Biochimica et Biophysica Acta, 731 (1983) 222–228
Elsevier

BBA 71669

NEUTRAL AMINO ACID TRANSPORT IN *LEISHMANIA* PROMASTIGOTES

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(Received February 8th, 1983)

Key words: Amino acid transport; Neutral amino acid; Countertransport; (Trypanosome, Leishmania)

Neutral amino acid transport was investigated in *Leishmania* promastigotes. Proline and alanine transport occur against their concentration gradient although there is a very rapid (40% at 30 min) conversion of proline to alanine. Uptake of these amino acids occurs by a sodium-independent route which is completely eliminated by addition of CCCP or KCN. K_m values for proline and alanine are 80 μM and 63 μM with V_{max} values of 6.4 and 7.2 nmol/min per mg dry weight, respectively. Countertransport of proline, alanine and phenylalanine was measured by loading the cells with a variety of neutral amino acids and proline analogs, followed by CCCP addition. The effect of aminooxyacetic acid, an inhibitor of alanine aminotransferase (EC 2.6.1.2), on proline and alanine countertransport was also examined. The results obtained are consistent with the presence of at least two systems for neutral amino acid transport in *Leishmania* promastigotes.

Introduction

Protozoan parasites of the genus *Leishmania* (order Kinetoplastidia, family Trypanosomatidae) are the causative agents of leishmaniasis, a tropical disease, second in importance only to malaria. The life cycles of such organisms are completed in two different hosts, a vertebrate and an insect (for review, see Ref. 1). Knowledge of the membrane physiology of *Leishmania* is essential for developing more effective chemotherapeutic drugs against this desease.

Studies of amino acid transport across the plasma membrane of *Leishmania* have been generally carried out in relation to the nutritional requirements of the cultured forms of these organisms [2,3]. Thus, it has been shown by Krassner and Flory [4] that the absence of carbohydrates from the growth medium of *Leishmania tarentolae* is fully compensated by the presence of proline. In fact, promastigote cultures of this parasite, presumably physiological equivalents of the insect stage, oxidize proline at a high rate [5]. However, no attempts have been made to characterize the number and kinds of transport systems for amino acids present in these microorganisms.

In the present work, we report an investigation of some aspects of the transport of neutral amino acids, including proline, across *Leishmania* sp. promastigotes. By countertransport studies we show the presence of two systems for neutral amino acid transport. Such systems have specificities very similar to systems A and L of vertebrate cells. However, in contrast to this system, both components of the neutral amino acid transport into *Leishmania* promastigotes are independent of the presence of sodium in the external medium.

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Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone: C_{in}, amino acid internal concentration; C_{ex}, amino acid external concentration.

Materials and Methods

Materials. ^{14}C-labelled amino acids were purchased from The Radiochemical Centre Amersham, Bucks., U.K. Non-radioactive amino acids, thioproline, azetidine-2-carboxylate, (methylamino)isobutyric acid, aminooxyacetic acid, CCCP, KCN and 2,4-dinitrophenol were obtained from Sigma Chemical Co. (St. Louis, MO). All labelled and unlabelled solutes used as substrates for transport experiments were L compounds without exception.

Preparation of Leishmania promastigotes for transport experiments. Leishmania sp., strain NR was cultured in a liquid medium [6] with the following composition: 9 g NaCl; 0.4 g KCl; 4 g glucose; 7.5 g sodium phosphate (dibasic); 2 g lactalbumin (Difco); 10 g triptose (Difco); 2 g liver extract (Difco); 5 g yeast extract (Difco) per liter of glass distilled water adjusted to a pH of 7.22. Just before use, fetal calf serum (Gibco Laboratories) was added aseptically to the flask to give a final concentration of 5% (v/v). The history of strain NR is described in Ref. 7. *Leishmania* promastigotes were harvested between four and seven days when the cells had reached late logarithmic phase (approx. $(2.5–4.0) \cdot 10^7$ cells/ml) by centrifugation at $900 \times g$ for 10 min at 4°C. The cells were then washed with 50 mM Tris-maleate at pH 7.22 containing 210 mM NaCl and 7 mM KCl. After washing, *Leishmania* promastigotes were suspended in the Tris-maleate buffer to a density of $(3.5–4) \cdot 10^7$ cells/ml. This cell density corresponds to a dry cell weight of 0.37 mg/ml. Coulter counter measurements of promastigote volume gave values of 42 ± 4 μm^3/cell By using this value and an estimation of cell water content of 80%, a figure of 4.1 μl/mg dry weight was calculated. This value was used to calculate internal solute concentrations in all uptake measurements.

In sodium-free experiments, all NaCl in the Tris-maleate buffer was replaced by choline chloride at the same concentration. In this case, analysis by flame photometry of the sodium content of the buffer solution in which *Leishmania* promastigotes were finally suspended indicated that its concentration did not exceed 300 nequiv./ml.

Uptake experiments. Amino acid transport was measured by following the uptake of ^{14}C-labelled amino acids using a rapid filtration technique. Appropiate volumes of cell suspension (3–5 ml) were equilibrated for 15 min at 28°C in a shaking bath. When inhibitors were used, they were added after this period of incubation and the incubation was continued for 5 min. At zero time, the radioactive amino acid was added at a final concentration of 0.1 mM (1 mCi/mmol). At specific intervals, 0.5 ml aliquots were withdrawn and mixed with a 5 ml 20% (w/v) ice-cold sucrose solution and rapidly filtered through Millipore filters (0.8 μm pore size). A sample was withdrawn immediately after adding the isotope to determine the non-specific adsorption to the cell membrane and filter. Membrane filters were dried under an infrared lamp and dissolved in Bray's scintillation solution [8]. The amount of radioactivity was counted in a Packard TriCarb Liquid Scintillation Spectrometer, model 3385. Results are reported as nanomoles of amino acid per mg dry weight and as intracellular concentration. All experiments were carried out in duplicate using two different batchs of cells.

Incorporation into proteins. The amount of radioactive material incorporated into proteins was determined after the addition of labelled amino acid (0.1 mM, 2 mCi/mmol), to promastigotes prepared as described above. Aliquots were withdrawn at 10 min time intervals: part of this suspension (0.5 ml) was filtered as previously described to determine total uptake, the rest was extracted for 30 min with 1 ml of 10% ice-cold trichloroacetic acid. To this solution, 5 ml of 20% ice-cold sucrose were added and filtered through a Millipore filter (0.8 μm pore size). The radioactivity measured for the trichloroacetic acid-insoluble material represents the incorporation of amino acid into cellular material. The amount of amino acid or its products in the amino acid pool was calculated by substracting the amount of radioactivity in the trichloroacetic acid-insoluble fraction from the total uptake.

Metabolism of proline. Ascending thin-layer chromatography in cellulose powder coated plates was used to determine the extent to which proline was metabolized during the course of an uptake experiment. The cells $(12.5 \cdot 10^7$ cells/ml) were incubated with [^{14}C]proline (0.2 mM, 10 mCi/mmol). Samples of 1 ml were taken at different

intervals, diluted in 10 ml of 20% ice-cold sucrose and filtered as before but washed twice with 5 ml of 20% sucrose. The membrane filters with the trapped cells were then immersed in 5 ml of 60% ethanol and heated at 50°C for 10 min. This suspension was sonicated in a Bronson sonicator (4 μ amplitude) to disrupt the membrane filter and cells and centrifuged at $1000 \times g$ for 15 min. The supernatant was collected and reduced to a constant volume (1 ml) by evaporation.

5 μl of each extract were placed on TLC plates (0.25 mm, 20 × 20 cm) and chromatographed with a solvent consisting of phenol/H_2O (75 : 25, w/w) for 7 h [9]. Unlabelled proline, aspartic acid, glutamic acid, and alanine were used as controls. After drying the plates, the thin layer was removed by scratching in 5-mm sections and counted in Bray's solution. Amino acids controls were identified by using the ninhydrin-collidine reagent [9].

Countertransport experiments. Washed promastigotes were incubated with 50 mM unlabelled amino acid for 30 min at 28°C in the presence of 100 μg/ml cycloheximide. The cells were then rapidly washed to remove external amino acid and resuspended in the Tris-maleate buffer to a final density of $(10–15) \cdot 10^7$ cells/ml. At zero time, countertransport was started by adding 1 μCi of the appropriate amino acid (250 mCi/mmol) and 15 μM CCCP. The time course of radioactive amino acid uptake was determined by following the same procedure described. Control experiments were carried out in parallel, without any unlabelled amino acid for loading the cells.

Results

Amino acid transport across Leishmania promastigotes

When *Leishmania* promastigotes are incubated with radioactive amino acids such as [^{14}C]proline or [^{14}C]alanine the time course of influx of radioactivity into the cell is linear during the first 5 min followed by a slower rate of entrance which is near steady state at about 30 min. At such a time, the isotope concentration inside the cell varies between 40–75-times the external concentration for proline (depending on the batch of cells used) and 60–100-times the external concentration for alanine (Fig. 1). By contrast, the observed accumulation ratios for amino acids such as phenylalanine, (methylamino)isobutyric acid or leucine are much lower (1.5–3-times the external solute concentration) than those measured for alanine or proline (Fig. 1).

The relation between the initial rate of solute uptake (measured during the first 4 minutes) and the external radioactive solute concentration is that predicted by the Michaelis-Menten equation with K_m and V_{max} of 80 μM and 6.4 nmol/min per mg dry wt. for proline and 63 μM and 7.2 nmol/min per mg dry wt. for alanine.

In order to investigate to what extent the accumulation of radioactive solute inside the cell represents amino acid incorporation into proteins, the amount of radioactivity found in the trichloroacetic acid-insoluble fraction was measured as a function of time.

The results obtained indicate that only 6% of total radioactive proline was incorporated into the

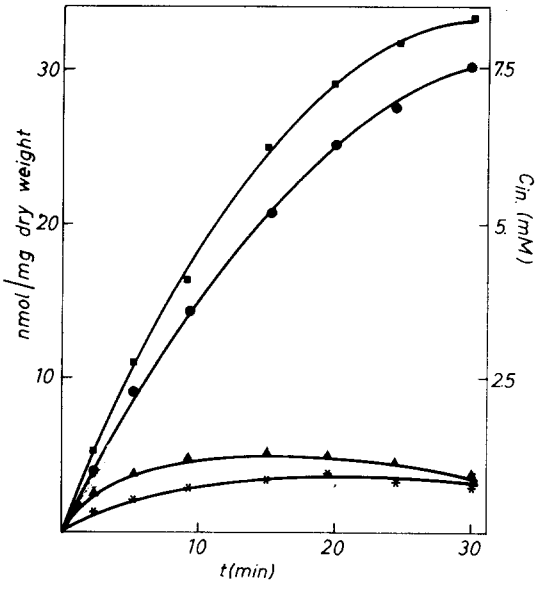

Fig. 1. Time course of amino acid uptake of *Leishmania* promastigotes at 28°C. Transport assay was carried out as described under Materials and Methods. The data shown are average values of three separate experiments. ●———●, proline; ■———■, alanine; ▲———▲, phenylalanine, *———*, MeAIB. In all cases, amino acid external concentration (C_{ex}) was 0.1 mM (1 mCi/mol) unless otherwise stated. C_{in}, amino acid internal concentration.

cell proteins at 30 min although it increased to 33% at 150 min. Additionally, when *Leishmania* promastigotes were incubated with radioactive proline in the presence of cycloheximide, the results indicated than less that 10% of intracellular proline was incorporated into proteins at 150 min.

The time course of proline conversion into other amino acids was measured by following the appearance of labelled solutes in TLC plates. At 5 min after adding radioactive proline to the external medium, about 19% of the radioactivity was found in glutamic acid but no other amino acid was labelled. At 15 min a small amount of radioactivity also appeared in alanine (7%), a proportion that increased to near 40% at 30 min. However, when *Leishmania* promastigotes were preincubated with 100 μM aminooxyacetic acid, an inhibitor of alanine aminotransferase, the radioactivity found in alanine at 5 min (7%) remained constant throughout the experiment.

The results described indicate that only about half the total radioactivity accumulated in *Leishmania* promastigotes at 30 min can be ascribed to proline accumulation. If now we assume that such amount is homogeneously distributed in the cell internal water, a solute gradient of 20–30-fold the external concentration can be calculated. This calculation shows clearly that proline influx into the cell is carried out against its concentration gradient. The active nature of this amino acid uptake was also indicated by the observation that the influx of proline and alanine was completely inhibited by 2 mM KCN, 15 μM CCCP or 0.1 mM 2,4-dinitrophenol.

Specificity of proline transport across Leishmania promastigotes

The extent by which proline transport across *Leishmania* promastigotes was inhibited by other amino acids and proline analogs is presented in Table I. It can be seen that a proline analog such as azetidine-2-carboxylate and alanine are the most potent inhibitors of proline influx into the cell. Less effective solutes are glycine, thioproline, serine and methionine in this order, with leucine and phenylalanine being ineffective inhibitors. The results shown also indicate that proline inhibits transport of alanine to the same extent that this amino acid inhibits proline transport.

TABLE I

EFFECT OF PROLINE ANALOGS AND OTHER AMINO ACIDS ON [^{14}C]PROLINE AND [^{14}C]ALANINE UPTAKE BY *LEISHMANIA* PROMASTIGOTES

Amino acid added (1 mM)	% inhibition of initial uptake	
	[^{14}C]Proline	[^{14}C]Alanine
None	100	100
Azetidine-2-carboxylate	15	38
Thioproline [a]	31	58
Proline	–	15
Alanine	16	–
Methionine	52	–
Serine	46	32
Glycine	28	–
Leucine	84	88
Phenylalanine	79	85

[a] Thiazolidine-4-carboxylic acid.

Effects of sodium substitution on amino acid accumulation

In order to study the effect of a sodium gradient across the cell membrane on the rate and the extent of amino acid accumulation, *Leishmania* promastigotes were incubated in an external medium where all sodium was substituted by choline. The absence of Na^+ in the external medium has absolutely no influence on the uptake or in the accumulation ratio of proline, alanine, phenylalanine or (methylamino)isobutyric acid.

Effect of CCCP on proline accumulation

When *Leishmania* promastigotes were incubated with CCCP prior to adding radioactive proline, there was no amino acid accumulation inside the cell, only solute equilibration along its concentration gradient. In order to investigate further such an effect, CCCP was added 8 min after proline accumulation inside the cell had been started (Fig. 2). It can be seen that there is a very rapid decrease in the radioactivity inside the cell reaching values near to the external concentration. A similar effect was obtained by adding at this time unlabelled proline (2 mM). However, in this case, after a few minutes there was a new increase in proline uptake into the cell. When unlabelled proline (2 mM) was added together with CCCP, proline efflux was even greater than in the former

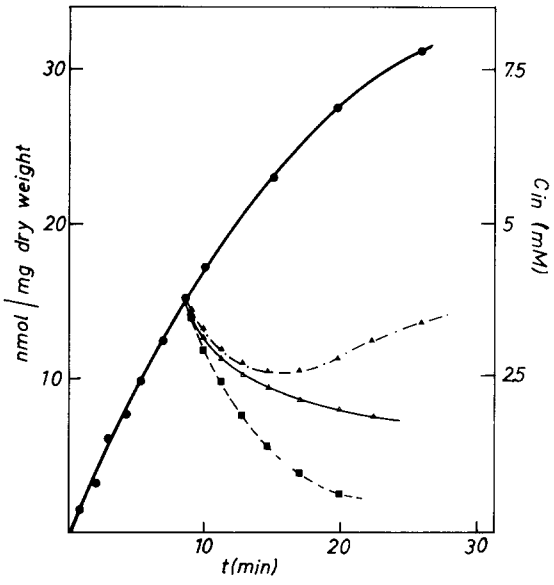

Fig. 2. Exchange of intracellular [^{14}C]proline with external proline. At the time indicated (8 min), the following additions were made. ▲·–·–·▲, 2 mM nonradioactive proline; △———△, 15 μM CCCP; ■— — —■, 2 mM nonradioactive proline plus 15 μM CCCP; ●———●, control.

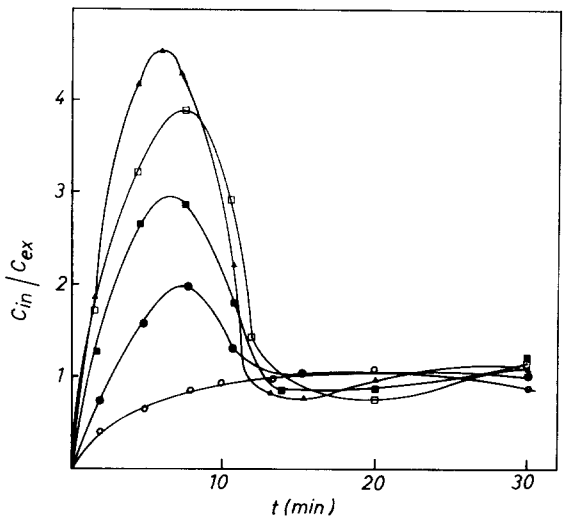

Fig. 3. Countertransport of proline. The cells were incubated with nonradioactive proline (50 mM) at different times (with 100 μg/ml cycloheximide), rapidly washed three times and resuspended at the same initial density. ○———○, noninincubated; ●———●, 5 min; ■———■, 10 min; □———□, 20 min; ▲———▲, 30 min. The countertransport was initiated by adding 15 μM CCCP and 1 μCi [^{14}C]proline (250 mCi/mmol).

two cases with the accumulation ratio decreasing rapidly to unity.

Countertransport

In the light of the previous experiment which indicated that CCCP uncouples amino acid accumulation into the cell without inhibiting the solute exchange system, it was thought to be of some interest to study the transient amino acid accumulation induced by another amino acid gradient exerted in opposite direction (solute countertransport). In the experiment shown in Fig. 3, *Leishmania* promastigotes were incubated with a constant amount of proline (50 mM) at different times, so that promastigotes became loaded with different proline concentrations. At zero time, CCCP and radioactive proline were added to the external medium and the time course of radioactivity incorporation into the cell measured (see Methods). Fig. 3 shows that at about 7 to 8 min there is a maximum in the accumulation of radioactivity, the magnitude of such maximum being proportional to the internal proline concentration.

Proline countertransport was also measured when *Leishmania* promastigotes were incubated with alanine, azetidine-2-carboxylate, thioproline, glycine and methionine (Table II). However, no proline countertransport was observed when leucine or phenylalanine were used as driving solutes. It can be seen in Table II that when alanine is the driving solute, proline countertransport was higher than any of the other amino acids tested including proline.

It is shown also in Table II that alanine countertransport with different driving solutes is very similar to proline countertransport. In order to determine to what extent this similitude of proline and alanine countertransport is due to the very rapid conversion of proline to alanine, the following experiments was carried out: *Leishmania* promastigotes were incubated with proline (50 mM) and 100 μM aminooxyacetic acid for 30 min previous to adding [^{14}C]proline or [^{14}C]alanine with CCCP as described. The result was that in both cases radioactivity accumulation is reduced by half (Table II). As expected, no effect on the maximal accumulation was measured when the incubation with aminooxyacetic acid was carried out in a

TABLE II

AMINO ACID COUNTERTRANSPORT IN *LEISHMANIA* sp.

t_{CT} is the time at which the maximum of countertransport was measured. r is accumulation ration (C_{in}/C_{ex}) of the radioactive amino acid measured at the maximum of the countertransport experiment. The concentration of aminooxyacetic acid (AOA) used was 100 μM.

Radioactive amino acid	Unlabelled amino acid (for loading)	t_{CT} (in min)	r
[^{14}C]Proline	proline	7– 8	4
	proline + AOA	13–15	2 –3
	alanine	6	4 –5
	alanine + AOA	6– 7	4 –5
	azetidine-2-carboxylate	7–10	3.5–4
	thioproline	10	3 –3.5
	glycine	10	3
	methionine	20	1.5–3
	serine	20	2
	leucine	no CT	–
	phenylalanine	no CT	–
[^{14}C]Alanine	alanine	5– 6	4.5–5
	alanine + AOA	5– 6	5
	proline	7– 8	4 –4.5
	proline + AOA	12–14	2
	methionine	20	2
	serine	20	2 –2.5
	leucine	no CT	–
	phenylalanine	no CT	–
[^{14}C]Phenylalanine	phenylalanine	5– 6	6.5–7
	leucine	6– 7	7
	methionine	15–20	3
	proline	no CT	–
	alanine	no CT	–

medium containing alanine instead of proline.

Finally, the magnitude of the countertransport of phenylalanine in the presence of different amino acids is shown in Table II. When phenylalanine or leucine was the driving solute, the rate and extent of the accumulation ratio of phenylalanine was even greater than the corresponding proline or alanine countertransport. On the other hand, no phenylalanine countertransport could be measured when proline or alanine was used as driving solute, but methionine drived proline or alanine countertransport as effectively as phenylalanine countertransport (Table II).

Discussion

The data presented for the uptake of neutral amino acids into *Leishmania* promastigotes show the active nature of this process as indicated by the extent of the accumulation inside the cell and its sensitivity to CCCP and KCN. Thus even though the characterization of amino acid transport in these cells is complicated by the very rapid metabolic rate of proline-alanine interconversion, conservative estimations of proline concentration inside the cell gave values than are 20–30-fold the external concentration.

An important characteristic of amino acid transport across *Leishmania* promastigotes membrane is its sodium independence clearly indicating that energization of amino acid movement is not occurring via a cotransport with this cation. On the contrary the very rapid effect of CCCP in bringing amino acid accumulation to a halt, suggests that this compound may act by collapsing a pH gradient across the cell membrane. The role played by H^+ cotransport in the specific translocation of amino acids and sugars across the plasma membrane of several microorganisms and yeast is well substantiated (for a review, see Ref. 10).

The results of the countertransport experiments indicate the presence of *Leishmania* promastigotes of at least two systems for neutral amino acids transport. Thus, the rate and extent of proline and alanine countertransport in the presence of different driving solutes follow the same order in which they inhibit proline influx into the cell (Table I and II). In addition, no countertransport of proline or alanine could be measured with phenylalanine or leucine, the solutes with less inhibitory properties on proline transport. The fact that no phenylalanine countertransport was observed when alanine or proline was the driving solute but only with phenylalanine, leucine or methionine, indicated the presence of a second system with different affinity characteristics from the former system. An important characteristic of this second transport system is that the overshoot it produces into the cell is greater than in the first system. This property is remarkable similar to that observed for the system L of amino acid transport in vertebrate cells [11]. Indeed, the amino acids transported by the two-transport system characterized in *Leish-*

mania promatigotes coincide with those reported for systems A and L of neutral amino acid transport [12] including the finding that methionine can share both transport systems [13]. However, it is well known that system A of vertebrate cells is Na^+ dependent [14].

The alanine and proline transport system in *Leishmania* promastigotes resembles the cysteine system characterized by Young and Ellory in sheep erythrocytes [15] which is also Na^+-independent. However, contrary to this system, preliminary results have indicated that proline uptake into the *Leishmania* promastigotes is not affected by basic amino acids such as lysine or histidine. Another feature of the amino acid transport system in *Leishmania* promastigotes is that CCCP uncouples translocation without inhibiting the exchange properties of the carrier system. This behaviour is known to occur in *Escherichia coli* [16] and elsewhere [17–19] but not in *Chlorella* [20] or *Trypanosoma cruzi* [21].

Acknowledgments

This work was supported in part by Consejo de Desarrollo Científico y Humanístico (Universidad Central de Venezuela). We are indebted to Dr. J. Urbina for helpful discussions.

References

1 Lainson, R. and Shaw, J. (1978) Nature 273, 595–600
2 Law, S.S. and Mukkada, A.J. (1979) J. Protozool. 26, 295–301
3 Mukkada, A.J. and Simon, M.W. (1977) Exp. Parasitol. 42, 87–96
4 Krassner, S.M. and Flory, B. (1971) J. Parasitol. 57, 917–920
5 Krassner, S.M. and Flory, B. (1972) J. Protozool. 19, 682–685
6 Camargo, E.P. (1964) Rev. Inst. Med. Trop. Sao Paulo. 6, 93–100
7 Bonay, P. (1980) Lic's Thesis. Escuela de Biologia, Universidad Central de Venezuela
8 Bray, G.A. (1960) Anal. Biochem. 1, 279–285
9 Johnson, A.B. and Strecker, M.J. (1962) J. Biol. Chem. 237, 1876–1882
10 Gunn, R.B. (1980) Annu. Rev. Physiol. 42, 249–259
11 Oxender, D.L. and Christensen, H.N. (1963) J. Biol. Chem. 238, 3686–3669
12 Thomas, E.L. and Christensen, H.N. (1971) J. Biol. Chem. 246, 1682–1688
13 Mathews, R.L. and Zand, R. (1979) Biochim. Biophys. Acta 554, 227–233
14 Christensen, H.N. (1975) Biological Transport, 2nd Edn., W.A. Benjamin, Reading, MA
15 Young, J.D. and Ellory, J.C. (1977) Biochem. J. 162, 33–38
16 Winkler, H.H. and Wilson, T.H. (1966) J. Biol. Chem. 241, 2200–2211
17 Wilson, D.B. (1978) Annu. Rev. Biochem. 47, 933–965
18 Harold, F.M. (1976) Curr. Topics. Bioenergetics. 6, 83–149
19 Hamilton, W.A. (1975) Adv. Microb. Physiol. 12, 1–31
20 Komor, E., Hass, D. and Tanner, W. (1972) Biochim. Biophys. Acta 266, 649–660
21 Urbina, J. (1980) Acta Cient. Venez. 31, Suppl. 1, 197–198