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Transport of aspartic acid, arginine, and tyrosine by the opportunistic protist *Pneumocystis carinii*

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Abstract

In order to improve culture media and to discover potential drug targets, uptake of an acidic, a basic, and an aromatic amino acid were investigated. Current culture systems, axenic or co-cultivation with mammalian cells, do not provide either the quantity or quality of cells needed for biochemical studies of this organism. Insight into nutrient acquisition can be expected to lead to improved culture media and improved culture growth. Aspartic acid uptake was directly related to substrate concentration, Q_{10} was 1.10 at pH 7.4. Hence the organism acquired this acidic amino acid by simple diffusion. Uptake of the basic amino acid arginine and the aromatic amino acid tyrosine exhibited saturation kinetics consistent with carrier-mediated mechanisms. Kinetic parameters indicated two carriers ($K_m = 22.8 \pm 2.5$ μM and $K_m = 3.6 \pm 0.3$ mM) for arginine and a single carrier for tyrosine ($K_m = 284 \pm 23$ μM). The effects of other L-amino acids showed that the tyrosine carrier was distinct from the arginine carriers. Tyrosine and arginine transport were independent of sodium and potassium ions, and did not appear to require energy from ATP or a proton motive force. Thus facilitated diffusion was identified as the mechanism of uptake. After 30 min of incubation, these amino acids were incorporated into total lipids and the sedimentable material following lipid extraction; more than 90% was in the cellular soluble fraction.

Keywords: Amino acid transport; Facilitated diffusion; Simple diffusion; *Pneumocystis*

1. Introduction

Pneumocystis carinii is an opportunistic extracellular pathogen of the lung alveolus, and the etiologic agent of *P. carinii* pneumonia (PcP). The infection causes morbidity and mortality in immunosuppressed patients such as those with AIDS. Pentamidine and a combination of trimethoprim and sulfamethoxazole (TMP/SMZ, Bactrim) are the main treatments of choice for PcP, but these can cause severe side effects in some patients [1]. Research on the organism has been hampered by the lack of an axenic culture protocol that provides large numbers of organisms to study [2]. Axenic long-term cultivation has recently been achieved [3], but additional work is required to improve organism proliferation and culture growth. With only a few exceptions [4], most endogenous metabolic and biosynthetic processes as well as exogenous nutrient acquisition mechanisms have yet to be examined in *P. carinii*. The slow progress in the development of axenic cul-

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenyl hydrazone; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; KCN, potassium cyanide; Q_{10}, temperature coefficient; SHAM, salicylhydroxamic acid

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ture methods has in part been responsible for this lack of information. Examination of the pathogen's membrane transport processes may reveal attractive chemotherapeutic targets. Such studies can also provide data on the mechanisms by which exogenous nutrients are taken up, and this kind of information may be useful for improving culture media formulations.

Amino acid influx and efflux are essential for cellular homeostasis because they play important roles in a multitude of fundamental functions such as protein and lipid synthesis, osmoregulation, cell growth and development, and energy production. In many cell types, selective permeability of biomembranes to water-soluble solutes such as amino acids have been demonstrated, and have shown to be facilitated by protein carrier molecules. Cells commonly have a carrier system for acidic amino acids, and a separate one for the basic amino acids. More than one carrier of the neutral amino acids is often detected in organisms. Prokaryotes [5], the yeasts *Saccharomyces* [6,7] and *Schizosaccharomyces* [8–11], *Leishmania* [12,13], *Trypanosoma* [14,15], and mammalian cells [16] possess several amino acid transporters; most that have been studied function by energy-requiring active mechanisms that transport these compounds against a chemical gradient.

Recently it was shown that, unlike many other lower eukaryotes analyzed, *P. carinii* did not take up the neutral amino acids leucine, glutamine, and serine by active transport. These compounds were transported by facilitated diffusion, and utilized a common carrier system (M. Basselin, Y.H. Qiu, K.J. Lipscomb, E.S. Kaneshiro, unpublished results). In the present study, the mechanisms by which *P. carinii* takes up aspartic acid (acidic), arginine (basic), and tyrosine (aromatic) were investigated to provide a better overview of how various groups of amino acids are transported by this important opportunistic pathogen.

2. Materials and methods

2.1. Chemicals

L-[3,5-^{14}C]Tyrosine (43 Ci/mmol) was purchased from Amersham (Piscataway, NJ, USA). L-[2,3,4,5-^{3}H]Arginine monohydrochloride (45 Ci/mmol) and L-[2,3-^{3}H]aspartic acid (28 Ci/mmol) were from American Radiolabeled Chemicals (Saint Louis, MO, USA). L-Aspartic acid, L-arginine, L-tyrosine, ouabain, valinomycin, choline chloride, lithium chloride, salicylhydroxamic acid (SHAM), carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), gramicidin, *N*,*N*′-dicyclohexylcarbodiimide (DCCD), and bovine serum albumin were from Sigma-Aldrich (Saint Louis, MO, USA). Potassium cyanide (KCN) and sodium azide were obtained from Fisher Scientific Co. (Springfield, NJ, USA).

2.2. Organisms

P. carinii f. sp. *carinii* was isolated from the lungs of rats that developed PcP using the Boylan and Current [17] corticosteroid-immunosuppressed animal model. Briefly, viral antibody-negative female Lewis rats (Harlan Sprague–Dawley, Indianapolis, IN, USA) were immunosuppressed with methylprednisolone acetate (Depo-Medrol; Upjohn Co., Kalamazoo, MI, USA) and were twice inoculated intratracheally with 10^{6}–10^{7} mixed-life-cycle stages of cryopreserved organisms [18]. Lung homogenates containing *P. carinii* that were used for inoculation into rats were prepared under aseptic conditions in the absence of antibiotics [18]. 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 according to the Kaneshiro et al. protocol [18]. Briefly, lung pieces were homogenized (Stomacher; Tekmar, Cincinnati, OH, USA) with the mucolytic sulfhydryl agent glutathione, 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 absence of host contaminants was demonstrated by microscopic, biochemical, and immunochemical analyses; the preparations were found to be 95–100% pure [18]. The organism preparations were also shown to be

> 99% and 100% pure with respect to potential bacterial and fungal contaminants, respectively. The *P. carinii* used for biochemical studies were prepared under normal open-laboratory bench conditions, thus the final preparations were analyzed for microbial contamination that occur during these routine isolation and purification procedures. No fungal colonies were detected in the analyses of six separate preparations after incubation overnight at room temperature (0% fungal contamination). The same preparations tested for bacterial contaminants were incubated at 37°C, and then the colonies were counted. The contamination from bacteria was calculated with respect to the number of *P. carinii* organisms. The results indicated an average of $0.065 \pm 0.033\%$ bacteria (> 99% *P. carinii*); the highest value observed was 0.19% bacteria (> 99% *P. carinii*).

These preparations contained 10–30% cystic forms and 80–95% viable organisms [19]. 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 ($n = 10$) [18]. In contrast, the volume of final purified *P. carinii* organism pellets isolated from a single infected rat (10^8–10^9 organisms) is 0.3–0.4 ml containing approximately 5 mg protein [18].

2.3. Amino acid uptake analyses and incorporation

Organisms (10^8–10^9) were centrifuged at $926 \times g$ for 10 min at 4°C and then the pellet was resuspended in a HEPES-buffered solution (100 mM HEPES, 1.8 mM $CaCl_2$, 0.85% NaCl) supplemented with 2% glucose at pH 7.4 at a density of approximately 5×10^7 organisms/ml. Organism suspensions (0.8 ml) were incubated with 2–5 μCi L-[3,5-^{14}C]tyrosine, 2–5 μCi L-[2,3,4,5-^{3}H]arginine HCl, 2–5 μCi L-[2,3-^{3}H]aspartic acid, plus or minus other non-radioactive amino acids at the concentrations indicated in the text. After 30 min at 37°C, the suspensions were centrifuged, washed twice with 10 ml of HEPES-buffered solution, centrifuged into a packed pellet and then the lipids were extracted. The 30-min incubation period was selected since uptake of amino acids is slow and shorter times did not result in sufficient radioactivity in cells to perform experiments using the same organism preparation for testing different incubation times, substrate concentrations, inhibitors and untreated controls. One-minute incubations with 1 μCi tritiated aspartate, arginine, or tyrosine resulted in only < 50–175 total cpm (10^7 organisms). Also, a 30-min incubation enabled the detection of incorporation into lipids and proteins. The effects of ionophores and inhibitors on amino acid transport was studied by incubating cells with the compounds for 10 min prior to performing the uptake assays.

Total lipids were extracted according to the method of Bligh and Dyer [19], and purified by biphasic partitioning as described by Folch et al. [20]. Aliquots of the pellet fraction, lower organic phase and upper aqueous phase were mixed with 5 ml of liquid scintillation fluid to determine the radioactivity incorporated into water-soluble material, extracted lipids, and material in the pellet obtained following lipid extraction. Counting efficiencies were 45 and 94% for [^{3}H] and [^{14}C], respectively.

Protein concentration was measured by the method of Lowry et al. [21] with bovine serum albumin as a standard. The results were expressed in pmol of amino acid/mg of *P. carinii* total proteins/min. Statistical analyses and plotting of data were performed using Microsoft Excel 2000. Values of apparent kinetic parameters were resolved using the method of Spears et al. [22] when two transporters were detected for a single amino acid. Temperature coefficient (Q_{10}) values were calculated by the van't Hoff equation $Q_{10} = (k_2/k_1)^{10/t_2 - t_1}$.

Inhibitor compound and amino acids that were tested for their effects on the uptake of arginine and tyrosine were not analyzed using broad ranges of concentration. For this report, other amino acids and inhibitor compounds were considered effective if they decreased the uptake of the amino acid of interest by 70% of control values. Inhibition by 70% of control, without increasing inhibitor concentration or other experimental conditions, was a sufficiently substantial value to be considered a real effect.

3. Results

3.1. Uptake of the acidic amino acid L-aspartic acid

The time course for the uptake of 1 mM aspartic acid at pH 7.6 was linear up to 45 min (Fig. 1A). Aspartate uptake was then measured for 30 min using 0.22 μM to 5 mM substrate at pH 7.4 and from 0.09 μM to 20 mM substrate at pH 5.5. The plot of the initial velocity against the concentration of aspartic acid was linear (Fig. 1B,C). The uptake of aspartic acid was directly related to the substrate concentration; saturation was not demonstrable. These results indicating adherence to Fick's Law showed that this acidic amino acid was taken up by *P. carinii* by simple diffusion; a carrier was not involved. The values of the diffusion constant could be calculated from the slopes of the linear plots at both pH values. The permeability of the plasma membrane appeared to be significantly affected by the extracellular pH, with the diffusion constant increasing strongly at pH 5.5. Addition of 2.5 and 5 mM glutamic acid did not inhibit the diffusion of 1 mM aspartic acid at pH 7.4 (93.1 and 91.5%, respectively of that without glutamate addition). Similar results were obtained at pH 5.5.

The effects of temperature on aspartate uptake were analyzed at pH 7.4 and pH 5.5. Cell suspensions were held on ice (4°C) and uptake was compared to those at 37°C. The Q_{10} values were 1.10 at pH 7.4 and 1.34 at pH 5.5, which are consistent with the effects of temperature on physical processes.

After 30 min of incubation, 91.3% of the radioactivity from aspartic acid was found in the cytosolic soluble fraction. Radioactivity from aspartate was incorporated into total extractable lipids (3.1%), and in the pellet comprised of proteins and other material after total lipids had been extracted (5.6%).

3.2. Transport of the basic amino acid L-arginine

Arginine uptake from 1- to 60-min incubation periods was measured; uptake was approximately linear during the first 35 min (Fig. 2A), therefore subsequent experiments were standardized to 30-min incubations. The uptake at different substrate concentrations was determined using 0.125 μM to 15 mM of arginine. The uptake was saturable (Fig. 2B), indicating that uptake was not by simple diffusion but was by facilitated transport mechanisms.

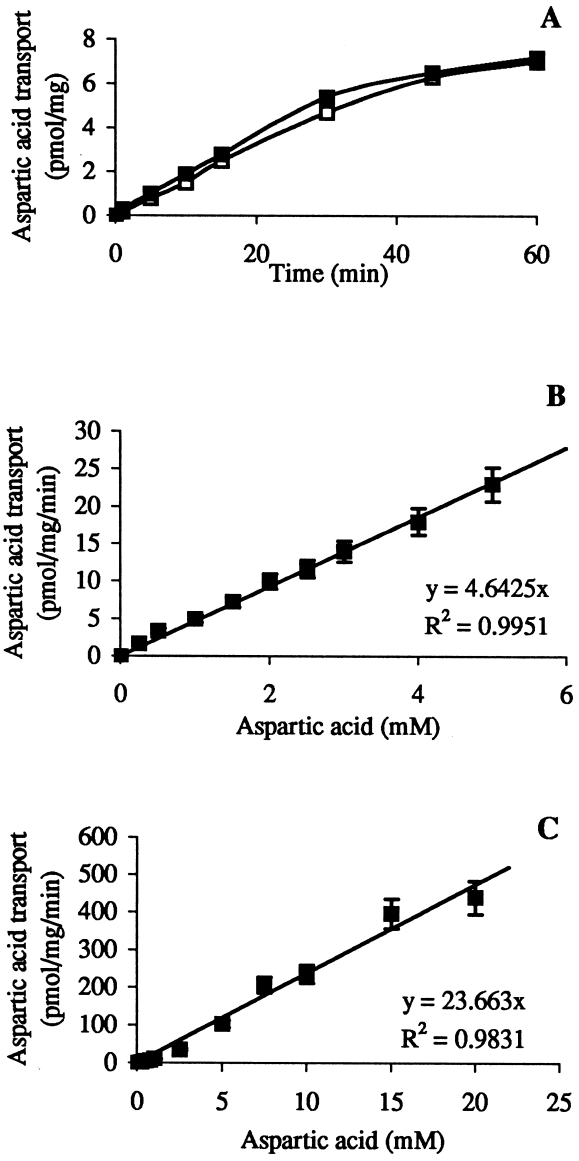

Fig. 1. Kinetics of aspartic acid transport by *P. carinii*. Time course of aspartic acid uptake was measured between 1 and 60 min at a substrate concentration of 1 mM, pH 7.4. Closed and open symbols represent values from two independent experiments (A). The rate of aspartic acid uptake was measured over the concentration range of 0.22 μM to 5 mM at pH 7.4 for 30 min at 37°C (B) and between 0.09 μM and 20 mM aspartic acid at pH 5.5 (C). Each point in B and C represents the means ± S.E.M. of three independent experiments.

Two separate arginine transport systems were detected by evaluating uptake rates using the Eadie–Hofstee plot (Fig. 2C). The K_m values for the high-

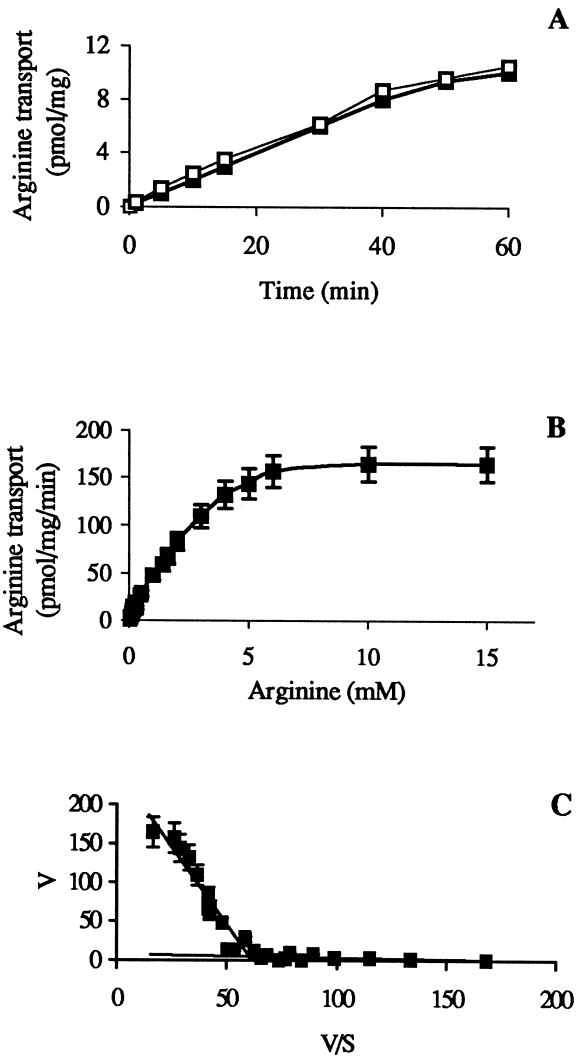

Fig. 2. Kinetics of arginine transport by *P. carinii*. (A) Time course of arginine uptake was measured between 1 and 60 min at a substrate concentration of 1.5 μM. Closed and open symbols represent two independent experiments. (B) The rate of arginine uptake was analyzed at pH 7.4 at varying substrate concentrations using 30-min incubations. (C) Eadie–Hofstee plots were used to determine the kinetic parameters for the two transporters. Each point represents the mean ± S.E.M. of three separate experiments.

affinity and low-affinity transporters were 22.8 ± 2.5 μM and 3.6 ± 0.3 mM, respectively. The velocities of the carriers at K_m were 2.15 ± 0.07 and 128 ± 7 pmol/mg protein/min, respectively. The V_{max} values were 2.45 ± 0.17 pmol/mg protein/min and 217 ± 9 pmol/mg protein/min, respectively. The K_m values for lysine uptake in the yeasts *Agaricus* (32 μM) [23] and *Schizosaccharomyces pombe* (26 μM) [9], the flagellated protozoan parasites *Leishmania* (15 μM) [13], *Trypanosoma* (25 μM) [14], and *Giardia* (15 μM) [24] were comparable to that of the high-affinity *P. carinii* arginine transporter. The K_m value for lysine uptake by the low-affinity transporter in *S. pombe* was 1.1 mM [7], which was lower than that of the low-affinity *P. carinii* arginine transporter, but in both organisms the K_m values of the low-affinity basic amino acid transporters were in the millimolar ranges.

Temperature-sensitivity for arginine uptake by *P. carinii* was demonstrated. When the organism suspensions were held on ice, transport was inhibited. Uptake by the high-affinity transporter was only 15 ± 6% of that measured at 37°C, and uptake by the low-affinity transporter was 19 ± 5% of the 37°C control. The calculated Q_{10} values for the high- and low-affinity transporters for the basic amino acid arginine were 1.78 and 1.65, respectively. These were not like the low values typical of physical processes, such as that of uptake of the acidic amino acid aspartic acid shown above. However, these Q_{10} values were not typical of enzymatic and transport processes such as that clearly shown for tyrosine (see below). In addition to its interaction with its carrier, the charge on this basic amino acid may alter its translocation rate by physical interactions at or within the cell surface membrane.

After 30-min incubation with exogenous radiolabeled arginine, the pellet obtained after lipid extraction, which included cellular proteins contained 3.2% of the total radioactivity in the organisms. Radioactivity was also incorporated into total extractable cellular lipids (2.7%); however, most activity was found in the cytosolic soluble fraction (94.1%).

The basic amino acid lysine inhibited both of the arginine transporters (Table 1). The neutral amino acid leucine had a slight affect on arginine uptake; it inhibited only the high-affinity arginine transporter. Leucine inhibited this arginine transporter by 51% using a leucine/arginine ratio of 8.7. Another neutral amino acid glutamine was tested; it did not have a particularly notable effect on the high- and low-affinity arginine transport systems. The acidic amino acid aspartic acid and the aromatic phenylalanine exhibited no effects on the uptake of arginine by *P. carinii* (Table 1).

Arginine uptake by *P. carinii* was not altered by KCN, azide, and azide plus SHAM, indicating that

Table 1
Effects of amino acids on arginine uptake by *P. carinii*

Amino acid added	Arginine uptake (percent of control)	
	high-affinity carrier 1	low-affinity carrier 2
None (control)	100	100
Lysine	58 ± 2	71 ± 2
(200 μM)	56 ± 3	–
(15 mM)	–	48 ± 3
Glutamine	79 ± 2	91 ± 4
(200 μM)	81 ± 3	–
Leucine	77 ± 4	101 ± 5
(200 μM)	49 ± 2	–
Phenylalanine	108 ± 5	78 ± 3
Aspartic acid	102 ± 5	103 ± 2

Uptake by both the high-affinity and low-affinity arginine carriers were examined at substrate concentrations of 23 μM and 3.6 mM, respectively. Incubations of *P. carinii* suspensions with radiolabeled arginine was performed at pH 7.4 for 30 min in the absence or the presence of another amino acid at concentrations of 100 μM (high-affinity carrier 1) or 10 mM (low-affinity carrier 2). Results in each experiment were normalized to percent of control (velocity at K_m). Values are expressed as means of the percent of control values ± S.E.M. of three independent experiments.

uptake did not require energy from ATP generated from mitochondrial reactions (Table 2). However, it cannot be ruled out that there are sufficient levels of ATP in the cells generated from glycolysis to provide energy for arginine transport. These inhibitor compounds were also tested using 1-min incubations to examine arginine uptake under conditions that minimize the potential effects of radiolabeled arginine efflux from cells. The same results were obtained as those using 30-min incubations (data not shown). The inhibitor of the Na^++K^+ ATPase/pump ouabain and the K^+ ionophore valinomycin also did not have an effect on arginine uptake. Substitution of sodium ions by choline did not alter arginine transport (90 ± 6% and 113 ± 6% of control for the high- and low-affinity transporters, respectively). These data showed that arginine transport did not require sodium or potassium. The inhibitor of proton translocation DCCD and the proton ionophore CCCP both showed no inhibitory activity on arginine uptake by *P. carinii*, demonstrating that arginine uptake by this organism did not involve proton transport (Table 2).

3.3. Uptake of the aromatic amino acid L-tyrosine

Experiments on the rate of tyrosine uptake were performed using incubation times between 1 and 60 min (Fig. 3A). Uptake rate was constant up to 40 min of incubation, thus subsequent experiments on kinetic parameters and inhibition studies were standardized for 30-min incubation times. The rate of tyrosine uptake as a function of substrate concentration was tested from 1.4 μM to 5 mM. Saturation was observed indicating that tyrosine transport was carrier-mediated (Fig. 3B). The apparent kinetic parameters were calculated after linear transformation using Lineweaver–Burk and Eadie–Hofstee plots (Fig. 3C,D). The values were 284 ± 23 μM for K_m and 93.4 ± 0.6 pmol/mg protein/min for V_{max}. The uptake velocity for tyrosine at K_m was 35.2 ± 0.8 pmol/mg protein/min. The K_m for tyrosine was sim-

Table 2
Effect of inhibitors on arginine uptake by *P. carinii*

Inhibitor compound	Inhibitor concentration	Arginine uptake (percent of untreated control)	
		high-affinity carrier 1	low-affinity carrier 2
Azide	1 mM	98 ± 3	100 ± 6
Azide+SHAM	1 mM+1 mM	101 ± 3	95 ± 4
KCN	1 mM	96 ± 3	89 ± 2
Ouabain	1 mM	105 ± 4	101 ± 10
DCCD	100 μM	96 ± 5	117 ± 8
CCCP	50 μM	92 ± 6	83 ± 2
Valinomycin	10 μM	86 ± 3	119 ± 5

Organism suspensions were preincubated with the inhibitor compounds for 10 min prior to measuring uptake for a 10-min period. Arginine concentrations of 23 μM and 3.6 mM were used to analyze carrier 1 and carrier 2, respectively. Velocities were normalized to percent of control (absence of inhibitor). Values are expressed as means ± S.E.M. of percent control values from three independent experiments.

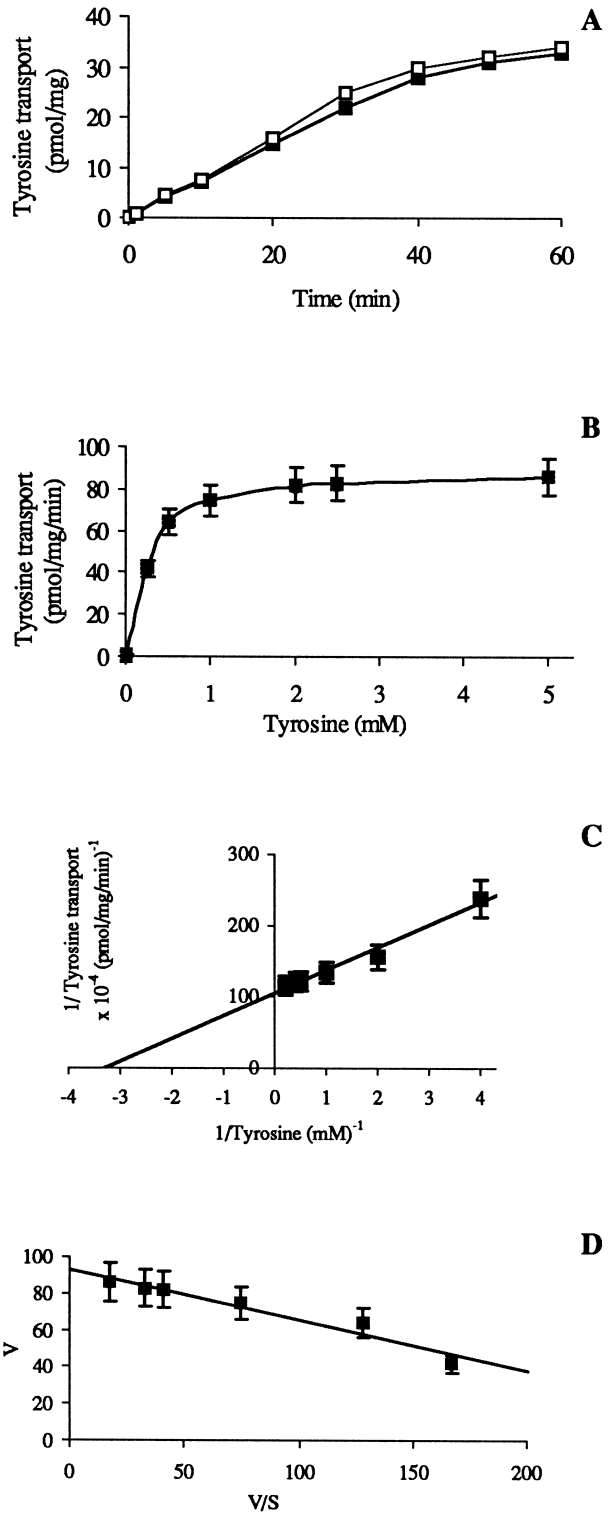

Fig. 3. Kinetics of tyrosine transport by *P. carinii*. (A) Time course of tyrosine transport was studied between 1 and 60 min using 5.55 μM tyrosine. Open and closed symbols represent values obtained in two different experiments. (B) The initial rate of tyrosine transport was measured at pH 7.4 for 30 min using varying substrate concentrations. (C) Kinetic parameters are shown by the double reciprocal Lineweaver–Burk plot. (D) Kinetic parameters illustrated by the Eadie–Hofstee plot. Each point in B, C and D represents the mean ± S.E.M. of three separate experiments
←

ilar to that found in the fission yeast *S. pombe*, which was reported to be about 200 μM [8].

When *P. carinii* organism suspensions were held on ice, transport was reduced to 8.3 ± 1.1% of controls measured at 37°C. The Q_{10} for the uptake process was 2.12, which was typical of those for enzyme reactions and carrier-mediated transport.

To determine the substrate specificity of the transporter, tyrosine uptake was studied in the presence of other L-amino acids. The aromatic amino acids phenylalanine and tryptophan inhibited tyrosine transport by 77 and 76%, respectively. These results indicate that these amino acids may be also taken up by *P. carinii* via the same basic amino acid transporter (Table 3). Interestingly, the neutral amino acid leucine had an equally high inhibitory effect and caused

Table 3
Effect of L-amino acids on L-tyrosine transport by *P. carinii*

Amino acid added (2.5 mM)	Tyrosine transport (percent of control)
None (control)	100
Tyrosine	14 ± 3
Phenylalanine	23 ± 3
Tryptophan	24 ± 2
Leucine	29 ± 4
Valine	36 ± 2
Serine (1.25 mM)	55 ± 3
Glutamine	73 ± 3
(5 mM)	52 ± 4
Proline	95 ± 4
Arginine	101 ± 4
Aspartic acid	116 ± 5

Uptake of radiolabeled tyrosine was determined using 280 μM tyrosine (K_m) at pH 7.4 for 30 min with or without another amino acid. Velocity of tyrosine uptake was expressed as percentage of the velocity observed in the absence of another amino acid (control). Values are means ± S.E.M. of three independent experiments.

a decrease in tyrosine transport by 71%. Other neutral amino acids, valine, serine and glutamine had less effects decreasing tyrosine transport by 64, 45 and 27%, respectively. These results suggest that tyrosine may use a neutral amino acid transporter system or that neutral and aromatic amino acids can share a common transporter. The basic amino acid arginine, the acidic amino acid aspartic acid, and the cyclic amino acid proline did not alter tyrosine transport (Table 3).

The requirements for tyrosine transporter function were examined. Tyrosine uptake by *P. carinii* was not affected by either the Na^+/K^+ ATPase inhibitor ouabain or the K^+ ionophore valinomycin (Table 4). The HEPES-buffered solution used for incubations did not include K^+ salts. Incubation of organisms in a sodium-free buffered solution (substituted by 145 mM choline) did not alter tyrosine uptake (94% of control). Substitution of Na^+ by 145 mM lithium ions also had no effect (97% of control). These results indicated that tyrosine uptake by *P. carinii* did not require extracellular sodium or potassium.

Tyrosine uptake was unaffected by 1- or 30-min incubations with KCN or sodium azide, inhibitors that block cellular respiration and hence the production of ATP (data not shown). That *P. carinii* has a functional alternative oxidase system is supported by a number of experiments showing activity of the inhibitor SHAM on the organism [3,25,26]. Tyrosine uptake was not altered by treatment with SHAM in the presence of sodium azide. Together these results suggested that the uptake of tyrosine by *P. carinii* did not require energy from ATP generated by oxidative phosphorylation.

The proton ionophore CCCP, the cation ionophore gramicidin, and the H^+-ATPase inhibitor DCCD had only trivial effects on tyrosine transport, thus eliminating the possibility that tyrosine uptake was by active transport or by tyrosine–H^+ symport mechanisms (Table 4).

After 30 min incubation with radiolabeled tyrosine, radioactivity was incorporated into lipids (2.9%), the cellular pellet following lipid extraction (1.7%), and in the cytosolic soluble fraction (95.4%).

Table 4
Effect of inhibitors on tyrosine uptake by *P. carinii*

Inhibitor	Inhibitor concentration	Tyrosine uptake (percent of untreated control)
Azide	1 mM	96 ± 12
Azide+SHAM	1 mM+1 mM	93 ± 6
KCN	1 mM	85 ± 6
Ouabain	1 mM	108 ± 12
DCCD	100 μM	110 ± 9
CCCP	50 μM	90 ± 14
Valinomycin	10 μM	106 ± 16
Gramicidin	10 μg/ml	74 ± 8

Pneumocystis organism suspensions were incubated with the inhibitor compounds for 10 min, prior to measuring radiolabeled tyrosine uptake. In each experiment, velocities were normalized to percent of control (absence of inhibitor compound). Values are expressed as means ± S.E.M. of the percent of control values; $n = 3$.

4. Discussion

4.1. Amino acid uptake mechanisms in P. carinii

In many other cell types amino acid uptake occurs at a high rate and is aided by energy-requiring active transport mechanisms. Uptake of these compounds is frequently linked with the translocation of an inorganic ion. In contrast, aspartate, arginine and tyrosine uptake by *P. carinii* appear to be slow, and the results from a separate study on neutral amino acids (M. Basselin, Y.H. Qiu, K.J. Lipscomb, E.S. Kaneshiro, unpublished data) and those reported here are consistent with the absence of active transport mechanisms for concentrating these compounds against a chemical gradient into the cells. Thus far, evidence has been provided to support the following conclusions: (i) aspartic acid, an acidic amino acid enters the organism by simple diffusion; (ii) arginine, a basic amino acid is taken up by facilitated diffusion; (iii) tyrosine, an aromatic amino acid is transported by a carrier, and the mechanism is facilitated diffusion; (iv) neutral amino acids are transported by facilitated diffusion using a separate carrier system (M. Basselin, Y.H. Qiu, K.J. Lipscomb, E.S. Kaneshiro, unpublished data). With the exception of the acidic amino acids, entry of an individual amino acid into *P. carinii* is apparently mediated by a primary transporter system, but some amino acids can also use an alternate secondary carrier system. This suggestion is supported by the strong inhibition exerted

on a specific transporter system (e.g. the tyrosine carrier) by similar amino acids, but other types of amino acids also have effects. Also consistent with this suggestion is the detection of two transporters for the basic amino acid arginine. Leucine had moderate inhibitory effects on the high-affinity arginine uptake system (with no effects on the low-affinity system), and strongly inhibited tyrosine uptake, suggesting that this amino acid can enter cells via multiple carrier systems. Since tyrosine and arginine did not have an effect on leucine uptake (M. Basselin, Y.H. Qiu, K.J. Lipscomb, E.S. Kaneshiro, unpublished data), this supports the suggestion that the aromatic and basic amino acid transporters are distinct from the primary carrier utilized by leucine and other neutral amino acids.

4.2. Comparison of the uptake rates of amino acids and other nutrients

Pneumocystis efficiently takes up lipid substrates [27]. Uptake rates and apparent kinetic parameters for palmitate and oleate suggest the mechanism may be by facilitated transport [28], but detailed experiments were not performed to determine whether uptake of these fatty acids was uphill, required energy or inorganic ions. Recently, the essential nature of *S*-adenosylmethionine (SAM) in *P. carinii* nutrition was demonstrated [29]; the organisms rapidly utilized this compound, which is needed in several metabolic pathways. Uptake of SAM involved two carrier systems, which actively transported the compound into cells against a chemical gradient. Proton-gradient-driven active transport operates in the uptake of amino acids into many organisms [5–14,16]. The gene for a putative cell surface H^+-ATPase was identified in *P. carinii* [30], and more recently, it was reported that H^+-ATPase activity at the cell surface regulates intracellular pH [31] and plays a significant role in the regulation of the cell surface membrane potential of this organism [32]. The involvement of a proton-motive force in nutrient uptake by *P. carinii* appears plausible, but this has yet to be experimentally demonstrated for any compound. In a separate study, we demonstrated that this putative *P. carinii* pump was not required for uptake of serine, glutamine or leucine. In this report, we eliminated the possibility that arginine, aspartic acid and tyrosine are taken up by mechanisms involving the H^+-ATPase/pump at the cell surface.

4.3. Nutritional aspects

Thus far, our results indicate that the uptake rates of individual amino acids by *P. carinii* are all slower than those of SAM [29] and fatty acids [28], and that simple diffusion and facilitated diffusion, but not active transport, are apparently sufficient. Only one gene whose product may facilitate amino acid uptake (amino acid permease) has been identified (by expressed sequence tag techniques) in the ongoing *Pneumocystis* Genome Project (www.uky.edu/projects/pneumocystis). Our experiments have identified at least three amino acid transporters. It is not known whether this expressed amino acid permease gene encodes any of the three amino acid carriers we have thus far identified in this organism.

The slow uptake of exogenous amino acids and the mechanisms operative in *P. carinii* may indicate that the organism synthesizes de novo most, if not all of its amino acids. Although experiments on amino acid synthesis in this organism have not been systematically investigated, the identification of the *arom* gene [33] suggests that, unlike mammals, *P. carinii* can synthesize its own aromatic amino acids. In *P. carinii*, the *arom* gene encodes five shikimic acid pathway enzymes. In other organisms examined, the product of this pathway is chorismate, which is a central precursor for various biosynthetic pathways including aromatic amino acids.

Even if *P. carinii* synthesizes de novo its amino acids, this does not preclude the possibility that exogenous amino acid supplementation can increase axenic culture growth rates. Although not taken up by active transport, exogenous amino acids are taken up and incorporated into the organism's distinct complex molecules such as proteins, glycoproteins, and lipids [4,28,34]. It is not surprising that serine was incorporated into *P. carinii* lipids [34] (M. Basselin, Y.H. Qiu, K.J. Lipscomb, E.S. Kaneshiro, unpublished data) since it is the direct precursor of phosphatidylserine and the long chain base of sphingolipids. However, all amino acids thus far examined appear to be at least partially utilized by the organism for lipid biosynthesis; radioactivity was found in the total lipid fraction in each case. In this study we

found that twice the amount of radioactivity from tyrosine was incorporated into total lipids compared to the pellet (which includes substantial amounts of protein) obtained by centrifugation following lipid extraction.

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