. Microbiol. 28 (1990) 1385–1394.
[3] S. Merali, U. Frevert, J.H. Williams, K. Chin, R. Bryan, A.B. Clarkson Jr., Continuous axenic cultivation of *Pneumocystis carinii*, Proc. Natl. Acad. Sci. USA 96 (1999) 2402–2407.
[4] C.H. Lee, N.L. Bauer, M.M. Shaw, M.M. Durkin, M.S. Bartlett, S.F. Queener, J.W. Smith, Proliferation of rat *Pneumocystis carinii* on cells sheeted on microcarrier beads in spinner flasks, J. Clin. Microbiol. 31 (1993) 1659–1662.
[5] M.T. Cushion, J.J. Ruffolo, M.J. Linke, P.D. Walzer, *Pneumocystis carinii*: growth variables and estimates in A549 and WI38 VA 13 human cell lines, Exp. Parasitol. 60 (1985) 43–54.
[6] E. Sloand, B. Laughon, M. Armstrong, M.S. Bartlett, W. Blumenfield, M. Cushion, A. Kalica, J.A. Kovacs, W. Martin, E. Pitt, E.L. Pesanti, F. Richards, R. Rose, P.D. Walzer, The challenge of *Pneumocystis carinii* culture, J. Eukaryotic Microbiol. 40 (1993) 188–195.
[7] J.A. De Stefano, J.D. Myers, D. Du Pont, J.M. Foy, S.A. Theus, P.D. Walzer, Cell wall antigens of *Pneumocystis carinii* trophozoites and cysts: purification and carbohydrate analysis of these glycoproteins, J. Eukaryotic Microbiol. 45 (1998) 334–343.
[8] D.J. Williams, J.A. Radding, A. Dell, K.H. Khoo, M.E. Rogers, F.F. Richards, M.Y. Armstrong, Glucan synthesis in *Pneumocystis carinii*, J. Protozool. 38 (1991) 427–437.
[9] E.L. Pesanti, C. Cox, Metabolic and synthetic activities of *Pneumocystis carinii* in vitro, Infect. Immun. 34 (1981) 908–914.
[10] M. Tangney, F.G. Priest, W.J. Mitchell, Two glucose transport systems in *Bacillus licheniformis*, J. Bacteriol. 175 (1993) 2137–2142.
[11] C. Parker, R.W. Hutkins, *Listeria monocytogenes* scott A transports glucose by high-affinity and low-affinity glucose transport systems, Appl. Environ. Microbiol. 63 (1997) 543–546.
[12] J.B. Russell, Low-affinity, high-capacity system of glucose transport in the ruminal bacterium *Streptococcus bovis*: evidence for a mechanism of facilitated transport, Appl. Environ. Microbiol. 56 (1990) 3304–3307.
[13] F. Bringaud, T. Baltz, African trypanosome glucose transporter genes: organization and evolution of a multigene family, Mol. Cell. Biol. 13 (1993) 1146–1154.
[14] S. Heiland, N. Radovanovic, M. Hofer, J. Winderickx, H. Lichtenberg, Multiple hexoses transporters of *Schizosaccharomyces pombe*, J. Bacteriol. 182 (2000) 2153–2162.
[15] K. Kirk, H.A. Horner, J. Kirk, Glucose uptake in *Plasmodium falciparum*-infected erythrocytes is an equilibrative not an active process, Mol. Biochem. Parasitol. 82 (1996) 195–205.
[16] M. Mueckler, Facilitative glucose transporters, Eur. J. Biochem. 219 (1994) 713–725.
[17] E. Tetaud, F. Bringaud, C. Chabas, M.P. Barrett, T. Baltz, Characterization of glucose transport and cloning of a hexose transporter gene in *Trypanosoma cruzi*, Proc. Natl. Acad. Sci. USA 91 (1994) 8278–8282.
[18] B.H. Ter Kuile, F.R. Opperdoes, Mutual adjustment of glucose uptake and metabolism in *Trypanosoma brucei* grown in a chemostat, J. Bacteriol. 174 (1992) 2929–2934.
[19] L.A. Knodler, P.J. Schofield, M.R. Edwards, Glucose transport in *Crithidia luciliae*, Mol. Biochem. Parasitol. 56 (1992) 1–14.
[20] G.A. Kimmich, J. Randles, D. Restrepo, M. Montrose, The potential dependence of the intestinal Na^+-dependent sugar transporter, Ann. NY Acad. Sci. 456 (1985) 63–76.
[21] J.C. Beck, B. Sacktor, The sodium electrochemical potential-mediated uphill transport of D-glucose in renal brush border membrane vesicle, J. Biol. Chem. 253 (1978) 5531–5535.
[22] Z. Xie, E. Turk, E.M. Wright, Characterization of the *Vibrio parahaemolyticus* Na^+/Glucose cotransporter, J. Biol. Chem. 275 (2000) 25959–25964.
[23] P.J. Henderson, Proton-linked sugar transport systems in bacteria, J. Bioenerg. Biomembr. 22 (1990) 525–569.
[24] S.H. Goh, H.B. LeJohn, Glucose transport in *Achlya*: characterization and possible regulatory aspects, Can. J. Biochem. 56 (1978) 246–256.
[25] M. Hofer, F.R. Nassar, Aerobic and anaerobic uptake of sugars in *Schizosaccharomyces pombe*, J. Gen. Microbiol. 133 (1987) 2163–2172.
[26] H. Lichtenberg-Frate, T. Naschen, S. Heiland, M. Hofer, Properties and heterologous expression of the glucose transporter GHT1 from *Schizosaccharomyces pombe*, Yeast 13 (1997) 215–224.
[27] R. Lagunas, Sugar transport in *Saccharomyces cerevisiae*, FEMS Microbiol. Rev. 104 (1993) 229–242.
[28] M. Parsons, B. Nielsen, Active transport of 2-deoxy-D-glucose in *Trypanosoma brucei* procyclic forms, Mol. Biochem. Parasitol. 42 (1990) 197–204.
[29] R.J.S. Burchmore, D.T. Hart, Glucose transport in amastigotes and promastigotes of *Leishmania mexicana mexicana*, Mol. Biochem. Parasitol. 74 (1995) 77–86.
[30] C.J. Boylan, W.L. Current, Improved model of *Pneumocystis carinii* pneumonia: induced laboratory infection in *Pneumocystis*-free animals, Infect. Immun. 60 (1992) 1589–1597.
[31] E.S. Kaneshiro, M.A. Wyder, L.H. Zhou, J.E. Ellis, D.R. Voelker, S.G. Langreth, Characterization of *Pneumocystis carinii* preparations developed for lipid analysis, J. Eukaryotic Microbiol. 40 (1993) 805–815.
[32] M. Basselin, K.J. Lipscomb, Y.H. Qiu, E.S. Kaneshiro, Transport of aspartic acid, arginine, and tyrosine by the opportunistic protist *Pneumocystis carinii*, Biochim. Biophys. Acta 1511 (2001) 349–359.
[33] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.
[34] M. Somogyi, Determination of blood sugar, J. Biol. Chem. 160 (1945) 69–73.
[35] G. Spears, J.G.T. Sneyd, E.G. Loten, A method for deriving kinetic constants for two enzymes acting on the same substrate, Biochem. J. 125 (1971) 1149–1151.

[36] M. Basselin, Y.H. Qui, K.J. Lipscomb, E.S. Kaneshiro, Uptake of the neutral amino acids glutamine, leucine and serine by *Pneumocystis carinii*, Arch. Biochem. Biophys. 391 (2001) 90–98.
[37] M.T. Cushion, F. Chen, N. Kloepfer, A cytotoxicity assay for evaluation of candidate anti-*Pneumocystis carinii* agents, Antimicrob. Agents Chemother. 41 (1997) 379–384.
[38] R. Docampo, N.M.J. VanderHeyden, M.M. Shaw, P.J. Durant, M.S. Bartlett, J.W. Smith, G. McLaughlin, An H^+-ATPase regulates cytoplasmic pH in *Pneumocystis carinii* trophozoites, Biochem. J. 316 (1996) 681–684.
[39] S. Merali, D. Vargas, M. Franklin, A.B. Clarkson Jr., *S*-Adenosylmethionine and *Pneumocystis carinii*, J. Biol. Chem. 275 (2000) 14958–14963.
[40] E.L. Pesanti, *Pneumocystis carinii*: oxygen uptake, antioxidant enzymes, and susceptibility to oxygen-mediated damage, Infect. Immun. 44 (1984) 7–11.
[41] C.Y. Jung, A.L. Rampal, Cytochalasin B binding sites and glucose transport carrier in human erythrocyte ghosts, J. Biol. Chem. 252 (1977) 5456–5463.
[42] N. VanderHeyden, G.L. McLaughlin, R. Docampo, Regulation of the plasma membrane potential in *Pneumocystis carinii*, FEMS Microbiol. Lett. 183 (2000) 327–330.
[43] M.M. Shaw, T.R. Kleyman, M.S. Bartlett, J.W. Smith, Sodium transport inhibitors decrease proliferation of *Pneumocystis carinii* in short-term culture, J. Eukaryotic Microbiol. 41 (1994) 110S.
[44] J.E.G. Barnett, G.D. Holman, K.A. Munday, Structural requirements for binding to the sugar-transport system of the human erythrocyte, Biochem. J. 131 (1973) 211–221.
[45] G.W. Gould, H.M. Thomas, T.J. Jess, G.I. Bell, Expression of human glucose transporters in *Xenopus oocytes*: kinetic characterization and substrate specificities of the erythrocyte, liver, and brain isoforms, Biochemistry 30 (1991) 5139–5145.
[46] E.M. Wright, Renal Na(+)-glucose cotransporters, Am. J. Physiol. 280 (2001) F10–F18.