Biochimica et Biophysica Acta, 735 (1983) 67–76
Elsevier

BBA 71861

STOICHIOMETRY OF H^+/AMINO ACID COTRANSPORT IN *NEUROSPORA CRASSA* REVEALED BY CURRENT-VOLTAGE ANALYSIS

DALE SANDERS [a], CLIFFORD L. SLAYMAN [a] and MARTIN L. PALL [b]

[a] *Department of Physiology, Yale School of Medicine, 333 Cedar St., New Haven, CT 06510 and* [b] *Molecular Genetics, Heald Hall, Washington State University, Pullman, WA 99164 (U.S.A.)*

(Received May 9th, 1983)

Key words: Cotransport; Amino acid transport; Proton stoichiometry; Current-voltage analysis; (N. crassa)

Coupling of ions to the uptake of neutral and basic amino acids via a general amino acid transport system (System II), was studied in a mutant of *Neurospora crassa (bat mtr)* which lacks other transport systems for these solutes. All amino acids tested – including ones bearing no net charge – elicited rapid membrane depolarization, as expected for ion-coupled transport. (Since amino acid transport in *Neurospora* is not dependent on extracellular Na^+ or K^+, the associated ion is presumed to be H^+.) Although the ^{14}C-labeled amino acid fluxes through System II are largely independent of the identity of the amino acid, the depolarization caused by basic amino acids (L-lysine and L-ornithine) is 60–70% greater than that for neutral amino acids (e.g. L-leucine). This difference is consistent with a constant H^+/amino acid stoichiometry of 2, the extra charge for lysine and ornithine being that on the amino acid itself, so that the charge ratio basic : neutral amino acids is 3 : 2. When actual membrane charge flow associated with amino acid uptake was compared with measured ^{14}C-labeled amino acid influx, ratios of 2.07 charges/mol L-leucine and 3.40 charges/mol L-lysine were obtained, again in accord with a constant translocation stoichiometry of $2H^+$/amino acid. The advantages of this electrical method for estimating H^+/solute stoichiometry in cotransport are discussed in relation to more familiar methods.

Introduction

Many microbial and plant cells maintain across their plasma membranes a proton electrochemical gradient ($\Delta\bar{\mu}_{H^+}$) reaching -200 to -300 mV [1–4]. It is now well accepted that the resulting downhill and inward flow of H^+ can be coupled to transport and accumulation of a wide variety of solutes [5,6]. A crucial parameter in the description of any such proton-coupled transport system is its stoichiometric coefficient, n (the ratio of protons : solute transported with each turn of the transport cycle), which strongly influences system behavior, as described either by thermodynamic or by kinetic data. But, particularly with proton transport systems, the difficulties of determining transport stoichiometry by chemical methods are formidable: factors such as spurious pathways, metabolic shifts and electrically compensating proton fluxes all can make counting of protons or determination of equilibrium conditions uncertain.

However, since the majority of proton-coupled transport systems are electrophoretic, such difficulties can be skirted by measuring the charge flow which accompanies solute uptake. (Although such an approach requires transporting cells which are large enough to puncture with microelectrodes, new electrophysiological techniques [7–9] should substantially reduce the severity of this constraint in the near future.) In the present experiments, we have used conventional electrophysiological techniques, to examine current : solute ratios in amino

acid uptake by the large-celled mycelial fungus, *Neurospora crassa*.

In addition to its size, this organism offers the advantage of transport-restricted mutants. This is particularly important for studies of amino acid transport, since in eukaryotic cells many amino acids can be transported via several transport systems [10–13]. We have used a strain of *Neurospora (bat mtr)* possessing only one transport system which handles basic and neutral amino acids. That is designated System II and has been described as a general amino acid transport system [14]. (Both the specific basic amino acid system (System III) and the specific neutral amino acid system (System I) are deleted in this strain [14–18].) System II handles basic and neutral amino acids at approximately equal maximal velocities, and – as we demonstrate below – with a stoichiometric coefficient (n) which is fixed and independent of the amino acid species transported.

Materials and Methods

Biological material

All experiments were carried out with a *bat mtr* strain of *Neurospora*, FGSC No. 2276 (allele isolate numbers can-37; Pm-22), obtained from the Fungal Genetics Stock Center, Arcata, CA. For experimental preparations, conidia were harvested from 6- to 8-day agar cultures (25°C) and inoculated into liquid or onto agar containing nitrogen-free Vogel's medium, plus 2% sucrose (w/v) and 0.2% proline (w/v). (Nitrogen-free medium was prepared by omitting the 25 mM NH_4NO_3 in Vogel's medium N [19].

Measurement of fluxes

Conidia were inoculated at 10^5/ml into 1-liter Erlenmeyer flasks containing 200 ml of liquid medium and placed on a reciprocating shaker (180 strokes/min) at 25°C. After 48 h, the cells were harvested on cheesecloth, washed thoroughly with distilled water, and resuspended at a density of approx. 1 mg dry weight/ml in diluted ($0.3\times$) N-free minimal medium (i.e., no NH_4NO_3) containing 1% (w/v) glucose, 0.2% proline and 2.3 mM $CaCl_2$ (pH 5.8) (test medium). (L-Proline is transported by a specific proline carrier, not by the general amino acid system (Pall, M.L., unpublished data), as in yeast [20]. Use of proline, rather than ammonium, as a nitrogen source results in full derepression of System II (Pall, M.L., unpublished data).) The suspensions were then preincubated in a water-bath shaker at 25°C for 30–60 min, after which 10-ml samples were removed with a Tipet (A.H. Thomas Co., Philadelphia, PA) and added to 1 μmol of ^{14}C-labeled amino acid (final concentration 100 μM) in a 125-ml Erlenmeyer flask. Cells were shaken vigorously at 25°C for appropriate times, collected on glass-fiber filters (Whatman GF/A), and washed three times with distilled water at 0°C. The pellets were extracted and counted in a toluene/Triton (X-100)-based scintillation fluid containing 10% H_2O.

Flux data are expressed on a (liter cell water)$^{-1}$ basis, converting from (kg dry wt.)$^{-1}$ with the factor 2.54 [21]. Flux and electrical data are presented in the form mean ± S.E.

Electrical measurements

Conidia of the *bat mtr* strain were germinated as described previously [22] on scratched cellophane underlayed with agar containing the NH_4^+-free growth medium. After about 30 h at 25°C, a portion of the hyphal mat and cellophane was removed, washed in the N-free test medium, and mounted in a lucite chamber. Electrical measurements were made by means of glass microcapillary electrodes filled with 1 M Na_2SO_4. (The sulfate filling solution, rather than conventional KCl, was used to avoid possible membrane damage from elevated cytoplasmic Cl^- [9], although control experiments showed these particular preparations to be insensitive to the pipette electrolyte, presumably because of the large cell/hyphal size (15–20 μm diameter, over 1 cm length).)

The voltage response to amino acids was found to be transient (see Fig. 2), and the peak response was attenuated by gradual addition of amino acids. These facts necessitated fast chamber perfusion (15 ml/min through a chamber volume of 0.7 ml) and selection of cells near the entry port, so that the test amino acid could be introduced abruptly (in less than 1 s).

The current-voltage (I–V) characteristics of the membrane were determined by a three-electrode method described previously [23]. Briefly, one

microelectrode was used to record the actual membrane potential (V_0), and a second was placed nearby to pass a measured current (i). These two electrodes were connected by a microprocessor-driven voltage-clamp circuit, which could deliver a bipolar staircase of pulses (each 160 ms long) spanning the range −300 to 0 mV. A third electrode also measured the membrane potential (V_1) at a distant point (usually approx. 100 μm away from the V_0- and i-electrodes). V_1 was compared with V_0 in order to calculate current spread along the hyphal fiber and membrane current density (I_m, in units of μA/cm^2) in the vicinity of the V_0-electrode. (For ease of calculations, the branch geometry of each hypha to be studied was simplified by careful selection and/or by crush-killing of major branches near V_0 and V_1.) The plot of I_m versus V_0 defines the membrane I-V relationship.

Normally, the membrane I-V relationship was determined just before addition of an amino acid, and again as soon as the voltage response had reached its maximum. The resulting two curves were fitted by a non-linear least-squares method [24], either to a parabolic function, or (for short voltage spans) to a linear function. The calculated difference I-V curve (during amino acid minus control) was taken to represent the current-voltage relationship for the amino acid transport system. Thus current (net charge flux) through System II could be read off for any desired membrane potential within the accessible span.

Estimation of the stoichiometric coefficient

The stoichiometry for H^+/amino acid transport was estimated in two ways. (1) From the ratio of the currents elicited by neutral versus basic amino acids, plus the fact that one additional charge is carried on a basic amino acid, the lowest common integral stoichiometry was calculated. This method has the advantage that the measurements can be made on a single cell type, but it assumes common stoichiometry for the two classes of amino acid. (2) From a comparison of isotopic amino acid fluxes (measured in shaking culture cells) with the current flow (measured on hyphae), an absolute stoichiometry was estimated. This method does not depend on the assumption of common behavior by different amino acids, though the data must be normalized on a cell volume basis (not surface area), as has previously been done to compare activity of other transport systems in the two cell types [23].

Results

The general amino acid transport system in *Neurospora* is a single transport system by both kinetic [14] and genetic [25] criteria. It has a high affinity for neutral amino acids: for example the K_m for uptake of L-leucine is 4 μM. For basic amino acids, the affinity is even higher: e.g., the K_m for L-lysine is 0.9 μM. In the experiments reported here, all flux and electrical measurements were carried out at saturating velocity for System II, obtained by testing each amino acid at 100 μM.

Tracer influx of amino acids via System II

Initial time-courses for influx of three ^{14}C-labeled amino acids (leucine, lysine and ornithine) by *bat mtr* are plotted in Fig. 1. Isotope uptake is linear for at least 1.5 to 2 min after the addition of saturating concentrations of amino acid. More remarkably, the (maximal) amino acid flux is nearly independent of the identity of amino acid transported, as is demonstrated numerically in Table I

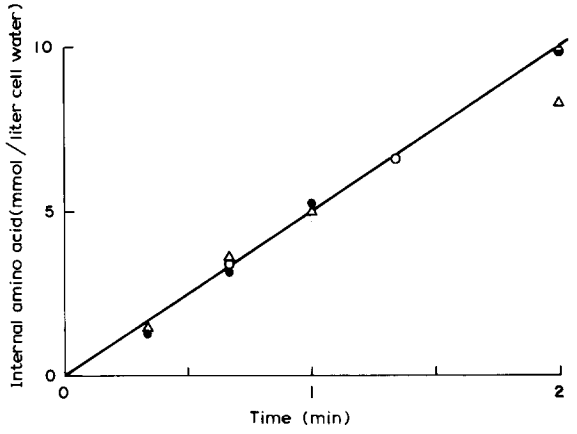

Fig. 1. Uptake of ^{14}C-labeled amino acids in *bat mtr*. Key: ●, L-leucine; △, L-lysine; ○, L-ornithine, all applied at 100 μM. Pooled data (except for lysine sample at 2 min) were fitted by least-squares to a linear function constrained to pass through the origin. Mean uptake rate is 5.01 ± 0.05 mmol/liter cell water per min. Delay in mixing of amino acids with cells might account for the slight tendency for the 20 s samples to lie below the fitted line.

TABLE I

^{14}C FLUXES AND MEMBRANE DEPOLARIZATION IN *bat mtr*

Amino acid	^{14}C flux (% control, Leu) [a]	Depolarization (% control, Leu) [b]	Weighted depolarization [c]
L-Leucine	100 ± 12	100 ± 10	1.00 ± 0.11
L-Phenylalanine	99 ± 3	78 ± 3	0.79 ± 0.03
L-Valine	102 ± 3	103 ± 5	1.01 ± 0.04
α-Aminoisobutyrate	61 ± 5	54 ± 5	0.89 ± 0.05
L-Ornithine	87 ± 3	144 ± 8	1.66 ± 0.05
L-Lysine	92 ± 4	147 ± 5	1.60 ± 0.04

[a] L-Leucine flux = 5.43 mmol/kg cell water per min. Values for all amino acids are means of three determinations ± S.E.
[b] Depolarization by Leu = 34.2 ± 3.8 (11) mV. Other values for depolarizations are means of between four and ten observations.
[c] Weighted depolarization defined as: depolarization (%)/flux (%).

(column 1) for five naturally occurring L-amino acids. Fluxes of the three neutral and two basic amino acids all fall within 10% of that for L-leucine, which was taken as the reference point. The one synthetic amino acid tested, α-aminoisobutyrate, did show a significantly lower flux, perhaps related to the H missing from its α-carbon atom.

Membrane depolarization by amino acids

Amino acids elicit rapid membrane depolarization when added to the medium bathing *Neurospora* (Fig. 2A). We take the speed of depolarization to be indicative of transport-associated current entering the cell, an interpretation which is reinforced by two further observations: (i) it occurs as well with non-metabolized amino acids (i.e., α-aminoisobutyrate) as with physiologic ones; and (ii) the triple mutant *pm bng*, which is deficient in all major amino acid transport systems [25,26], fails to show a marked electrical response to added leucine or lysine (data not shown). The fact that depolarization is associated with influx even of species having no net charge at the prevailing pH (e.g., leucine) can be taken as evidence for cotransport of amino acids along with at least one cation. In accordance with the findings for other amino acid cotransport systems in non-animal cells [5,11,27–29], this cation appears to be H^+, since there is no significant effect of extracellular K^+ or Na^+ concentration on the isotope flux through the general amino acid transport system (data not shown).

Fig. 2A shows that depolarization is biphasic in the sustained presence of amino acid, even though influx of labeled amino acid is constant (Fig. 1) over the period of 1–2 min spanned by the electrical trace. Transient behavior similar to this is typical of cotransport systems; it is thought to reflect a compensatory increase of ion extrusion by associated ion pumps [30–35]. An important implication of such electrical behavior is that only the initial electrical signal is likely to be quantitatively related to amino acid transport. (In the absence of a really high-speed flow/mixing device, we must assume that the time required to reach peak depolarization reflects mainly the time needed for amino acid to reach saturation at the transport sites.) For this reason, all further experiments were conducted with short pulses of amino acids, and

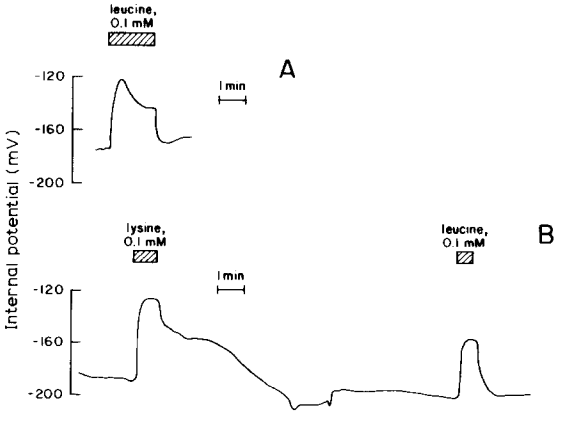

Fig. 2. (A) Membrane depolarization in *bat mtr* by 100 μM L-leucine, demonstrating transience of response. (B) Comparative depolarizations by L-lysine and L-leucine on the same hypha of *bat mtr*.

electrical parameters (voltage and current) were determined at peak depolarization. This protocol has the additional advantage of minimizing the cumulative effects of amino acid absorption during any single experiment.

The effects of both a basic and a neutral amino acid on membrane potential are compared in Fig. 2B. Lysine depolarized the membrane by 62 mV, whereas leucine, tested 12 min later, depolarized only 45 mV. Since the fluxes of the two amino acids should have been identical (Fig. 1), and since the larger depolarization by lysine occurred independently of the order in which amino acids were tested, the depolarization seems dependent simply on net charge (Table I, column 2). The mean depolarizations by both lysine and ornithine were about 50% greater than those produced by leucine, phenylalanine, or valine. Importantly, the more slowly transported analogue, α-aminoisobutyric acid, elicited only about 50% of the depolarization seen with leucine.

If depolarization is indeed a transport-linked event, the voltage displacements should be weighted according to the magnitude of measured amino acid flux. This calculation is summarized in Table I, column 3. It can be seen that α-aminoisobutyric acid gives a weighted voltage displacement which is not significantly different from that of leucine or valine. Phenylalanine does generate a smaller signal (20%) than leucine, but the statistical significance is dubious ($P < 0.03$). On the other hand, the two basic amino acids elicit weighted depolarizations 60–70% greater than that for leucine ($P < 0.001$)

By assuming a simple relationship between voltage displacement and transport current – an assumption which is tested below – we can make a direct comparison between the fluxes of charge entering the cell along with the two kinds of amino acid. The average weighted depolarization for ornithine and lysine is 1.63 ± 0.04, and that for the four neutral amino acids is 0.93 ± 0.07. The ratio between these is 1.75, which is close to an integral ratio of 5 : 3, or even 3 : 2. Since lysine and ornithine each carry a single net positive charge, at the extracellular pH for these experiments, a charge-transport ratio of 3 : 2 would be consistent with a transport stoichiometry of 2 H^+ per amino acid molecule taken up, regardless of the identity of the amino acid.

Ion currents accompanying amino acid transport

Voltage measurements and calculations of the kinds reported above are subject to one potentially serious criticism: that displacements of membrane potential need not necessarily be proportional to charge flow. Such proportionality obtains only if membrane resistance (or conductance, 1/resistance) is constant over the observed range of voltage displacements. But since the membrane conductance of *Neurospora* is known to change with membrane potential [23], actual measurement of the membrane current which accompanies amino acid flux through System II is essential. An efficient strategy for making this measurement is to impose currents from an extrinsic source (flowing, for example, between an intracellular microelectrode and a bath electrode), and to plot the amount of current delivered against the actual membrane potential obtained, over as wide a voltage range as possible. By performing this maneuver twice – before and during (at peak depolarization) amino acid activation of System II – and then subtracting the two, we obtain the current-voltage relationship for the amino acid transport system, and can read off the exact amount of current which must flow to give the observed peak depolarization.

A typical *I-V* plot for the *bat mtr* strain of *Neurospora*, obtained in the absence of added amino acids, is shown by the upper curve (control) in Fig. 3. This kind of control *I-V* relationship, which was obtained in all 23 trials (11 different hyphae) of *bat mtr*, turns out to be more nearly linear than had been expected from wild-type *Neurospora* (see Ref. 23). The condition mimics that reported for carbon-starved hyphae [36], and there may be an indirect physiological effect of the ammonium starvation which occurs during proline-growth of *Neurospora*. Obviously, such linearization of the membrane *I-V* relationships reduces the quantitative error which can arise in the direct comparison of amino acid flux and displacement of membrane potential (see above).

The second (lower) plot in Fig. 3 was obtained at the peak of lysine-induced depolarization. Its intercept on the abscissa, which represents the membrane potential of the cell with no injected current, shifted 44 mV rightward in lysine, equal to the actual peak depolarization. The two plots are almost parallel, and the difference curve – which gives the *I-V* relationship for lysine

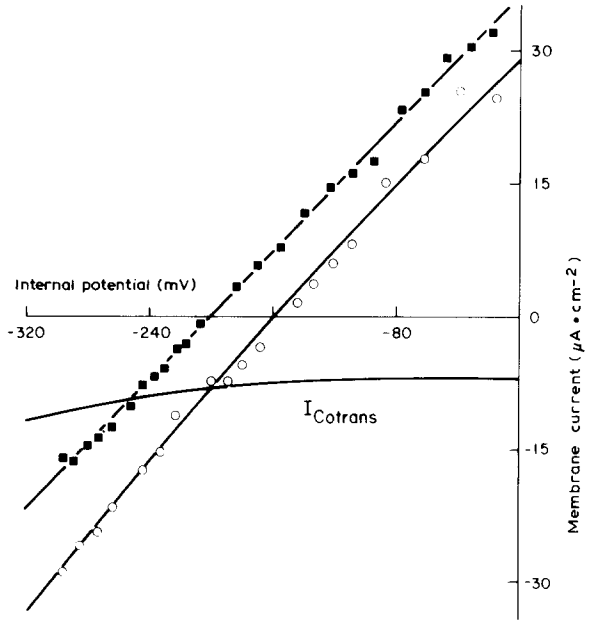

Fig. 3. Membrane current-voltage curves measured 32 s before the addition of 100 μM L-lysine (control, ■), and at the peak of the lysine-induced depolarization (○). Lines through the data points are least-squares fits according to the quadratic function $I_m = a_0 + a_1\Delta\psi + a_2\Delta\psi^2$, with I_m the membrane current and $\Delta\psi$ the internal potential. '$I_{Cotrans}$' is the difference of the two membrane *I-V* curves. Coefficients are as follows:

Curve	a_0	a_1	a_2
Control	35.9	0.18	$-1.5 \cdot 10^{-5}$
+lysine	29.4	0.18	$-4.5 \cdot 10^{-5}$

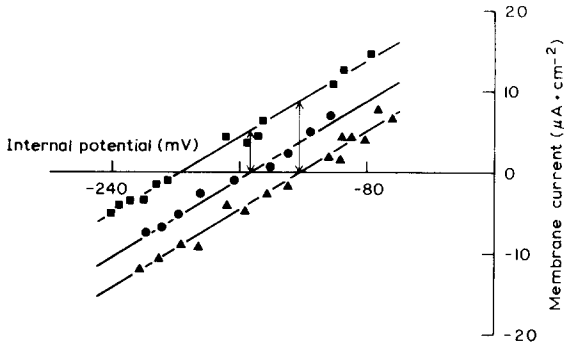

Fig. 4. Determination of amino acid-associated current for comparison with isotope flux experiments. The center portions of membrane current-voltage curves, obtained as described in Materials and Methods and the legend to Fig. 3, were fitted by least-squares to linear functions of the form $I_m = a_0 + a_1\Delta\psi$. Key: ■, control; ●, +L-leucine; ▲, +L-lysine. Vertical lines represent the current (sign inverted) due to the amino acid at the appropriate cell resting potential (5.0 $\mu A/cm^2$ for leucine and 8.9 $\mu A/cm^2$ for lysine). Coefficients are as follows:

Curve	a_0	a_1
Control	23.2	0.118
+leucine	18.6	0.121
+lysine	14.7	0.121

transport, runs parallel to the voltage axis over most of its length, with an indication of slightly greater current at more negative voltage. In other words, although amino acid uptake is indeed accompanied by an electric current, the process is only slightly sensitive to the actual membrane potential (over the range 0 to −300 mV). Similar results have been reported for amino acid transport in *Riccia* [37] and for glucose transport in *Neurospora* [36]. The meaning of this result will be taken up in the Discussion.

In the context of determining the charge : amino acid stoichiometry, however, we are interested primarily in the behavior of the *I-V* curves over the voltage range between the control membrane potential and peak depolarization, since this is the range over which chemical measurements of amino acid flux must be made. We therefore focussed further analysis on those particular segments of the current-voltage curves, as is shown in Fig. 4. In all cases, straight lines were fitted to the data, and in most sets of trials the fitted slopes for all *I-V* curves in the set did not differ significantly. Current flow induced by a particular amino acid could then be calculated from the vertical displacement between the control *I-V* curve and that obtained during peak depolarization. This displacement was measured at the membrane potential existing exactly at peak depolarization, indicated in Fig. 4 by the vertical arrows for leucine and for lysine. A summary of the calculations for all trials is given in Table II. As predicted from the voltage effects alone, the ratio of currents with lysine : leucine was close to 3 : 2. For comparison with the measured amino acid influx, current was converted to charge flow in milliequivalents/liter cell water (= current × surface : volume ratio/Faraday). The resultant stoichiometry, charge : [^{14}C]lysine was 3.40, and charge : [^{14}C]leucine was 2.07, again consistent with

TABLE II

PROPERTIES OF THE GENERAL AMINO ACID TRANSPORT SYSTEM FROM CURRENT-VOLTAGE STUDIES

^{14}C-labeled amino acid fluxes taken from Table I. Electrical data (in the form mean ± S.E.) are from three hyphae, representing six trials for leucine and five trials for lysine.

	Leucine	Lysine	Ratio
Depolarization (mV)	32 ±5	52 ±4	1.63±0.14
Current ($\mu A \cdot cm^{-2}$)	4.7 ±1.0	7.1 ±0.5	1.51±0.15
Charge flow (mequiv.$\cdot l^{-1} \cdot min^{-1}$)	11.2 ±2.3	17.0 ±1.2	
^{14}C-labeled amino acid flux (mmol$\cdot l^{-1} \cdot min^{-1}$)	5.4 ±0.6	5.0 ±0.2	
Charge/^{14}C-labeled amino acid (equiv.$\cdot mol^{-1}$)	2.07±0.22:1	3.40±0.10:1	

the notions that two protons are moved in with each amino acid molecule, and that a basic amino acid itself carries one additional charge. (Such a direct comparison between unidirectional flux (isotope) and net flux (current) is valid for amino acid transport in *Neurospora*, because the unidirectional efflux – even at high internal concentrations – is less than 5% of unidirectional influx [38]. For transport systems exhibiting significant self-exchange, this approach would not be valid [39].)

Discussion

Evaluation of methods for measuring stoichiometry in H^+ cotransport

Net fluxes. Most problems of determining H^+/solute stoichiometry arise because unidirectional H^+ fluxes cannot be measured by chemical means. Consequently, many investigators – following the lead of West and Mitchell [40] – have simply measured solute-dependent net H^+ uptake, using experimental conditions which minimize regulated changes of proton flux through other pathways. In the case of electrophoretic cotransport systems, however, the method can only give a lower limit for n because of compensation through the electric circuit.

This point can perhaps be made most clearly by an example. Suppose that, before activation of a particular H^+-solute cotransport system, the normal state of the membrane is such that any enforced voltage change drives half its current via K^+ ions, and half via H^+ ions. Initially, upon activation of the cotransport system (when the pump has not had time to adjust) protons entering on the cotransport system must be balanced half by decreased K^+ entry (or increased exit) and half by decreased H^+ entry (or increased exit) through the leaks. This means that if the real value of n is 2, then the apparent value – which must be equivalent to the changed K^+ flux – will be 1. (The argument applies to all measurements made after the membrane potential has shifted to its new (depolarized) value, for which the time is ultimately set in the range of 5–50 ms by charging of membrane capacitance.) This problem, unlike that of preventing later adjustments of pump or leak pathways [40,41], is unavoidable in any membrane system having appreciable proton permeability.

It seems possible, indeed, that variability in such charge compensation by H^+ (resulting from variability in background proton permeability) among different biological membranes might be responsible for reported differences of n in closely-related transport systems. Among yeasts, for example, the general amino acid permease in *Saccharomyces* shows $n = 2$, whereas in *Candida* the value is 1 [11,41,42]. An excellent illustration of this argument is the demonstration [43] that the stoichiometry for Na^+/glucose cotransport in intestinal epithelium had been underestimated (as $n = 1$) for over 10 years; a value of $n = 2$ is obtained if the voltage-sensitive background Na^+ flux is short-circuited with valinomycin/K^+.

Equilibrium. The other common method for determining n requires the transport system to reach equilibrium, for which condition the transport mass reaction

$$n\,H_o^+ + S_o^z \rightleftharpoons n\,H_i^+ + S_i^z \qquad (1)$$

yields

$$(n\Delta\bar{\mu}_{H^+}) + (\Delta\bar{\mu}_{S^z}) = 0 \qquad (2)$$

where $\Delta\bar{\mu}$ is the electrochemical potential difference across the membrane for H^+ and substrate S^z, and z is the valence of S. Expansion and rearrangement of these terms gives

$$n = \left[\frac{RT}{F}\ln\frac{[S^z]_o}{[S^z]_i} - z\Delta\psi\right] \Big/ \left[\Delta\psi - \frac{RT}{F}\ln\frac{[H^+]_o}{[H^+]_i}\right] \qquad (3)$$

In principle, this method requires only measurement of the respective solute concentrations, and of membrane potential ($\Delta\psi$). A major problem, however, is that of insuring equilibrium – rather than simply steady state – for the system. When transport of either the substrate or the driver ion also occurs through other pathways, even small ones, very large errors in the steady-state poise can be overlooked. Furthermore, even when the fluxes of S and H^+ are tightly coupled, quasi-steady states can exist well displaced from true equilibrium, especially in systems showing acute trans-inhibition (Ref. 44, and below). Again, only a lower kinetic limit on n is established by the method.

Net charge transport. The electrophysiological approach used here is not subject to either of these major errors. The determination is a kinetic one, inherently free of the equilibrium assumption; and because the strain of *Neurospora* chosen is genetically blocked for transport of (neutral, basic) amino acids through other pathways, the measured amino acid flux can be attributed over 99.5% to System II. Furthermore, since electroneutrality is maintained by charges flowing from an external source (in fact, the measuring amplifier), the identity of membrane ions flowing normally to compensate the cotransported proton influx is immaterial. The *I-V* method has also been used in conjunction with isotopic measurements to evaluate n for H^+/glucose cotransport in *Neurospora* ($n = 1$; Ref. 45) and H^+/Cl^- cotransport in *Chara* ($n = 2$; Ref. 46).

One limitation of the *I-V* technique, however, is obvious: it is applicable only to large microbial cells which permit direct electrophysiological measurements. Indeed, for the experiments on *Neurospora*, we must compare the currents in mature hyphae (grown on agar) with isotope fluxes in germinated conidia (grown in suspension cultures), then normalize for the different surface area/volume ratios. It is therefore satisfying that both the direct comparison of leucine (or lysine) flux with electric current, and the comparison of leucine depolarization with lysine depolarization lead to the same value of n ($= 2$). The latter comparison was made on only one cell type, and did not depend on knowledge of the absolute current or flux. Recently, methods have been developed in this laboratory which will facilitate measurement

TABLE III

THERMODYNAMIC CHARACTERISTICS OF THE GENERAL AMINO ACID TRANSPORT SYSTEM OF *NEUROSPORA*

The following values were used for substituting into the equations: $n = 2$; $z = 0$ (neutral) or $+1$ (basic); $[H^+]_i = 63$ nM ($\equiv$ pH 7.2: Ref. 3); $[H^+]_o = 1.58\ \mu$M ($\equiv$ pH 5.8); $[S^z]_i = [S^z]_o = 100\ \mu$M; $\Delta\psi = -150$ mV.

Parameter	Unit	Form of Eqn. 3	Amino acid	
			Neutral	Basic
Free energy available for transport	mV	$(n+z)\Delta\psi + \frac{RT}{F}\ln\left[\frac{[H^+]_i^n}{[H^+]_o^n}\frac{[S^z]_i}{[S^z]_o}\right]$	−465	−615
Equilibrium solute accumulation ratio	ratio	$\frac{[H^+]_o^n}{[H^+]_i^n}\cdot\exp\left[-\frac{(n+z)F\Delta\psi}{RT}\right]$	$7.7\cdot10^7$	$2.7\cdot10^{10}$
Reversal potential	mV	$\frac{RT}{(n+z)F}\cdot\ln\left[\frac{[H^+]_o^n}{[H^+]_i^n}\frac{[S^z]_o}{[S^z]_i}\right]$	+83	+55

of membrane current and isotope fluxes on the same cell type [9].

Stoichiometry, energetics and the shape of the I-V curve

Since the value of n is known, thermodynamic parameters for the transport of neutral and basic amino acids can be calculated, and these are listed in Table III. The free energy available for amino acid uptake is very large indeed under the conditions of these experiments, and the theoretical solute accumulation ratio is likewise enormous.

The transport reversal potential, or (opposing) membrane potential which would be required to reduce net transport to zero, can be calculated from a rearrangement of Eqn. 3 (see Table III). If the calculation is made soon after activation of System II, say when $[S]_i = [S]_o$, then the reversal potential would be +83 mV for leucine, and +55 mV for lysine (cell interior positive). Now, usually – though not always – voltage-sensitivity in a carrier-type transport system appears in the neighborhood of the reversal potential [44,47]. At membrane potentials well displaced from the reversal potential, the current-voltage curve saturates and becomes flat. Thus, it is clear why System II is nearly voltage-insensitive (Fig. 3): the reversal potentials for both leucine and lysine transport are well out of the range of accessible membrane potentials. A fundamental corollary of this result is that any experimental attempt to demonstrate that the transport of amino acids is electrophoretic would be doomed to failure if the response of flux to membrane potential (between 0 and −300 mV) were the only criterion.

Stoichiometry, kinetics and intrinsic control: why does $n = 2$?

Given the extremely high free energy available for amino acid accumulation, we can ask a teleological question: why does the cell use 2 H^+ to drive uptake of amino acids instead of 1 H^+, which would dissipate only half the energy stored in electrochemical form as a transmembrane H^+ gradient? Indeed, although the free energy available for amino acid uptake would be reduced, the maximum accumulation ratio for a neutral amino acid would still be $9 \cdot 10^3$, and for a basic amino acid $3 \cdot 10^6$.

The stoichiometry of 2 maintains the transport system very far from equilibrium, since it is unreasonable to expect an actual solute accumulation ratio in the range 10^7–10^{10}. Perhaps the best reason for this arrangement is the classical one given for those scalar reactions of intermediary metabolism which have similar characteristics [48]: displacement from equilibrium facilitates homeostatic regulation while leaving reaction rates relatively insensitive to variation of driving force per se. It has already been shown (Fig. 3) that transport is insensitive to membrane potential over a wide range. Similarly, at high external pH, where the chemical component of the H^+ gradient favors efflux, there should still be a strong inward driving force on amino acid transport because of the membrane potential. But if n were unity, equilibrium would be approached both for large membrane depolarizations and for elevated external pH, so that transport would slow accordingly. Clearly, however, with $n = 2$, the cell should be able to maintain a high, fixed rate of solute transport in the face of such perturbations of free energy difference across the transport system.

One striking characteristic of transport systems displaced far from equilibrium is sensitivity to internal ligand concentration [44]. Net flux can be reduced almost to zero as the internal ligand concentration rises, even in the presence of a large driving force. This 'transinhibition' should have an autoregulatory effect ('intrinsic control': Ref. 49) on cytoplasmic levels of the transported ligands: amino acids and H^+. Several other cotransport systems demonstrate acute sensitivity to internal H^+ concentration: H^+/hexose cotransport in *Chlorella* [50]; H^+/Cl^- cotransport in *Chara* [33,51]; H^+/glycine transport in yeast (Ballarin-Denti, A. and Slayman, C.L., unpublished data). At least in the latter two instances, kinetic modelling has demonstrated that the observed sensitivity can be generated only if $n > 1$. Thus, although the energy consumption for transport must be higher on a molar basis with $n = 2$, than with $n = 1$, shutting down of transport as internal solute concentration reaches the required level both ensures that the energy drain is not persistent and protects the cell against cytoplasmic acidification. In this context, then, it will be of interest to determine the response of the general amino acid

transport system of *Neurospora* to changes of internal pH.

Overall maximum current flow through the general amino acid transport system is about 5 $\mu A/cm^2$ (Table II), compared with a normal H^+ pump rate of 20 $\mu A/cm^2$. The pump accounts for 25–50% of steady-state ATP turnover in *Neurospora* [23]. This means that, even in the initial, rapid stages of amino acid uptake, accumulation of amino acids will not require much more than 10% of cellular energy turnover.

Acknowledgements

This work was supported by NIH grants GM-15858 (to C.L.S.) and GM-24368 (to M.L.P.).

References

1 Maloney, P.C. (1982) J. Membrane Biol. 67, 1–12
2 Padan, E., Zilberstein, D. and Schuldiner, S. (1981) Biochim. Biophys. Acta 650, 151–166
3 Sanders, D. and Slayman, C.L. (1982) J. Gen. Physiol. 80, 377–402
4 Smith, F.A. and Raven, J.A. (1979) Annu. Rev. Plant Physiol. 30, 289–311
5 Eddy, A.A. (1978) in Current Topics in Membranes and Transport (Bronner, F. and Kleinzeller, A., eds.), Vol. 10, pp. 279–360, Academic Press, New York
6 West, I.C. (1980) Biochim. Biophys. Acta 604, 91–126
7 Hammill, O.P., Marty, A., Neher, E., Sackmann, B. and Sigworth, F.J. (1981) Pflügers Arch. 391, 85–100
8 Felle, H., Porter, J.S., Slayman, C.L. and Kaback, H.R. (1981) Biochemistry 19, 3585–3590
9 Blatt, M.R. and Slayman, C.L. (1983) J. Membrane Biol. 72, 223–234
10 Guidotti, G.G., Borghetti, A.F. and Gazzola, G.C. (1978) Biochim. Biophys. Acta 515, 329–366
11 Eddy, A.A. (1982) Adv. Microb. Physiol. 23, 1–78
12 Wolfinbarger, L. (1980) in Microorganisms and Nitrogen Sources (Payne, J.W., ed.), pp. 63–87, Wiley & Sons, London
13 Blackman, M.S. and McDaniel, C.N. (1981) Plant Physiol. 66, 261–266
14 Pall, M.L. (1969) Biochim. Biophys. Acta 173, 113–127
15 Lester, G. (1966) J. Bacteriol. 91, 677–684
16 Pall, M.L. (1970) Biochim. Biophys. Acta 203, 139–149
17 Stadler, D.R. (1966) Genetics 54, 677–685
18 Thwaites, W.M. and Pendyala, L. (1969) Biochim. Biophys. Acta 192, 455–461
19 Vogel, H.J. (1956) Microbial Genet. Bull. 13, 42–43
20 Magaña-Schwencke, N., Schwencke, J. (1969) Biochim. Biophys. Acta 173, 313–323
21 Slayman, C.W. and Tatum, E.L. (1964) Biochim. Biophys. Acta 88, 578–592
22 Slayman, C.L. (1965) J. Gen. Physiol. 49, 69–92
23 Gradmann, D., Hansen, U.-P., Long, W.S., Slayman, C.L. and Warncke, J. (1978) J. Membrane Biol. 39, 333–367
24 Marquardt, D.W. (1963) J. Soc. Indust. Appl. Math. 11, 431–441
25 Rao, E.Y.T., Rao, T.K. and DeBusk, A.G. (1975) Biochim. Biophys. Acta 413, 45–51
26 DeBusk, R.M. and DeBusk, A.G. (1980) J. Bacteriol. 143, 188–197
27 Kinraide, T.B. and Etherton, B. (1980) Plant Physiol. 65, 1085–1089
28 Lüttge, U., Jung, K.-D. and Ullrich-Eberius, C.I. (1981) Z. Pflanzenphysiol. 102, 117–125
29 Robinson, S.P. and Beevers, H. (1981) Planta 152, 527–533
30 Slayman, C.L. and Slayman, C.W. (1974) Proc. Natl. Acad. Sci. U.S.A. 71, 1935–1939
31 Komor, E. and Tanner, W. (1976) Eur. J. Biochem. 70, 197–204
32 Novacky, A., Fischer, E., Ullrich-Eberius, C.I., Lüttge, U. and Ullrich W.R. (1978) FEBS Lett. 88, 264–267
33 Sanders, D. (1980) J. Membrane Biol. 53, 129–141
34 Gunter-Smith, P.J., Grasset, E. and Schultz, S.G. (1982) J. Membrane Biol. 66, 25–39
35 Kinraide, T.B. and Etherton, B. (1982) Plant Physiol. 69, 648–652
36 Hansen, U.-P. and Slayman, C.L. (1978) in Membrane Transport Processes (Hoffman, J.F., ed.), Vol. 1, pp. 141–154, Raven Press, New York
37 Felle, H. (1981) Planta 152, 505–512
38 Pall, M.L. and Kelly, K.A. (1971) Biochem. Biophys. Res. Commun. 42, 941–947
39 Chapman, J.B. (1982) J. Theor. Biol. 95, 665–678
40 West, I.C. and Mitchell, P. (1973) Biochem. J. 132, 587–592
41 Seaston, A., Inkson, C. and Eddy, A.A. (1973) Biochem J. 134, 1031–1043
42 Eddy, A.A., Philo, R., Earnshaw, P. and Brocklehurst, R. (1977) in Biochemistry of Membrane Transport (Semenza, G. and Carafoli, E., eds.), FEBS Symposium No. 42., pp. 250–260, Springer, Berlin
43 Kimmich, G.A. and Randles, J. (1980) Biochim. Biophys. Acta 596, 439–444
44 Sanders, D., Hansen, U.-P., Gradmann, D. and Slayman, C.L. (1983) J. Membrane Biol., in the press
45 Slayman, C.L., Slayman, C.W. and Hansen, U.-P. (1977) in Transmembrane Ionic Exchanges in Plants (Thellier, M., Monnier, A., Demarty, M. and Dainty, J., eds.), pp. 115–122, CNRS, Paris
46 Beilby, M.J. and Walker, N.A. (1981) J. Exp. Bot. 32, 43–54
47 Hansen, U.-P., Gradmann, D., Sanders, D. and Slayman, C.L. (1981) J. Membrane Biol. 63, 165–190
48 Newsholme, E.A. and Start, C. (1973) Regulation in Metabolism, pp. 8–27, Wiley, London
49 Sanders, D. (1984) in Chloride Transport Coupling in Cells and Epithelia (Gerencser, G.A., ed.), Elsevier Science Publishers, Amsterdam, in the press
50 Komor, E., Schwab, W.G.W. and Tanner, W. (1979) Biochim. Biophys. Acta 555, 524–530
51 Sanders, D. and Hansen, U.-P. (1981) J. Membrane Biol. 58, 139–153