Biochimica et Biophysica Acta, 978 (1989) 119–133
Elsevier

BBA 74270

Kinetic properties, nutrient-dependent regulation and energy coupling of amino-acid transport systems in *Penicillium cyclopium*

Werner Roos

Department of Cellular Physiology, Biotechnikum of the Martin Luther University, Halle (G.D.R.)

(Received 12 April 1988)

Key words: Amino acid transport; Proton/amino acid symport; Kinetics; Energy coupling; Permease; (*P. cyclopium*)

In submerged grown hyphae of *Penicillium cyclopium* the activities of seven transport systems could be distinguished which share in the uptake of L-arginine, L-glutamic acid, L-phenylalanine and L-leucine. They include the specific systems a (accepting L-arginine and L-lysine), b (L-phenylalanine, L-tyrosine), c (L-glutamic acid) and d (L-leucine), system I (a 'general amino-acid permease') and the low-affinity systems II and III, which accept acidic or basic amino acids, respectively, but also L-phenylalanine. In nutrient-sufficient cells, systems I, II and III remain repressed; uptake is dominated by the specific systems b, c, d and a, the latter reaching its maximum activity. Nitrogen starvation is the most powerful signal for the development of systems I, II and III, whereas, in carbon-starved cells, systems b, c and d reach maximum activities. The development of the general amino-acid permease in nitrogen-starved cells requires both translational and – with a few hours delay – transcriptional events as indicated by the influence of cycloheximide and 5-fluorouracil. The uptake of all amino acids is accompanied by a transient acidification of the cellular interior. Short-time preaccumulation of several anions, such as citrate, α-oxo-glutarate, glutamate (but not glutamine), increases the initial rate of amino-acid uptake at a pH above the optimum. Uncouplers inhibit the uptake not only under aerobic but also under anaerobic conditions, where the ATP content is not influenced by these compounds. These findings point to an H^+/amino acid symport, which is tightly connected with the recycling of the incoming protons by the plasmalemma H^+-ATPase.

Introduction

In eukaryotic microorganisms the uptake of amino acids is catalyzed by a multiplicity of carriers with different, overlapping degrees of specificity: some of them accept only a distinct amino acid or analogs of closely related structure, others transport classes of amino acids with a common structural feature (e.g., presence or absence of γ-NH_2 or γ-COOH groups), the broadest specificity being offered by the 'general amino acid permease' (GAP) [1–4]. A similar heterogeneity is known for the regulation of carrier synthesis and activity which occurs in response to changing environments and metabolic needs. Integrative signal systems working in large groups of organism (e.g., N-regulation, C-catbolite repression) are complemented by individual mechanisms of adaptation [1–5]. As only a few prominent yeast and fungal species have been studied in sufficient detail, the principles underlying the coordinate regulation of transport systems and their adaptability are far from being overseen or generally understood. Therefore, it seems helpful to gain comparative data from hitherto untreated species adapted to specific environmental and metabolic conditions. The present paper describes the kinetic and some regulatory properties of amino-acid transport systems in the mould *Penicillium cyclopium* with special emphasis on the general amino-acid permease. *Penicillium cyclopium* excretes significant amounts of H^+, either together with tricarboxylate cycle anions (mainly citrate) or in exchange with the uptake of NH_4^+ [6]. The permease patterns must therefore be expected to represent some kind of adaptation to the acidic environment. The protection of the cell from the external acidity rests on a Donnan distribution of H^+ at the cytoplasmic surface and suggests a very tight recycling of protons by the plasma-

Abbreviations: C_i, intracellular content; J_s, flux at a given substrate concentration; J_{max}, maximum flux; S, substrate (transportant) concentration; K_T, half-saturation constant of transport; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; GAP, general amino-acid permease.

Correspondence: W. Roos, Department of Cellular Physiology, Biotechnikum of the Martin Luther University, Weinbergweg 15, 4050 Halle, G.D.R.

lemma H^+ pump [7]. The present data further substantiate this view by showing the incorporation of H^+/amino acid cotransport into the pH stat mechanism. Another reason for studying amino-acid transport in *P. cyclopium* is the formation by this fungus of benzodiazepine alkaloids from phenylalanine and anthranilic acid. This process, which has been studied as a model system of cellular differentiation (cf. Ref. 34 and literature therein), is regulated via the transport and intracellular compartmentation of phenylalanine [34,35].

Materials and Methods

Chemicals

L-[U-^{14}C]Arginine, L-[U-^{14}C]lysine, L-[U-^{14}C]glutamic acid, L-[U-^{14}C]leucine and L-[U-^{14}C]phenylalanine were purchased from UVVR (Prague, C.S.S.R.), L-[U-^{14}C]tyrosine, [1,5-^{14}C]citric acid and 2-keto[5-^{14}C]glutaric acid were purchased from the Amersham International (U.K.). Unlabelled transportants and other chemicals were of analytical grade, glucose and sucrose according to AB 2-DDR (G.D.R. Pharmacopoeia).

Organism and growth conditions

Penicillium cyclopium Westling SM 72 was maintained on agar slants containing 275 mM glucose/35 mM ammonium tartrate/0.9 mM KH_2PO_4/0.67 mM KCl/0.62 mM $MgSO_4$/0.18 mM $FeSO_4$/0.06 mM $ZnSO_4$, with Cu, Co, Mn and Mo as trace elements, pH adjusted to 5.0 with H_2SO_4. After 4 d growth, conidia were brushed off and inoculated (approx. 10^7 spores/ml) into liquid media of the above composition, but with only 110 mM glucose plus 110 mM sucrose and 0.03% Triton X-100, which facilitates the microfilamentous growth of the hyphae. Submerged cultures (100 ml in 300 ml Erlenmeyer flasks) were grown on rotary shakers (250 rpm) at 24°C.

Preparation of cells for transport studies

After 2 d growth, an 100 ml submerged culture was diluted with 1 liter water and filtered through a nylon sieve to remove hyphal pellets. The fine, 'hairy' grown hyphae were washed twice by resuspension in 250 ml water and suction filtration and finally resuspended (250 mg fresh weight/100 ml) in half-concentrated culture liquid. The latter contained either all nutrients, no ammonium tartrate (nitrogen-starvation experiments) or neither glucose nor sucrose with equimolar NH_4Cl instead of ammonium tartrate (carbon starvation). The suspensions were aerated in gas washer flasks at 24°C for 7 h.

Transport assays

Hyphae from 20 ml of the above suspensions were filtered on a paper filter, resuspended in 18 ml uptake buffer (usually 60 mM maleate (pH 3.5)) and aerated in a filter funnel through its sintered glass bottom. After 30 s, 2.0 ml of a solution containing the labelled transportant, inactive amino acids and inhibitors, etc. were added. 5-ml samples were taken at 30 s, 2 min and 4 min of incubation, pipetted into 10 ml ice-cold uptake buffer in a filter funnel (23 mm diameter), rapidly filtered through paper filters, and the cells were washed by suction with 15 ml water at 0°C. The wet cell pellet was extracted with 2.0 ml ethanol 90% (v/v) overnight in a reciprocal shaker. 0.2 ml of the ethanolic supernatant were used for the determination of free amino acids or ammonia, if necessary. The remaining suspension was mixed with 7 ml Tritisol scintillant [8] and the radioactivity was counted in a Tricarb Liquid Scintillation Spectrometer (Packard 6220). The scintillation cocktail was then sucked away, the cells washed twice with 10 ml ethanol 96% (v/v) and resuspended in 2.0 ml of 10% sodium dodecyl sulfate solution. After reciprocal shaking overnight, the extracted protein in the supernatant was determined by the method of Lowry et al. [9]. The specific content of labelled amino acids taken up by the cells was then calculated on the basis of the protein content.

Each labelled amino acid shows a characteristic amount of adsorption to the washed cell pellet, which is completed within the first seconds of incubation and gives rise to a distinct y intercept of the C_i vs. t curve. This intercept is nearly identical with the amount of label which remains bound to inactivated cells (10 min at 100°C or 10 min pretreatment with 10 mM azide) after the above procedure. The adsorption of this amount is completed after the first seconds of incubation; therefore the linear slope of the uptake curve between 0.5 and 4 min of incubation is considered to represent the initial rate of uptake. During this period, neither a measurable efflux nor metabolism of the transportants could be detected.

The J values given in this paper represent the mean for 3–5 determinations. Within the same experiment, the average S.D. ($n = 5$) ranged from 9% at a substrate concentration of $5 \cdot 10^{-6}$ M to 14% at $5 \cdot 10^{-3}$ M. At substrate concentrations above 10^{-2} M the variance of J values increased considerably so that reproducible data could not easily be obtained by the method used.

Determination of K_T values

Half-saturation constants of transport were calculated from initial velocities of uptake according to the direct linear plot of Eisenthal and Cornish-Bowden [10]. For the kinetic separation of biphasic J_s/S plots, the direct linear plot was used in the following iteration procedure running on a desk computer ('low substrate concentrations' are those values between $2 \cdot 10^{-4}$ M and $5 \cdot 10^{-5}$ M; 'high substrate concentrations' between 10^{-4} M and $5 \cdot 10^{-3}$ M; five J_s/S pairs are used to construct a direct linear plot):

(i) construct a direct linear plot with the fluxes at low substrate concentrations (direct linear plot I) and calculate K_T and J_{max} as well as the fluxes for high substrate concentrations; subtract the latter fluxes from the initial rates measured in this concentration range;
(ii) with the corrected fluxes at high substrate concentrations, construct a direct linear plot II and calculate K_T, J_{max} as well as the fluxes for low substrate concentrations; subtract the latter fluxes from the initial rates measured in this concentration range;
(iii) with the corrected fluxes at low substrate concentrations, construct a new direct linear plot I, and so on...

The accuracy of the fit is indicated by the variance of the intercepts of a distinct direct linear plot, this values decreased to a nearly constant level after 4–8 iterations. The method has proved to be rapid and reliable, especially less sensitive against 'outliers' and different degrees of experimental error compared with least-square-fitting procedures.

Analytical procedures

Free amino acids were determined in ethanolic cell extracts (see above) either as ninhydrin-positive material or after separation with the Amino Acid Analyzer AAA 881 (Mikrotechna, Prague). Ammonia was determined in the same extracts with the phenol/hypochlorite method [13].

ATP was extracted from the cells by suspending 12 mg fresh weight in 1 ml glycine buffer (0.1 M (pH 11.0)) at 0 °C, freezing immediately at −20 °C and heating the frozen samples for 10 min in a boiling water bath. The determination of ATP was done using the luciferin/luciferase method [11] using the above-mentioned Tricarb Liquid Scintillation Spectrometer.

The average intracellular pH was estimated by the distribution of the acid dye Bromophenol blue [12]. The intracellular water volume was determined as the difference between the total water of a hyphal pellet (dry loss) and the extracellular water. The latter was estimated as the partial volume which is accessible to the nonpenetrating solute, human serum albumin.

Results and Discussion

Multiplicity of carriers

The rates of uptake of L-arginine, L-phenylalanine, L-glutamic acid and L-leucine into submerged grown hyphal cells strongly respond to the presence of nitrogen – as well as of carbon – sources in the growth medium. At the end of the logarithmic growth phase (i.e., after 2 d batch cultivation), the initial velocities are relatively low (around 10^{-10} mol/min per mg protein at $5 \cdot 10^{-6}$ M substrate concentration), but increase considerably during preincubation of the cells either with complete nutrients or with nutrient-starved media.

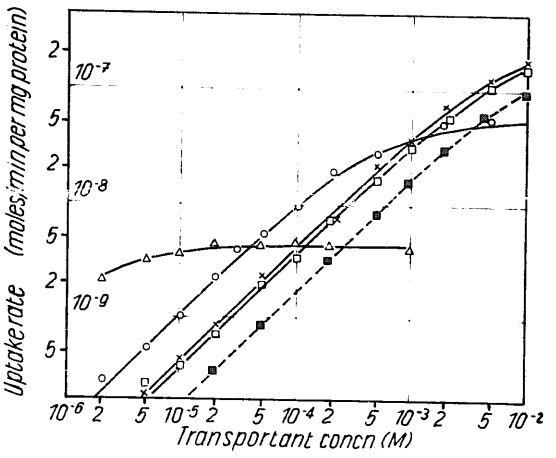

Fig. 1. Concentration dependence of initial uptake rates of L-arginine (△), L-glutamic acid (○), L-phenylalanine (×) and L-leucine (□) after 6 h preincubation of 2-days-old hyphae with complete medium. As described in the text, curves △, × and ○ represent the activities of transport systems a, b and c, respectively; curve ■- - - - -■, which represents the activity of system d is obtained by subtracting the leucine flux via systems c (calculated with the data from Table IIA) from the total flux of this amino acid. Note that both scales are logarithmic.

Cells in nutrient-sufficient media. Fig. 1 shows the dependence of the initial uptake rates of the four amino acids on their external concentration, as measured in hyphal cells growing with complete nutrients. The curves of arginine and glutamic acid uptake fit well with single Michaelis-Menten terms, whereas uptake of L-phenylalanine and L-leucine show a similar tendency but cannot completely be saturated within the range of usable substrate concentrations (see Materials and Methods).

The inhibition patterns of uptake (Table I) are indicative of the activities of four different carriers:

(1) Uptake of labelled L-arginine and L-phenylalanine is substantially inhibited only by the same amino acid and its structural analogs L-lysine or L-tyrosine, respectively. Similar results were found in reversed experiments, i.e., inhibition of L-lysine uptake by L-arginine and of L-tyrosine uptake by L-phenylalanine (data not shown). Therefore, the existence of two separate, specific permeases accepting L-arginine and L-lysine (named system a) and L-phenylalanine and L-tyrosine (named system b) has to be assumed. It cannot be excluded that L-glutamic acid is a low-affinity substrate of system b.

(2) Uptake of L-leucine and L-glutamic acid show overlapping specificities, as indicated by their mutual inhibition of uptake. In addition to the different inhibitory power of L-glutamic acid and L-leucine (Table I), there are several reasons to assume two transport systems for these amino acids:
(i) leucine uptake is not completely inhibited by increasing glutamic acid concentrations (Fig. 2). At $5 \cdot 10^{-4}$ M, only about 50% of leucine transport are sensitive to inhibition by L-glutamic acid;

TABLE I

Uptake of L-arginine, L-glutamic acid, L-phenylalanine and L-leucine in the presence of unlabelled amino acids into cells from nutrient-sufficient cultures

Initial uptake rates were determined in the presence of a 10-fold excess of unlabelled amino acids.

Competitor (L-isomer)	Transportant (L-isomer, [U-^{14}C]):	Uptake rate (% of level without competitor)			
		arginine (10^{-5} M)	glutamic acid ($5 \cdot 10^{-4}$ M)	phenylalanine ($5 \cdot 10^{-4}$ M)	leucine ($5 \cdot 10^{-4}$ M)
None		(100)	(100)	(100)	(100)
Arginine		11	100	80	100
Lysine		17	100	78	99
Histidine		95	n.d.	n.d.	n.d.
Ornithine		81	n.d.	100	n.d.
Glutamic acid		95	16	84	56
Aspartic acid		n.d.	90	n.d.	94
Leucine		93	60	99	64
Alanine		96	63	100	88
Phenylalanine		96	83	59	98
Tyrosine		98	90	65	100
Tryptophan		100	97	100	102

n.d., not determined.

(ii) after 48 h growth the sensitivity of leucine uptake towards L-glutamic acid, L-alanine and L-aspartic acid is negligible and develops only during incubation with complete nutrients in parallel with the increase of glutamic acid uptake. During this period the uptake rate of L-leucine at $5 \cdot 10^{-4}$ M is nearly doubled (Fig. 2). Tentative measurements have shown an increase of alanine and aspartic acid transport at the same time; both are inhibitable by L-glutamic acid.

Most probably, at the end of the logarithmic growth phase, a leucine-specific, glutamic acid-insensitive permease is present (system d), which maintains its activity when growth is enhanced by substitution of consumed nutrients. The latter conditions trigger the development of a new permease, which accepts L-glutamic acid, L-alanine, L-leucine and L-aspartic acid (system c, affinity decreasing in this order).

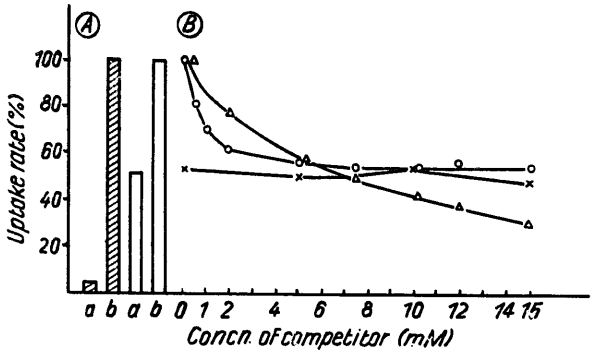

Fig. 2(A). Relative uptake rates of L-glutamic acid (hatched columns) and L-leucine (open columns) measured after 48 h cultivation (a) and after additional 6 h preincubation with 50% complete nutrient solution (b). Transportant concentrations $5 \cdot 10^{-4}$ M. (B) Effect of increasing concentrations of L-glutamic acid on the uptake rate of L-[U-^{14}C]leucine, $5 \cdot 10^{-4}$ M; ×, cells after 48 h cultivation; ○, cells after additional 6 h preincubation with 50% complete nutrients. △, for comparison: effect of increasing L-leucine concentrations on the uptake rate of the labelled L-leucine. Uptake rates (mol/min per mg protein): L-leucine, $100 = 1.65 \cdot 10^{-8}$; L-glutamic acid, $100 = 2.7 \cdot 10^{-8}$.

It is further shown in Table I that systems b and c are weakly inhibited by high concentrations of amino acids, which are unlikely to be substrates of these systems, i.e., phenylalanine uptake by L-arginine, and glutamic acid uptake by L-phenylalanine *. These inhibitions cannot be overcome by increasing substrate concentrations, which points to an nonspecific influence on the carrier activity rather than a competition for uptake.

Table IIA lists the kinetic constants of the carrier activities measured in nutrient sufficient cells; Fig. 1 gives the corresponding J_s/S plots for their main transportants.

The increase of transport rates via systems a, b and c during incubation with complete nutrients occurs in the presence of relatively high cellular contents of free amino acids and ammonia, which do not substantially change under these conditions (Fig. 3). The development of transport activity may therefore represent new synthesis of permease proteins rather than the release from transinhibition of permease activity by intracellular substrates.

* L-Arginine cannot be a substrate of system b, as arginine uptake is not inhibited by L-phenylalanine and shows no bipashic concentration dependence. L-Phenylalanine is not a substrate of system c, as the degree of inhibition of phenylalanine uptake by L-glutamic acid does not increase with the activity of system c during preincubation in complete medium.

TABLE II

Kinetic constants of transport systems separated in submerged grown hyphae of Penicillium cyclopium

For the determination of K_T and J_{max} see Materials and Methods. Experimental data used for the determination of K_T and J_{max}: Systems a, b and c: J_s/S plots of uptake of L-arginine, L-phenyl-alanine and L-glutamic acid, respectively. System d: J_s/S plot of leucine uptake via system d (Fig. 1, ■- - - - -■), obtained by subtracting from the total leucine flux the leucine uptake via system c. The latter is calculated from the glutamic acid-sensitive portion of leucine uptake (e.g., at $5 \cdot 10^{-4}$ M) and K_i (Leu) of glutamic acid uptake. Systems I, II and III: J_s/S plots of utpake of L-arginine, L-glutamic acid and L-phenylalanine after subtracting the fluxes via systems a, b, c and d according to Table IV.

System	Substrate(s)	K_T (M)		J_{max} (mol/min per mg protein)
A: specific systems, assayed in nutrient-sufficient cells				
a	L-Arginine, L-lysine (L-ornithine)	Arg:	$1.9 \cdot 10^{-6}$	$4.4 \cdot 10^{-9}$
b	L-Phenylalanine, L-tyrosine	Phe:	$6.6 \cdot 10^{-3}$	$2.8 \cdot 10^{-7}$
c	L-Glutamic acid	Glu:	$4.8 \cdot 10^{-4}$	$5.5 \cdot 10^{-8}$
	(L-leucine, L-alanine)	Leu [a]:	$3.0 \cdot 10^{-3}$	$6.0 \cdot 10^{-8}$
d	L-Leucine (L-alanine)	Leu:	$1.3 \cdot 10^{-2}$	$2.3 \cdot 10^{-7}$
B: less specific systems, assayed in nitrogen-starved cells				
I	α-Amino-α-carboxylic acids,	Arg:	$1.2 \cdot 10^{-5}$	$3.2 \cdot 10^{-8}$
	L-isomer preferred	Glu:	$2.4 \cdot 10^{-5}$	$3.1 \cdot 10^{-8}$
	(General amino acid permease)	Phe:	$1.4 \cdot 10^{-5}$	$6.3 \cdot 10^{-9}$
		Leu:	$2.4 \cdot 10^{-5}$	$1.5 \cdot 10^{-8}$
II	L-Arginine, L-phenylalanine	Arg:	$5 \cdot 10^{-3}$	$1.8 \cdot 10^{-7}$
III	L-Glutamic acid, L-phenylalanine	Glu:	$7 \cdot 10^{-3}$	$2.1 \cdot 10^{-7}$

[a] K_i (Leu) of L-glutamic acid uptake.

Nitrogen-starved cells. Preincubation of the cells with NH_4^+-free culture liquid gives rise to a biphasic concentration-dependence of uptake (Fig. 4A). Furthermore, the inhibition patterns shown in Table III indicate the dominating role of new carriers with a very broad specificity of transport. Nevertheless, the latter systems (a, b, c, d) were shown to contribute to the total uptake in nitrogen-starved cells as well. The share of these carriers of the total fluxes was estimated by experiments described in Table IV. In principle, initial uptake rates of L-arginine, L-glutamic acid, L-phenylalanine and L-leucine were measured in the presence of a large excess of such unlabelled amino acids, which are not transported via the same specific systems as the labelled transportant (cf. inhibition pattern in Table I),

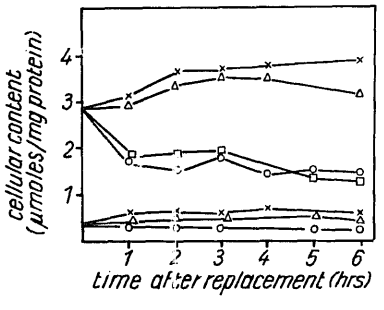

Fig. 3. Cellular contents of free amino acids (upper curves) and NH_4^+ (lower curves). After 2 d growth, the medium was replaced by half-concentrated culture liquid containing: ×, complete nutrients; ○, △, □, no NH_4^+; △, 50 μg/ml cycloheximide; □, 100 μg/ml 5-fluorouracil; ○, no inhibitors.

but would effectively compete for the less specific carriers expected. The resulting tracer fluxes, which represent mainly the activity of the specific carriers were then corrected for the remaining, largely reduced share of the unspecific systems. At low substrate concentrations – usually $2 \cdot 10^{-6}$ M and 10^{-5} M – this approach allowed the separation of distinct portions of the fluxes of L-arginine, L-phenylalanine, L-glutamic acid and L-leucine, which indeed show similar specificities and orders of affinities as reported above for systems a, b, c, and d, respectively (Table IV). They were therefore regarded to represent the activity of the latter carriers and used to calculate their fluxes over the entire concentration range (using the K_T values obtained with nutrient-sufficient cells). When these fluxes are subtracted from the total uptake rates, the characteristics of the newly developed, less specific systems become apparent:

The concentration-dependence of L-leucine uptake is converted into a single Michaelis-Menten term, suggesting that only one among the new carriers transports this substrate. In contrast, the corresponding J_s/S plots of transport of L-arginine, L-phenylalanine and L-glutamic acid remain biphasic (Fig. 4B). With the aid of a computerized iteration procedure (cf. Materials and Methods), each of the latter curves could be separated into two Michaelis-Menten terms with half-saturation constants around 10 μM and 1 mM, respectively. The existence of multiple transport systems thus indicated is supported by different inhibition patterns of uptake within both concentration ranges (Table IIIA, B).

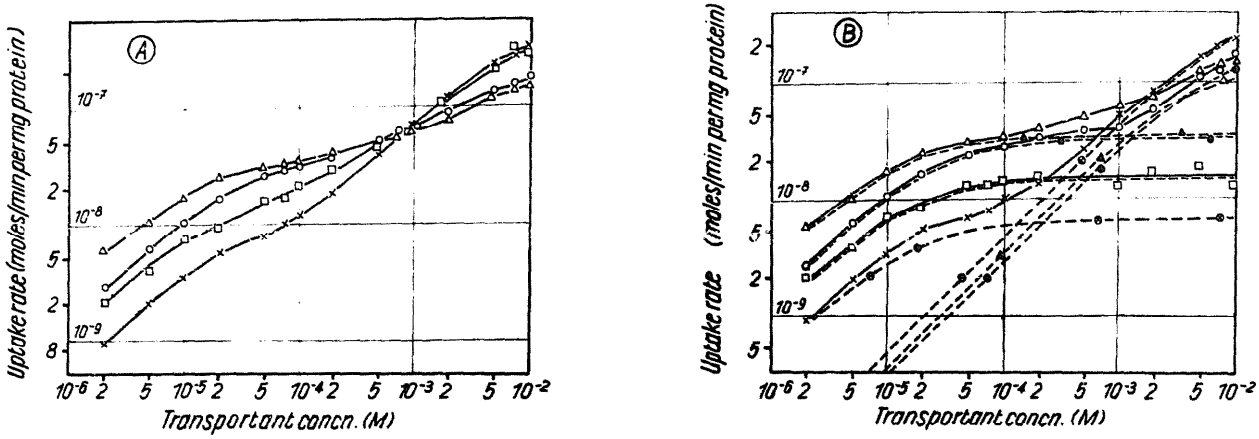

Fig. 4. Concentration dependence of initial uptake rates of L-arginine (A), L-glutamic acid (○), L-phenylalanine (x) and L-leucine (Cl) in hyphal cells after 6 h of nitrogen starvation. (A) Total fluxes; (B), full lines, total fluxes minus the fluxes via the specific systems a, b, c or cl (measured and calculated according to Table IV): broken lines, Michaelis-Menten graphs adjusted to the experimental points either as a single term (L-leucine uptake) or as a sum of two terms (uptake of t-arginine (A), L-glutamic acid (●) and L-phenylalanine (⊗)). Note that both scales are logarithmic.

At low substrate concentrations

At concentrations below 10^{-5} M, all amino acids tested are effective inhibitors of uptake of labelled L-arginine, L-phenylalanine, L-glutamic acid and L-leucine, provided that the a-amino and α-carboxyl group are unsubstituted, the L-isomer being the preferred species (Table IIIA). The mutual inhibition of uptake within pairs of the four amino acids was studied in more detail performing parallel experiments with both transportants labelled in turn (Table V). The results show a reasonable coincidence between the extent of inhibition and the relative rate of the concurrent uptake of the inhibitor. The K_i values were found to be similar with the K_T of uptake of the inhibiting amino acid which likewise points to a competitive type of inhibition.

These findings clearly suggest that the high-affinity component of uptake represents a single system with

TABLE III

Uptake of L-arginine, L-glutamic acid, L-phenylalanine and L-leucine in the presence of unlabelled amino acids into cells from nitrogen-starved cultures

Competitor A: $5 \cdot 10^{-5}$ M B: $5 \cdot 10^{-3}$ M	Uptake rate (% of level without competitor)						
	A transportant $5 \cdot 10^{-6}$ M (L-isomer, [U-^{14}C])				B [a] transportant $5 \cdot 10^{-3}$ M @-isomer, [U-^{14}C])		
	arginine	glutamic acid	phenyl-alanine	leucine	arginine	glutamic acid	phenyl-alanine
None	(100)	(100)	(100)	(100)	(100)	(100)	(100)
L-Arginine	26	25	37	30	69	97	64
L-Lysine	28	20	22	21	68	100	70
L-Glutamic acid	47	40	51	44	96	73	86
L-Aspartic acid	41	28	42	37	100	70	84
L-Glutamine	32	17	32	30			
L-Leucine	38	31	41	40	102	93	102
L-Alanine	37	30	42	40			
L-Phenylalanine	34	27	38	37	76	76	77
L-*N*-Methylphenyl-alanine	99	100	98	98			
L-Phenylalanine methyl ester	97	99	99	98			
D-Phenylalanine	56	55	59	61			
L-Tryptophan	51 30	28	58 32	60	84	79	82
L-Proline	98	97	100	99			

[a] These data represent fluxes via low-affinity systems only; fluxes via systems I, a, b or c, respectively, were calculated using the appropriate J_{max}, K_T and/or K_i (i.e. K_T of inhibitor) and subtracted from the measured total fluxes.

TABLE IV

Differentiation of tracer fluxes via specific and less specific carriers in nitrogen-starved cells

Tracer flux J_1 is measured in the presence of an excess of amino acid(s) ($5 \cdot 10^{-3}$ M) which inhibits unspecific carriers, but not (severely) the specific ones. Flux J_2 represents the rest flux of tracer, if both specific and unspecific carriers are clogged by an excess ($5 \cdot 10^{-3}$ M) of unlabelled transportant. The difference $J_1 - J_2$ is due to the activity of specific carriers, provided that the unspecific carriers are inhibited to the same degree by the inhibitor and by the transportant. To select inhibitor amino acids which meet this requirement, the labelled transportant was used in parallel experiments at $5 \cdot 10^{-3}$ M plus either the same amino acid or the potential inhibitor, both at the same concentration. From the resulting flux the activity of the specific system used by the tracer [b] was substracted to give the flux via the unspecific systems which was clogged either by the inhibitor or the unlabelled transportant. With the inhibitors used here, i.e. L-phenylalanine and the mixture of L-arginine and L-glutamic acid (each $5 \cdot 10^{-3}$ M), both data show reasonable coincidence. The difference $J_1 - J_2$ must be corrected for the inhibition of fluxes via the specific carriers by the excess of the used inhibitor (small, unspecific inhibition) or unlabelled transportant (competitive inhibition). These inhibitions are quantified by the factors C or U, respectively, where 1 = 100% inhibition. Both factors were determined with cells from nutrient-sufficient cultures, i.e. in the absence of unspecific systems. The flux via a specific carrier is then given by $J = (J_1 - J_2)/(C - U)$. All fluxes appear in % of uninhibited flux. (To keep the table readable, standard symbols of amino acids are used for the L-isomers; a, b, c or d, either as index or in parenthesis refer to the specific systems.)

	Transportant (L-isomer, [U-^{14}C], $2 \cdot 10^{-6}$ M)							
	arginine		glutamic acid		phenylalanine		leucine [a]	
Flux J_1 (flux with additional inhibitors)	+ Phe	12.0	+ Phe	5.0	+ Arg/Glu	12.0	+ Phe	6.4
	+ Leu	11.5	+ Arg	5.1	+ Leu	12.1	+ Glu	4.7 (J_3)
	+ Glu	12.2	+ Leu	3.1	+ Ala	12.4	+ Ala	4.5
	+ Lys	1.0	+ Ala	3.9	+ Tyr	10.7	+ Trp	6.8
			+ Asp	4.6			+ Arg	6.1
Flux J_2 (inhibition of specific systems)	+ Arg	0.8	+ Glu	2.0	+ Phe	10.2	+ Leu	4.1
	C(a) =	1.0	C(c) =	0.9	C(b) =	0.43	C(d) =	0.28
	U(a) =	0.04	U(c) =	0.15	U(b) =	0.15	U(d) =	0.0
Flux via specific system	J_a =	11.7	J_c =	3.9	J_b =	6.4	J_c =	2.2
							J_d =	4.3

[a] Uptake of leucine occurs via system c as well as system d. First, the flux via system c (glutamic-acid-sensitive) is determined: $J_c = (J_1 - J_3)/(0.91 - 0.15) = 2.2\%$. The inhibition of the phenylalanine-resistant flux J_1 by leucine allows the estimation of J_d as the inhibition by leucine of J_c (49%) and J_d (28%) can be measured in nutrient sufficient cells. Hence, $J_1 - J_2 = 2.3\% = 0.49\, J_c + 0.28\, J_d$ and $J_d = 4.3\%$.

[b] i.e., flux $J_1 - J_2$ from this table, calculated for the final substrate concentration with K_T from Table IIB.

the broad substrate specificity of a 'general amino-acid permease' as described also in several other fungi [3,4,14,15]. This permease in *P. cyclopium* is named system I in the following. It is further characterized by the ratio of maximum fluxes shown with individual transportants: substrates with hydrophobic side-chains (L-phenylalanine, L-leucine, L-tryptophan) allow markedly lower maximum fluxes as compared with more hydrophilic transportants (L-arginine, L-glutamic acid), whereas K_T is not seriously influenced in this respect (see Table IIB). One may therefore assume that K_T mainly mirrors the binding affinity between carrier and

TABLE V

Inhibition of L-arginine uptake by L-glutamic acid, L-phenylalanine and L-leucine compared with the individual fluxes of the competitors

In the first set of experiments, uptake of L-[U-^{14}C]arginine was followed in the presence of unlabelled competitors; in a parallel set the uptake of the labelled competitors was assayed in the presence of unlabelled L-arginine under identical conditions. Each amino acid concentration was 10^{-4} M. Transport via system I is calculated by subtracting from the total flux the fluxes via other systems involved in the transport of the labelled substrate, i.e., the low-affinity systems and the specific systems a (L-arginine), b (L-phenylalanine), c (L-glutamic acid, L-leucine) and d (L-leucine). The latter fluxes could be calculated directly from their known concentration dependence (see Figs. 1 and 3), since in the used concentration range the competitors cause no reduction of tracer fluxes via carriers other than system I.

Transportant: L-[U-^{14}C]arginine				
Competitors:	None	L-glutamic acid	L-phenylalanine	L-leucine
L-Arginine flux via system I	2.95	2.2	1.75	1.9
Inhibition of this flux:		0.75	1.2	1.1
Inhibition in % of J_{max} (Arg)		23	37	33
Competitor: L-arginine				
Transportant: [U-^{14}C]		L-glutamic acid	L-phenylalanine	L-leucine
Flux via system I		0.9	0.22	0.48
Flux in % of J_{max} (Arg)		29	35	32

TABLE VI

Activities of individual transport systems in cultures of different physiological states

The individual transport activities were estimated from the fluxes of labelled transportants as follows (see also text). Systems a, b, c or d: fluxes measured with transportants 5 μM in the presence of 5 mM phenylalanine, glutamic acid or arginine (cf. Table IV); system I: fluxes measured with transportants 5 μM minus the fluxes via systems a, b, c or d, respectively. In all these cases (a, b, c, d and I) fluxes at 5 mM were calculated from the fluxes at 5 μM using the K_T values from Table II. Systems II and III: fluxes measured with transportants 5 mM minus fluxes via systems I, a, b, c or d, respectively; fluxes at 5 μM estimated from the latter data using K_T values from Table II.

Transportant (L-isomer [U-^{14}C])	Uptake rate (10^{-3} mol/min per mg protein)					
	Nutrient regime C,N-sufficient		N-starved		C-starved	
Transport system	5 μM	5 mM	5 μM	5 mM	5 μM	5 mM
Arginine, total flux	3.2	4.5	11	120	4	38
a	3.2	4.5	0.9	1.3	1.4	1.8
I	0	0	9.9	34	2.7	9.2
II	0	0	0.15	75	0.1	25
Phenylalanine, total flux	0.25	120	2.0	230	1.4	502
b	0.25	120	0.15	75	0.8	460
I	0	0	1.9	6.3	0.5	1.7
II + III	0	0	0.2	140	0.1	53
Leucine, total flux	0.2	160	4.0	290	1.8	480
c + d	0.2	160	0.3	280	0.6	475
I	0	0	3.8	13	1.0	3.4
Glutamic acid, total flux	0.55	48	6.0	130	2.4	125
c	0.55	48	0.25	22	1.0	89
I	0	0	5.5	19	1.6	5.4
III	0	0	0.1	82	0.1	30

0, uptake not measurable.

transportant, whereas J_{max} is limited by the rate of translocation, the latter being slowed down by hydrophobic residues.

At high substrate concentrations

At concentrations above 10^{-3} M, uptake rates are inhibitable only to a limited degree as no saturating excess of inhibitors can be realized within the range of usable concentrations. Nevertheless, the attainable inhibition patterns (Table IIIB) indicate that

(i) L-arginine and L-glutamic acid do not mutually inhibit their uptake;

(ii) uptake of L-phenylalanine is inhibited by both L-arginine and L-glutamic acid and vice versa;

(iii) inhibitors, of arginine uptake (basic amino acids) as well as of glutamic acid uptake (aspartic acid) all interfere with the uptake of L-phenylalanine; and

(iv) L-leucine does not interfere with the uptake of L-arginine, L-glutamic acid and L-phenylalanine.

Hence, it appears that there are two independent systems transporting basic or acidic amino acids. They are both used by L-phenylalanine and L-tyrosine, but not by L-leucine. The contribution of both a glutamic-acid-sensitive and an arginine-sensitive component to the uptake of L-phenylalanine is confirmed by observations made at low L-phenylalanine concentrations (and thus with more accurate transport rates): if the uptake of labelled L-phenylalanine ($5 \cdot 10^{-5}$ M) via system I is blocked by an excess of L-leucine ($5 \cdot 10^{-3}$ M), the total tracer flux drops to 45%, which represents mainly the low-affinity component *. This flux is further reduced to 37% by either 10^{-2} M L-arginine or 10^{-2} M L-glutamic acid, but decreases to 29% when a mixture of these competitors (each $5 \cdot 10^{-3}$ M) is added. It seems therefore reasonable to suggest two low-affinity systems to be active in nitrogen-starved cells **. They are named system II (transporting acidic amino acids and L-phenylalanine) and system III (transporting basic amino acids and L-phenylalanine) in the following. It should be realized that – for experimental reasons – the kinetic properties of these transport activities are so far only rough estimates as compared with the more precisely characterized systems I, a, b, c, and d.

* The leucine-resistant tracer flux (45% of total flux) occurs via the low-affinity uptake, 30% and system b, 15%. As shown earlier, the latter system is only weakly inhibitable by L-glutamic acid or L-arginine.

** Nevertheless, mutual noncompetitive effects of L-arginine, L-glutamic acid and/or L-phenylalanine on their uptake cannot totally be excluded. (An assay of the concurrent flux of the inhibiting amino acid (analogous to the experiment described in Table V) is complicated due to the relatively high share of fluxes via systems I, a, b, or c, respectively).

TABLE VII

Uptake of L-arginine in the presence of unlabelled amino acids into cells from carbon-starved and nitrogen-starved cultures

Total uptake rates of L-[U-^{14}C]arginine were measured in the presence of unlabelled competitors: from these data, fluxes via system I were calculated by subtracting the fluxes via system a. The latter were calculated from their known contribution to the total flux (cf. Table VI) and the K_T (Arg) from Table IIA.

Competitor ($5 \cdot 10^{-5}$ M)	Fluxes of L-arginine ($5 \cdot 10^{-6}$ M) (% of level without inhibitor)			
	Physiological state of culture			
	N-starved		C-starved	
	total flux	system I	total flux	system I
None	(100)	(100)	(100)	(100)
L-Arginine	26	25	23	28
L-Glutamic acid	47	41	64	45
L-Aspartic acid	41	34	60	39
L-Leucine	38	31	53	29
L-Alanine	37	30	51	26
L-Phenylalanine	34	27	52	27
L-Tyrosine	51	46	66	48

Table II summarizes the kinetic data of all transport systems characterized so far in *P. cyclopium.*

Carbon-starved cells

Preincubation of the hyphae with culture liquid free of carbon sources results likewise in a multiplicity of transport activities. The specific transport systems take a much higher share of the total uptake compared with nitrogen-starved cells. Therefore the newly developed, unspecific carriers in carbon-starved cells are assayed with some lower accuracy. Nevertheless, there are various indications that most if not all carriers are similar to the systems characterized in nitrogen-starved hyphae.

Specific carriers. In the same way as described above (see Table IV), distinct portions of the fluxes of labelled L-arginine, L-phenylalanine, L-glutamic acid and L-leucine could be separated which are selectively inhibited by substrates of the specific systems a, b, c and d, respectively. When these fluxes are used to construct J_s/S plots with the K_T values known for the specific systems (Table IIB) the curves predict that at high-substrate concentrations the specific carriers largely dominate the uptake of L-leucine and of L-phenylalanine. Table VI, column 6 shows the values calculated for 5 mM substrate concentration. They were confirmed when the specific fluxes of these two transportants at $5 \cdot 10^{-3}$ M were measured in the presence of $5 \cdot 10^{-2}$ M L-arginine; in a typical experiment, the fluxes of L-phenylalanine and L-leucine reached 90 or 106%, respectively, of the predicted values. Accordingly, K_T of arginine transport in the presence of 10^{-2} M phenylalanine amounts to $2.5 \cdot 10^{-6}$ M, which is close to K_T(Arg) of system a ($1.9 \cdot 10^{-6}$ M). It seems therefore reasonable to suggest that systems a, b, c and d are active in carbon-starved cells, too.

System I. After subtraction from the total uptake of the transport rates of L-arginine and L-glutamic acid via the specific systems, the remaining fluxes yield a biphasic J_s/S plot which can be separated into two Michaelis-Menten terms. The individual K_T values of arginine transport – $1.5 \cdot 10^{-5}$ M and approx. $5 \cdot 10^{-3}$ M – are in good agreement with those found for systems I and II, respectively, in nitrogen-starved cells. At low substrate concentrations, the remaining transport activity shows the broad substrate specificity known of system I (Table VII). A characteristic feature of system I is the ratio of fluxes of L-arginine, L-glutamic acid, L-leucine and L-phenylalanine at a distinct substrate concentration (at $5 \cdot 10^{-6}$ M, this order is roughly 10 : 5.5 : 4 : 1.5, cf. Fig. 4). This relationship remains unchanged if the unspecific portions of uptake of these amino acids are compared between nitrogen-starved and carbon-starved cells, even though their total fluxes differ widely (Table VI). These findings clearly suggest the presence of system I in carbon-starved cells.

Systems II and III. A carrier similar to system II (accepting both L-arginine and L-phenylalanine) manifests itself as a distinct, phenylalanine-sensitive portion of arginine flux in the presence of excess L-glutamic acid, which practically eliminates the tracer flux via system I. Analogously, a carrier with properties like system III (accepting both L-glutamic acid and L-phenylalanine) can be identified as a phenylalanine-sensitive portion of glutamic acid flux if system I is clogged by an excess of L-arginine.

The fluxes via the above-mentioned systems in carbon-starved cells are compiled in Table VI, columns 5 and 6.

Overview: Nutrient-dependent development of permease patterns

Table VI gives an overview of the activities of the observed permeases in cells preincubated under various

nutritional conditions. The following characteristics can be seen:

(i) Nitrogen deficiency is the most powerful signal for the development of systems I, II and III, which are not detectable in nutrient-sufficient cells. Carbon starvation causes a markedly lower increase of activity of this permeases. This finding can only partially be due to reduced energy supply but points mainly to the inhibitory effect of ammonia, as withdrawal of NH_4^+ from carbon-starved cultures causes a significant, though somewhat smaller, increase of transport (data not shown).

(ii) The specific systems a–d are obviously under the influence of several, partially overlapping regulatory circuits. Carbon starvation provides the most effective signal for the simultaneous development of all of these permeases, which implies that carbon catabolite repression may exert a general control and coordinate their expression with that of metabolic chains utilizing amino acids as carbon sources instead of glucose. In addition, systems a, c and d develop in the absence of ammonia. The regulation of system a deserves special mention as its activity attains the highest levels in cultures growing fast in the presence of both ammonia and glucose. This may reflect a special requirement for arginine of growing cells.

As a consequence of the kinetic properties of the above permeases and their nutrient-dependent regulation the total uptake capacity available for individual amino acids is specifically adjusted to different metabolic situations (Table VI, total fluxes).

At low substrate concentrations the highest uptake rates are realized by nitrogen-starved cells, which reflects mainly the kinetic and regulatory properties of the dominating system I. The uptake capacity for high concentrations develops differentially: arginine transport increases preferentially during nitrogen limitation, transport of L-phenylalanine and L-leucine mainly during carbon-limitation, whereas glutamic acid transport similarly responds to both starvations. From these data the outline of a cellular strategy can be imagined which subordinates the uptake of low-concentation amino acids in general as well as of basic amino acids at higher concentrations to the control of nitrogen anabolism or catabolism, respectively. On the other hand, neutral amino acids at higher concentrations are preferentially used in demand for C-sources. Transport of acidic amino acids underlies both principles to a similar extent.

pH dependence of uptake of L-arginine, L-glutamic acid, L-phenylalanine and L-leucine

As can be deduced from the pH profiles of uptake shown in Fig. 5, all transport systems assayed in *P. cyclopium* allow maximum initial rates around pH 3.5. At this pH, which coincides with the prevalent acidity

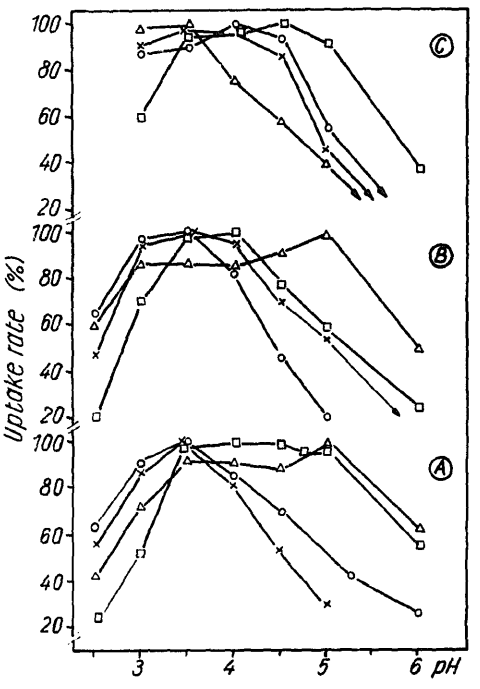

Fig. 5. pH dependence of initial uptake rates of L-arginine (△), L-glutamic acid (○), L-phenylalanine (×) and L-leucine (□) under various conditions: (A) nutrient-sufficient cells, substrate concentration $5 \cdot 10^{-6}$ M, i.e., uptake mainly via systems a, c, b, d, resp.; (B) nitrogen-starved cells, substrate concentration $5 \cdot 10^{-6}$ M, i.e., uptake mainly via system I; (C) nitrogen-starved cells, substrate concentration $5 \cdot 10^{-3}$ M, i.e., uptake mainly via systems II and III. Maleate buffers, 60 mM were used throughout. Data are given in % of the maximum uptake rate of each amino acid.

of the culture medium (pH 3.2–3.6 in 2 d growing cultures), neutral and acidic amino acids exist mainly as zwitterionic molecules; basic amino acids bear an extra positive charge. At pH values above 3.5, characteristic differences become apparent.

System I is represented by the fluxes of the four amino acids at $5 \cdot 10^{-6}$ M in nitrogen-starved cells. The steep decline of uptake of L-glutamic acid at pH > 3.5 contrasts with the broader optimum of transport of L-phenylalanine and L-leucine. At pH 4.5 for instance, 61% of glutamic acid, but less than 1% of phenylalanine or L-leucine are anionic. This suggests that system I strongly prefers zwitterionic species to anionic transportants. Similar properties are known from the GAPs of *Penicillium chrysogenum* [15] and *Neurospora crassa* [16]. A specific property of system I is indicated by the very broad optimum of L-arginine transport (between pH 3 and 5). As in this region almost all transportant molecules are cationic, it seems reasonable to suggest the involvement of a side-chain-recognizing anionic site, e.g. an α-carboxyl of glutamic acid ($pK_a = 4.28$). The existence of such an anionic group would further explain the exclusion from system I of transportants with anionic side-chains.

The pH dependence of the low-affinity transport systems dominating at high substrate concentrations

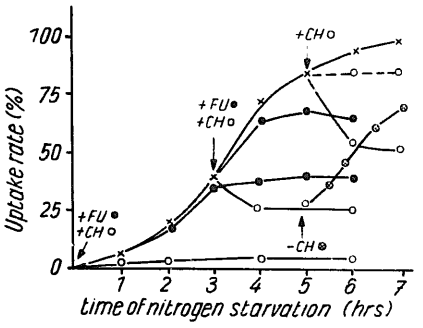

Fig. 6. Development of transport activity of system I during nitrogen starvation, influence of inhibitors of gene expression. After 48 h growth, hyphae were harvested and resuspended in ammonia free culture liquid (see Materials and Methods) and initial uptake rates of L-phenylalanine, $5 \cdot 10^{-6}$ M, were followed over 7 h. At the times indicated by arrows, 50 µg/ml cycloheximide (CH) or 100 µg/ml 5-fluorouracil (FU) were added. ×, control; ○, cultures with cycloheximide; ●, cultures with 5-fluorouracil; ⊗, culture treated for 2 h with cycloheximide, after removal of the drug; ○------○, cultures with cycloheximide, glucose and phosphate omitted. Data are given in % of maximum uptake rates of nitrogen-starved cultures.

($5 \cdot 10^{-3}$ M) is clearly different from that of the high-affinity transport. First, uptake of $5 \cdot 10^{-3}$ M glutamic acid shows maximum rates between pH 3 and 4.5, which might indicate that system III – unlike system I – transports both the anionic and zwitterionic species. Second, L-arginine uptake declines with pH 3.5 and thus points to an individual property of system II not shown by system I (lack of the putative anionic binding site?). Third, the broad optimum of L-leucine transport clearly differs from the leucine transport via system I but coincides with the pH dependence of system d (L-leucine uptake into nutrient-sufficient cells). This finding is consistent with the result of the kinetic analysis, i.e., the absence of another low-affinity carrier for L-leucine except system d, in nitrogen-starved cells.

The pH dependence of the specific systems a and c resembles that of the transport of L-arginine and L-glutamic acid, respectively, via system I. This might indicate similarities of the ionic properties between these high-affinity systems and the GAP.

Regulation of synthesis and turnover of GAP

The increase of transport activity of system I (assayed with L-[U-^{14}C]arginine, $5 \cdot 10^{-6}$ M) during nitrogen starvation is clearly influenced by inhibitors of gene expression (Fig. 6).

Cycloheximide ($2 \cdot 10^{-4}$ M) immediately stops the increase of activity at any time, this effect being fully reversible by withdrawal of the drug. It may reflect either the stop of synthesis of protein(s) required for the permease function or the maintenance of transinhibition of a preexisting permease by endogenous amino acids. Indeed, cycloheximide prevents the decrease of the content of free amino acids, which occurs at the beginning of NH_4^+ starvation. However, this decline lasts about 1 h (cf. Fig. 3), whereas the development of maximum transport activity is completed only after 7 h, suggesting that the release from transinhibition may not play a significant role during the development of GAP activity in NH_4^+-starved cells. 5-Fluorouracil ($8 \cdot 10^{-4}$ M) likewise leads to a complete stop of the increase of transport activity. The mode of action of this drug is primarily in the inhibition of transcriptional processes and was demonstrated also in our fungus [17]. In contrast to cycloheximide, 5-fluorouracil causes some reduction, but not cessation of the incorporation of amino acids into protein. Within 1–4 h, the incorporation of L-[U-^{14}C]leucine into the solubilized protein (see Materials and Methods) did not drop below 80% of control cells. Accordingly, the drug does not significantly influence the measurable decline of free cellular amino acids during NH_4^+ starvation (Fig. 3).

Hence, the decrease of free amino acids below the level of growing cells is not a sufficient prerequisite for the development of GAP activity. It seems rather that this process requires both transcriptional and translational events. The inhibitory effect of 5-fluorouracil proceeds by different lag phases: when added at the beginning of NH_4^+ starvation, the activity increase proceeds unchanged over approx. 3 h, whereas at later stages of derepression the inhibitory effect is detectable already 1 h after administration of the drug. This 1 h delay is identical with the lag phase required to reach the maximum inhibition of protein synthesis caused by 5-fluorouracil.

Taken together, these findings indicate that the increase of transport activity during NH_4^+ starvation reflects the de novo synthesis of functional protein(s) of the GAP. This process, which is repressed in the presence of ammonia and glucose, proceeds in two phases. The initial phase, which comprises nearly one-third of maximum expression of permease activity, is independent of transcriptional processes and represents most probably the translation of preexisting, stable mRNA. Further expression requires transcriptional processes which are sensitive to 5-fluorouracil. The existence and translational activity of stable mRNA is of general significance in the differentiation of filamentous fungi and has been demonstrated for our object too [18].

The block of protein synthesis caused by cycloheximide allows some insight into the turnover of the GAP system. If cells from growing cultures are suspended in NH_4^+-free culture liquid or buffered glucose solutions the drug not only stops the permease development but triggers a rapid decrease of permease activity. Within 1 h the transport rate drops to about two-thirds of the original value and then remains constant over several hours. This decline does not occur – but, instead, a halt of further increase – if cycloheximide is added in the absence of glucose from the medium or after the culture has reached the stationary phase (Fig. 6). In each case,

cycloheximide causes a net degradation of protein, resulting in an increase of free amino acids (cf. Fig. 3). Therefore a transinhibition by these protein-derived amino acids should not be the reason for the decline of GAP activity, which occurs only in cells of high metabolic activity.

Most probably under these conditions the permease or some of its components undergoes a rapid turnover. The limited inactivation seen under cycloheximide might suggest that the degrading enzymes themselves have a short half-life and are inactivated within about 1 h after the stop of translation.

In our experiments with *P. cyclopium* the effect of cycloheximide on the GAP was neither reduced by concomitant addition of 10 mM Ca^{2+} nor enhanced in the presence of 2 mM phosphate. This is in contrast to earlier findings reported with *Penicillium chrysogenum*, where a very rapid decrease of the GAP activity (in 1–2 min) after addition of cycloheximide was ascribed to the liberation of membrane-bound Ca^{2+} required for the transport process [25].

H^+/amino acid cotransport

As in the other filamentous fungi investigated so far, uptake of amino acids in *P. cyclopium* displays characteristic features of active transport. External amino acids are concentrated to considerable cellular concentrations. The accumulation ratio (based on the cellular water content) ranges from several thousands at low outside concentrations to near 10 at 10^{-2} M solutions [34]. The initial rates of uptake of all amino acids show a strong temperature dependence with an optimum at 27°C and an activation energy around 10^4 kJ/mol. The following experiments characterize the mode of energy coupling of system I (assayed with L-[U-^{14}C]phenylalanine) in nitrogen-starved cells.

The uptake is highly sensitive to uncouplers and inhibitors of ATP synthesis (Table VIII). At low concentrations of the energy poisons, there exists a linear correlation between the reduction of ATP levels and the inhibition of transport. At high concentrations of uncouplers, transport is totally inhibited, though a significant percentage of ATP synthesis * is maintained (most probably via glycolysis).

As to be expected, ATP production under anaerobic conditions is unaffected by uncouplers. In contrast, uptake rates are inhibited by uncouplers to a similar degree, under both aerobic and anaerobic conditions. Furthermore, addition of glucose + phosphate increases the uncoupler-resistant percentage of ATP in aerobic cells without an effect on transport rates (Table IX), ATP is therefore very unlikely to be the direct energy source of amino-acid transport. Instead, the data sug-

* As the turnover of ATP is very high (after addition of $2 \cdot 10^{-5}$ M CCCP the ATP content drops to 12% within 40 s) the data in values which are measured 1 min after addition of inhibitors represent mainly newly made ATP.

TABLE VIII

Transport activities and ATP content in N-starved cells in the presence of metablic inhibitors and uncouplers

Inhibitors were added together with the transportant L-[U-^{14}C]phenylalanine ($5 \cdot 10^{-6}$ M) and initial uptake rates determined according to Materials and Methods. The ATP content was determined after 2 min. All data are in % of the values measured with inhibitor-free control cultures.

Inhibitor	Inhibitor concentration (M)	Cellular ATP content (%)	Uptake rate of L-phenylalanine (%)
2,4-dinitro-phenol	$7.5 \cdot 10^{-6}$	74	75
	10^{-5}	28	25
	10^{-4}	14	3
KCN	$5 \cdot 10^{-5}$	45	41
	10^{-4}	20	25
Na_3AsO_4	$3 \cdot 10^{-4}$	92	85
	10^{-3}	56	55
NaN_3	$2 \cdot 10^{-6}$	72	65
	$5 \cdot 10^{-6}$	32	34
	$2 \cdot 10^{-5}$	18	15
	10^{-4}	13	2
CCCP	$3 \cdot 10^{-6}$	34	30
	$5 \cdot 10^{-6}$	17	14
	$2 \cdot 10^{-5}$	11	1

TABLE IX

Effect of CCCP and azide on ATP content and transport activity under different metabolic conditions

Suspensions of N-starved cells in maleate buffer (50 mM, pH 3.5) were either aerated or flushed with argon for 30 min. During this time, glucose (1%) and NaH_2PO_4 (1 mM) were present in half of the samples. Inhibitors were added together with the transportant L-[U-^{14}C]phenylalanine, $5 \cdot 10^{-6}$ M. The ATP content was determined after 2 min, transport activity according to Materials and Methods. All data are in % of the maximum values measured with aerated samples containing glucose plus phosphate.

Conditions		Cellular ATP content (%)	Uptake rate of L-phenylalanine (%)
Buffer	air	90	91
+ CCCP, 10^{-6} M	air	29	30
+ NaN_3, $5 \cdot 10^{-6}$ M	air	32	34
Buffer	argon	57	34
+ CCCP, 10^{-6} M	argon	53	12
+ NaN_3, $5 \cdot 10^{-6}$ M	argon	55	10
Buffer glucose, phosphate	air	100	100
+ CCCP, 10^{-6} M	air	54	37
− NaN_3, $5 \cdot 10^{-6}$ M	air	59	35
Buffer glucose, phosphate	argon	56	30
+ CCCP, 10^{-6} M	argon	47	10
+ NaN_3, $5 \cdot 10^{-6}$ M	argon	52	11

gest that the uptake is energized by a H^+-gradient generated at the expense of cellular ATP.

H^+-coupled transport can be maintained only if the breakdown of the energizing protonmotive force (ΔpH and/or $\Delta\psi$) by the cotransported protons is prevented either by their continuous extrusion via the proton translocating ATPase or by charge-compensating fluxes of other ions. In hyphal cells of *P. cyclopium* the membrane potential is kept near zero (compared with $\Delta\psi$ values in the order of -100 or -200 mV in *Saccharomyces* and *Neurospora*, respectively [36,37], mainly because of a Donnan distribution of protons which establishes an acidic surface layer of cytoplasm (pH near 5) beneath the cellular membrane [7]. The situation provides an efficient protection of the cellular interior from an uncontrolled influx of protons from the usually acidic environment. The maintenance of an inwardly directed protonmotive force therefore requires a tight and immediate recycling of incoming protons by the H^+-ATPase of the cytoplasmic membrane. Indeed, inhibition of this proton pump by vanadate not only reduces amino-acid uptake but also increases the H^+ concentration of the surface layer [7]. It was therefore not surprising to find no measurable alkalization of the external medium during amino-acid accumulation. However, the intracellular pH shows a small but significant decrease during the initial phase of uptake, which tends to reverse during further accumulation (Table X). This effect is shown to a similar degree by neutral, acidic and basic amino acids. It indicates that in the initial phase of uptake a small portion of the symported protons is not recycled; the resulting transient acidification causes an increased rate of outward proton pumping, which then compensates for the proton influx. Compared with the net uptake of amino acid, the net influx of protons is, however, negligible each time.

The limiting role of the membrane potential as an energy source for amino-acid uptake is further subtantiated by the following findings. First, a small but significant influx of amino acids into ATP-depleted cells * can be initiated by addition of the chaotropic membrane traversing anion SCN^- (figures not shown), which compound clearly causes a negative $\Delta\psi$ [7]. This is in line with the classical findings about H^+-symports e.g., in *E. coli* [20]. Second, the short-time preaccumulation of several organic anions (notably glutamic, but also aspartic, α-oxoglutaric, oxaloacetic, citric and tartaric) to a distinct cellular level causes a remarkable stimula-

TABLE X

Extracellular and intracellular pH during uptake of glutamic acid, arginine and phenylalanine

Suspensions of N-starved cells (5 mg fresh weight/ml) containing Bromophenol blue ($5\cdot10^{-4}$ M) and glucose (1%) were aerated at 24°C. After addition of the indicated [U-^{14}C]amino acids ($5\cdot10^{-3}$ M) the pH was immediately adjusted to 3.9 with 0.01 M HCl or 0.01 M NaOH, respectively. At the times indicated, the accumulated amino acid, pH_{int} and pH_{ext} were determined in the same sample (cf. Materials and Methods). Data represent mean ± S.E. for three determinations.

Transportant	Time of incubation (min)	pH_{ext}	pH_{int}	Accumulated amino acid (μmol/ml cell water)
None	4	4.19±0.06	5.85±0.10	
	11	4.11±0.05	5.82±0.10	
	30	4.05±0.03	5.78±0.12	
L-Glutamic acid	4	3.95±0.05	5.48±0.12	19±4
	11	3.95±0.07	5.13±0.15	35±5
	30	3.93±0.04	5.65±0.13	71±6
L-Arginine	4	3.96±0.06	5.49±0.09	32±3
	11	4.00±0.05	5.65±0.10	54±5
	30	3.89±0.03	5.71±0.11	87±7
L-Phenylalanine	4	4.00±0.06	5.29±0.10	31±3
	11	3.98±0.08	5.45±0.12	39±3
	30	3.96±0.04	5.61±0.15	54±6

TABLE XI

Effect of preincubation with different anions of the initial rate of L-phenylalanine transport

N-starved cells were preincubated with the indicated acids in maleate buffer (50 mM, pH 3.5), then filtered and the cells resuspended in the same buffer (60 mM, pH 5.5) containing L-[U-^{14}C]phenylalanine ($5\cdot10^{-6}$ M). Initial uptake rates were determined as according to Materials and Methods. The accumulation of some anions during preincubation was measured in parallel runs where these compounds were present in ^{14}C-labelled form. After preincubation, these suspensions were treated as described for the determination of uptake rates.

Preincubation		Uptake rate of L-phenylalanine (% of level without preincubation)	Compound accumulated during preincubation (μmol/mg protein)
Substrate ($5\cdot10^{-3}$ M)	Time (min)		
Citric acid	7	128	0.19
α-Oxoglutaric acid	10	132	0.32
Oxaloacetic acid	10	130	n.d.
L-Glutamic acid	5	175	0.66
	10	149	0.77
	15	115	0.82
	30	92	0.96
L-Aspartic acid	10	145	0.80
L-Glutamine	5	95	0.61 [a]
	10	90	0.70 [a]
L-Phenylalanine	5	86	1.1
	10	80	1.9

[a] glutamic acid content.
n.d., not determined.

* Cells were treated with 10^{-4} M 2,4-dinitrophenol in uptake buffer 30 min prior to incubation and the inhibitor washed out carefully.

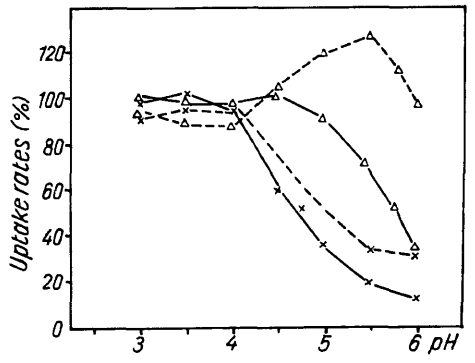

Fig. 7. Effect of short-time preincubation of nitrogen-starved cells with L-glutamic acid at the initial uptake rates of L-phenylalanine (×) and L-arginine (△), each $5 \cdot 10^{-6}$ M, at different pH. Broken lines, cells preincubated for 6 min with $5 \cdot 10^{-3}$ M L-glutamic acid in maleate buffer (60 mM, (pH 3.5)) and washed twice with water at 0 °C; full lines, cells preincubated with buffer alone. Maleate buffers 60 mM were used for the determination of uptake rates. Data are given in % of maximum uptake rates of either amino acid in control suspension.

tion of the following uptake of amino acids (Table XI). Under the experimental conditions, the preincubation with the above acids caused a similar adaptive response of pH_{int} as shown already with L-glutamic acid (see Table X) i.e., the decrease of the cellular pH is much slower than expected if the measured anion content represented accumulated free acids. Hence, in the initial phase of incubation, the incoming protons (irrespective whether they are transported as part of the undissociated acids or as H^+/anion symport) should be recycled and/or bound to cellular buffer systems. In each case there would be a tendency to increase anionic charges. If seen together with the above mentioned effects of SCN^-, it seems likely that in the initial phase of accumulation of the organic anions a more negative $\Delta\psi$ is transiently generated *. That such a process may indeed be responsible for the enhancement of initial rates of the following amino-acid uptake is demonstrated by preincubation with glutamine instead of glutamic acid: Even though the intracellular glutamate content reaches nearly the same level (via the glutaminase reaction) as after preincubation with glutamic acid, there is no stimulation of the subsequent amino-acid uptake. In this case, the charge of the glutamate anion is compensated by the concomitant generation of NH_4^+. A short preincubation with neutral or basic amino acids to a similar cellular content does not stimulate the subsequent amino-acid uptake. Above all, the stimulation of uptake occurs only at an external pH above 4.5 and increases with pH_{ext}, i.e., under conditions where the proton gradient declines and the protonmotive force consists mainly or exclusively of $\Delta\psi$ (Fig. 7). It seems therefore reasonable to suggest that preincubation with the above acids gives rise to an increased membrane potential (inside more negative) which accelerates the H^+/amino acid cotransport.

All the latter data are in line with our previous findings [7] and point to a tight coupling of H^+/amino acid symport with the proton extrusion by the ATPase of the cytoplasmic membrane. In this way the cotransported protons are immediately recycled, thus preventing dramatic effects on the cellular and extracellular pH.

* Of course, during longer incubations, not only CO_2 production but also charge-compensating fluxes of other ions [6] have to be expected. With $5 \cdot 10^{-3}$ M L-glutamic acid and citric acid, no significant metabolism has been observed within 10 min of uptake.

Conclusions

A detailed comparison of transport properties, even among related species, is often complicated due to differences between the cultivation conditions, growth and physiological state, heterogeneity, etc. of the assayed cultures. Nevertheless, the transport activities characterized here show some obvious similarities with, but also clear-cut differences from, the data known from other lower fungi, mainly *P. chrysogenum, Neurospora crassa, Saccharomyces cerevisiae*, and *Aspergillus nidulans*.

Obviously, a common equipment of these fungi is a GAP of high affinity, the physiological significance of which may lie in the absorption of small amounts of amino acids from the medium during nitrogen- and/or carbon-starvation ('scavenger systems', [14,16,21–24].). The pH optimum of the GAP (system I) in *P. cyclopium* (3.5) is much lower than those values reported from the comparable systems in the above fungi, thus reflecting an adaptation to the prevalent pH of culture. This difference may explain the much lower affinity for acidic amino acids of the GAP values in *P. chrysogenum* [25], *N. crassa* [26] and *Saccharomyces* [14]. At the pH optimum of uptake (between 5 and 6) acidic transportants exist mainly as anionic molecules, which in general are hardly accepted by the GAP. (As an exception, the GAP in germinating spores of *N. crassa* was reported to change some of its properties and then no longer discriminate against anionic transportants [28].) In the latter fungi, acidic amino acids are transported via separate systems, which accept both anionic and zwitterionic species and develop under similar conditions as the GAP [25,27,29]. In *P. cyclopium*, in the affinity range of the GAP, no such system could be detected. It would bear no obvious advantage, as at the usual culture pH (near 3.5), anonic molecules are a small minority compared with the abundant, zwitterionic species of L-glutamic and L-aspartic acid, which are good substrates of system I.

The acidic amino-acid transport system c of *P. cyclopium* has a lower affinity than the comparable

carriers in the other fungi. Compared with the well-characterized system II of *A. nidulans*, which is highly regulated by nitrogen sources [30], it differs by its high activity in nutrient-sufficient and carbon-starved mycelia. Among the more specific carriers, system a shows much resemblance to the basic amino acid permease of *N. crassa* [32] and *P. chrysogenum* [25] with regard to substrate specificity and affinity as well as the preferred development during growth with complete nutrients. A leucine-specific carrier like system d has only been reported from *S. cerevisiae*, but with a 10-fold higher substrate affinity [31]. System h appears to be a unique property of our object, as no specific carriers for aromatic amino acids have been reported from other fungi.

It was further surprising to find the tracer fluxes via both low-affinity systems II and III (transporting basic or acidic amino acids, respectively) were specifically influenced by phenylalanine. Transport systems with similar specificity and affinity have likewise not been characterized so far. Further work is required to show whether our data really reflect a sharing of the carriers or a competition of the high substrate fluxes for energy sources etc. It seems tempting to investigate whether there is from our data a connection between the concurrence of various phenylalanine-transporting systems and the incorporation of this amino acid into benzodiazepine (dipeptide) alkaloids [34].

References

1 Jennings, D.H. (1976) in Encyclopedia of Plant Physiology, Vol. 2 (Luettge, U. and Pitman, U.G., eds.), pp. 189–228, Springer, Berlin.
2 Eddy, A.A. (1982) Adv. Microb. Physiol. 23, 2–76.
3 Cooper, T.G. (1982) in The Molecular Biology of the Yeast Saccharomyces: Metabolism and Gene Expression (Strathern, J.N., Jones, E.W. and Broach, J.R., eds.), pp. 399–462, Cold Spring Harbor, NY.
4 Horak, J. (1986) Biochim. Biophys. Acta 864, 223–256.
5 Harder, W. and Dijkhuizen, L. (1983) Annu. Rev. Microbiol. 37, 1–23.
6 Roos, W. and Luckner, M. (1984) J. Gen. Microbiol. 130, 1007–1013.
7 Roos, W. and Slavik, J. (1987) Biochim. Biophys. Acta 899, 67–75.
8 Pande, P.S. (1976) Anal. Biochem. 74, 25–32.
9 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
10 Eisenthal, R. and Cornish-Bowden, A. (1974) Biochem. J. 139, 715–720.
11 Ellis, R.J. and Garner, C. (1980) Anal. Biochem. 105, 354–360.
12 Kotyk, A. and Janacek, K. (1975) Cell Membrane Transport, Principles and Techniques, p. 259, Plenum Press, New York.
13 Liemann, P. (1965) Zeitschr. Anal. Chem. 214, 66.
14 Grenson, M., Hou, C. and Crabeel, M. (1970) J. Bacteriol. 103, 770–777.
15 Benko, P.V., Wood, T.C. and Segel, J.H. (1969) Arch. Biochem. Biophys. 144, 168–183.
16 Pall, M.L. (1969) Biochim. Biophys. Acta 173, 113–127.
17 Nover, L. and Luckner, M. (1976) Nova Acta Leopoldina, Suppl. 7, 229–240.
18 Luckner, M., Lerbs, W. and Roos, W. (1986) in Regulation of Secondary Metabolite Formation (Kleinkauf, H., von Döhren, H., Dornauer, H. and Nesemann, G., eds.), pp. 133–156, VCH, Weinheim.
19 Hunter, D.R., Norberg, C.L. and Segel, I.H. (1973) J. Bacteriol. 144, 168–183.
20 West, I.C. (1980) Biochim. Biophys. Acta 604, 91–126.
21 Skye, G.E. and Segel, I.H. (1970) Arch. Biochem. Biophys. 138, 306–318.
22 Benko, P.V., Wood, T.C. and Segel, I.H. (1967) Arch. Biochem. Biophys. 122, 783–804.
23 DeBusk, R.M. and Ogilvie, S. (1985) J. Gen. Microbiol. 131, 905–912.
24 Cook, R.J. and Anthony, C. (1980) J. Gen. Microbiol. 120, 447–451.
25 Hunter, D.R. and Segel, I.H. (1971) Arch. Biochem. Biophys. 144, 168–183.
26 Wolfinbarger, L., Jr. and DeBusk, A.G. (1971) Arch. Biochem. Biophys. 144, 503–511.
27 Pall, M.L. (1970) Biochim. Biophys. Acta 211, 513–520.
28 Wolfinbarger, L., Jr. and Kay, W.W. (1973) Biochim. Biophys. Acta 330, 335–343.
29 Darte, C. and Grenson, M. (1975) Biochem. Biophys. Res. Commun. 67, 1028–1033.
30 Pateman, J.A., Kinghorn, J.R. and Dunn, E. (1974) J. Bacteriol. 119, 534–542.
31 Ramos, E.H., De Bongivanni, L.c. and Stoppani, A.O.M. (1980) Biochim. Biophys. Acta 599, 214–231.
32 Pall, M.L. (1970) Biochim. Biophys. Acta 203, 139–149.
33 Singh, D.P. and Le'John, H.B. (1975) Can. J. Biochem. 53, 975–988.
34 Roos, W. and Luckner, M. (1982) in Overproduction of Microbial Products (Krumphanzl, V., Sikyta, B. and Vanek, Z., eds.), pp. 111–126, Academic Press, London.
35 Luckner, M., Lerbs, W. and Roos, W. (1986) in Regulation of Secondary Metabolite Formation (Kleinkauf, H., von Döhren H., Dornauer, H. and Nesemann, G., eds.), pp. 133–156, VCH, Weinheim.
36 Boxman, A.W., Barts, P.W.J.A. and Borst-Pauwels, G.W.F.H. (1982) Biochim. Biophys. Acta 686, 13–18.
37 Sanders, D. and Slayman, C.L. (1982) J. Gen. Physiol. 80, 380–402.