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Inhibition of transport system $b^{0,+}$ in blastocysts by inorganic and organic cations yields insight into the structure of its amino acid receptor site

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The most conspicuous, Na^+-independent amino acid transport process in preimplantation mouse blastocysts was provisionally designated system $b^{0,+}$ because it accepts some cationic and zwitterionic amino acids about equally well as substrates. Although system $b^{0,+}$ is not Na^+-stimulated, it has not been determined if it is inhibited by Na^+, or if its activity is affected by most other ions. Therefore, we measured uptake of amino acids by blastocysts in isotonic solutions of different ionic and nonionic osmolites. Na^+-independent L-leucine uptake was unaffected by the ion concentration, but L-lysine transport was several-fold faster in isotonic solutions of non-electrolytes than in similar solutions of inorganic and organic salts or zwitterions. The K_m value for 'Na^+-independent' L-lysine transport was about 10-fold higher in isotonic salt solutions than in solutions of nonionic osmolites, whereas the K_m value for L-leucine transport was about the same in either type of solution. Therefore, inorganic and organic cations and the cationic portions of zwitterions appear to compete with cationic but not zwitterionic amino acids for system $b^{0,+}$ receptor sites. The cation, harmaline, was a particularly strong competitive inhibitor of 'Na^+-independent' L-lysine uptake by system $b^{0,+}$, even in isotonic salt solutions, whereas it inhibited L-leucine uptake noncompetitively. Moreover, harmaline appeared to compete with inorganic cations for the lysine receptor sites of system $b^{0,+}$. Harmaline also has been found by other investigators to competitively inhibit L-lysine uptake by the Na^+-independent system asc_1 in horse erythrocytes, whereas it noncompetitively inhibits alanine uptake by the same system. Similarly, harmaline noncompetitively inhibits L-alanine uptake by the Na^+-dependent system ASC in human erythrocytes, but it appears to compete for binding with L-alanine's cosubstrate, Na^+. In addition, others have found that the positively-charged side chains of cationic amino acids seem to take the place of Na^+ needed near side chains in order for zwitterionic amino acids to be transported by systems ASC and y^+. We conclude that system $b^{0,+}$ may be similar to systems asc_1, ASC and y^+, and that each of these systems may be a variant of the same ancestral transport process. We speculate that since it appears to accept a broader scope of substrates and to interact with a wider variety of cations than do systems asc_1, ASC or y^+, system $b^{0,+}$ may more closely resemble the proposed ancestral transport process than any of the other contemporary systems.

Introduction

Amino acid transport systems in avian and mammalian cells can be divided into three broad categories based on whether they prefer as substrates cationic, anionic or zwitterionic amino acids. Each of these categories can be subdivided into Na^+-dependent and Na^+-independent types of systems [1–3]. Better-known transporters that fall into some of these categories include the Na^+-dependent systems A and ASC and the Na^+-independent systems L and asc_1, each of which prefers zwitterionic amino acids as substrates. In contrast, the Na^+-independent system y^+ prefers cationic amino acids as substrates [1–3]. Recently, we discovered in preimplantation mouse blastocysts a Na^+-dependent system provisionally designated $B^{0,+}$ [4] and a Na^+-independent system called $b^{0,+}$ [5]. Unlike most other known transporters, each of the latter systems accepts

Abbreviations: BCO, 3-amino-*endo*-bicyclo[3.2.1]octane-3-carboxylic acid; BCH, 2-amino-*endo*-bicyclo[2.2.1]heptane-2-carboxylic acid.

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some cationic and zwitterionic amino acids about equally well as substrates. Moreover, the flux of L-lysine into 1-cell conceptuses via system $b^{0,-}$ appears to be inhibited by the Na^+ in isotonic NaCl to less than 20% of the flux into conceptuses in isotonic sucrose or mannitol [6]. For these reasons, classification of amino acid transport systems based on whether they are Na^+-dependent (i.e., Na^+-stimulated) and whether they prefer cationic or zwitterionic amino acids as substrates appears to become inadequate in some instances. Therefore, it also may be useful to begin to group some transport systems according to their structural similarities as far as these structures can be inferred.

It has been concluded for a few pairs of systems that the structures of their amino acid receptor sites are similar. Thomas and Christensen [7] concluded that there is a surprising parallel between the cation-binding component of the amino acid receptor sites of systems ASC and y^+. Each of these systems can receive the side chains of zwitterionic amino acids in association with Na^+ or the positively charged side chains of cationic amino acids, although with widely different relative selectivities [3]. Similarly, although system asc_1 is Na^+-independent for zwitterionic substrates, it seems to have a receptor site for cationic amino acids that resembles the receptor site of system ASC [8]. Therefore, all three of the systems, asc_1, ASC and y^+ may have structurally similar transporters. In this paper we present data consistent with the theory that the latter three systems and system $b^{0,+}$ in mouse blastocysts constitute a family of systems with structural similarities not shared by other amino acid transport processes.

Materials and Methods

The methods of obtaining blastocysts and measuring their ability to take up amino acids have been described recently [4,5,9,10]. For each independent experiment, blastocysts were obtained from a group of several 8–11 week old Swiss ICR mice (Harlan Sprague Dawley, Inc.) about 82 h after the mice had been induced to ovulate and mate utilizing gonadotropins (i.e., about 94 h after injection of human chorionic gonadotropin; Ref. 11). Some blastocysts were maintained in Brinster's medium [12] for up to six hours before their ability to take up an amino acid was measured. No change in transport activity has been observed during incubation of blastocysts in Brinster's medium at this stage of development.

At the total substrate concentrations we studied, L-lysine and L-leucine uptake increased in a virtually linear manner with time for at least 10 min. For uptake measured after 0.0, 2.5, 5.0 or 10.0 min in isotonic LiCl or sucrose, the correlation coefficients were $\geqslant 0.996$ and the coefficients of determination were $\geqslant 0.992$ ($P < 0.01$; Ref. 13; data not shown). Therefore, initial velocities of uptake were measured in blastocysts by incubating them with a ^{3}H-labeled amino acid (ICN Pharmaceuticals or Amersham; 20–60 Ci/mmol) and various concentrations of nonradioactive amino acids for 5 min at 37 °C [4,5,9,10]. After incubations, blastocysts were processed to determine how much radiolabel they had taken up [4,5,9,10]. Non-labeled amino acids and other chemicals were purchased from Sigma Chemical Co. (3-amino-*endo*-bicyclo[3.2.1]octane-3-carboxylic acid, BCO, was a gift from Professor Carmen Avendaño, Ref. 14). As in our prior studies [4,5,9,10], amino acids were sometimes dissolved in solutions referred to as phosphate-buffered NaCl or LiCl (75 mM total phosphate (pH 7.1)). In addition, in some experiments amino acids were dissolved in solutions we refer to as 'isotonic' solutions (i.e., 300 mosmolar), which contained only 0.75 mM phosphate, about 2 mM K^+ and about 1 mM Cl^-. The latter small amount of buffer was adequate to maintain the pH of the various isotonic solutions at values between 6.3 and 7.8 (usually between 6.5 and 7.5) during the course of our experiments as determined before and after the interval of uptake. Na^+-independent amino acid transport into blastocysts is unaffected by changes in the external pH between 6.3 and 8.0 [5]. Therefore, 0.75 mM phosphate buffer was sufficient to maintain the pH within this range yet allow study of transport at total ion concentrations as low as 4 mM. Transport by blastocysts in isotonic LiCl or NaCl (0.75 mM total phosphate) was indistinguishable from transport in phosphate-buffered LiCl or NaCl (75 mM total phosphate), respectively.

We frequently examine amino acid uptake by blastocysts at substrate concentrations near 1 μM for two principal reasons. First, this approach increases the probability that low-K_m, low-capacity transport systems will be detected even in the presence of higher-K_m, higher-capacity systems [15], without hampering the ability to detect higher K_m transport activities. Second, amino acid transport systems with K_m values for some substrates near 1 μM have been detected in preimplantation conceptuses [6,9], and the K_m value for L-lysine uptake by blastocysts may be as low as 1 μM under some conditions used in studies described here (see Results). In contrast, inhibition studies were conducted with concentrations of potentially inhibitory amino acids of up to 12 mM in order nearly to saturate inhibition of lysine uptake (such concentrations of potential inhibitors are commonly used in transport studies; e.g., Refs. 3 and 7). The experimental approach of testing the effects of potential inhibitors on uptake at relatively low substrate concentrations does not increase the possibility that contaminants rather than inhibitors will affect uptake. As for potential inhibitors themselves, the possible effects of contaminants depend primarily on the K_i values of any such contaminants regardless of whether the substrate concentration is at or far below the K_m

value for substrate uptake. Moreover, we regularly verify unanticipated inhibition with 'AB' or 'ABC' testing [1,2,15] to determine if the inhibitory substance behaves as a substrate in the same way that it behaves as an inhibitor at substrate and inhibitor concentrations of all relevant substances near their apparent K_m and K_i values [4,5,9]. Finally we have shown that uptake of amino acids at concentrations near 1 μM cannot be attributed to binding of the amino acids to the plasma membrane rather than to uptake; virtually no uptake or binding of any amino acid was detected in isotonic sucrose or phosphate-buffered LiCl after blastocysts had been killed by exposing them to ultrasound in a Bransonic 12 ultrasonic cleaner (Smith Kline Co.) for 5–25 min at 25–30 °C in 150 mosmolar phosphate-buffered NaCl also containing 10 mM KCN (data not shown). Although the latter treatment disrupted most conceptuses inside their zona pellucidae, it was not excessively harsh since some conceptuses survived the treatment as determined visually and by their ability to accumulate radiolabeled amino acids.

Blastocysts labeled in an isotonic solution composed of 300 mM mono- or disaccharide and 0.75 mM phosphate were found to 'float' rather than remain on the bottom of the culture dish when first placed in these solutions. The effects on L-lysine transport described below cannot be attributed to such 'floating', however. No effect of floating on transport was observed (a) when L-leucine, glycine or L-glutamate was the substrate (see results) or (b) when conceptuses were labeled with L-[^{3}H]lysine in isotonic salt solutions also containing the requisite amount of a high molecular weight polysaccharide (Ficoll 400, Pharmacia Fine Chemicals) to make them float (data not shown). Furthermore, none of the isotonic solutions caused blastocysts to shrink or swell noticeably during the 5-min uptake assay period. Parametric [13,16] and non-parametric [17–21] statistical methods were used to perform analysis of variance and to calculate values of kinetic parameters, respectively. We conclude that the numerically adjacent or paired values of kinetic parameters, which are presented in the tables, differ significantly when their 92–94% confidence intervals do not overlap, since even the 90% confidence intervals overlap somewhat when $P = 0.05$ in a 't' test [13].

Results

Effect of salts on amino acid transport

Replacement of Na^+ by uncharged osmolites produced a more than 3-fold increase in L-lysine influx into blastocysts (Fig. 1). In contrast, transport of L-leucine, glycine and L-glutamate was considerably slower when blastocysts were in isotonic solutions of uncharged osmolites instead of NaCl. Statistically significant exodus of L-lysine from blastocysts was not detected after 15 min of incubation in either isotonic NaCl or sucrose (Table I). Therefore, our results are due to more rapid L-lysine uptake in isotonic sucrose, rather than faster L-lysine exodus when blastocysts are washed for less than one min in isotonic NaCl. Analog inhibition studies of L-lysine transport confirmed the presence of system $B^{0,+}$, system $b^{0,+}$, and a cation-preferring transport process in blastocysts (Fig. 2), as anticipated from prior studies [4,5,9,10]. L-Lysine uptake was, however, several-fold more rapid via the 'Na^+-independent' system $b^{0,+}$ in isotonic sucrose than in isotonic NaCl (Fig. 2). The effect of salts and zwitterions on L-lysine uptake was marked even at relatively low ion concentrations (Fig. 3 and Table II). For this reasons, we performed experiments to determine the effect of salts on the values of K_m and V_{max} for L-lysine uptake via system $b^{0,+}$ while the less conspicuous, cation-preferring transport process was also active. L-Lysine transport via each of these systems alone can be determined by measuring L-lysine uptake in the presence and absence of 20 mM L-leucine, which selectively inhibits system $b^{0,+}$ [5]. However, this concentration of L-leucine might also inhibit L-lysine uptake in the same way that salts do when they are added to solutions of low ion concentration (e.g., Fig. 3). Nevertheless, the values of the kinetic parameters for total, mediated L-lysine uptake can be attributed mainly to system $b^{0,+}$ which is the predominant system for L-lysine uptake in blastocysts at this stage of development (Fig. 2 and Ref. 5). Similarly, Na^+-independent L-leucine transport is mainly via system $b^{0,+}$ in blastocysts [5]. We also compared the values of the kinetic parameters to those determined specifically for system $b^{0,+}$ in phosphate-buffered LiCl [5].

Competitive inhibition of L-lysine but not leucine transport by cations

The K_m value for L-lysine transport in blastocysts was about 10-fold larger in isotonic NaCl or phosphate-buffered LiCl than in isotonic sucrose, whereas the V_{max} value was about 50% larger in isotonic NaCl than in the other two solutions (Fig. 4; Table III). The higher V_{max} value in isotonic NaCl was probably due to the added contribution of the Na^+-dependent system $B^{0,+}$ which has a V_{max} value one quarter to one half as large as system $b^{0,+}$ at this stage of development [2]. A similar effect of ions on the K_m value was observed for L-lysine uptake in a single experiment in which choline chloride was used instead of NaCl to make the isotonic solution of high ionic strength (data not shown). Likewise, the K_i value for competitive L-lysine inhibition of L-leucine uptake via system $b^{0,+}$ (in the presence of 10 mM BCH, an analog of BCO, Refs. 2, 3, to inhibit system L) was 10 μM in isotonic sucrose (data not shown), whereas this value was 79 μM in an isotonic salt solution [5]. It is likely that this K_i value for L-lysine (i.e., 10 μM) is slightly higher than its

K_m value in isotonic sucrose (Table III) because 10 mM BCH was also present. The cationic portion of BCO and BCH may weakly (Fig. 2) but competitively inhibit L-lysine interaction with system $b^{0,+}$ in the same way that all cations seem to do (see Discussion).

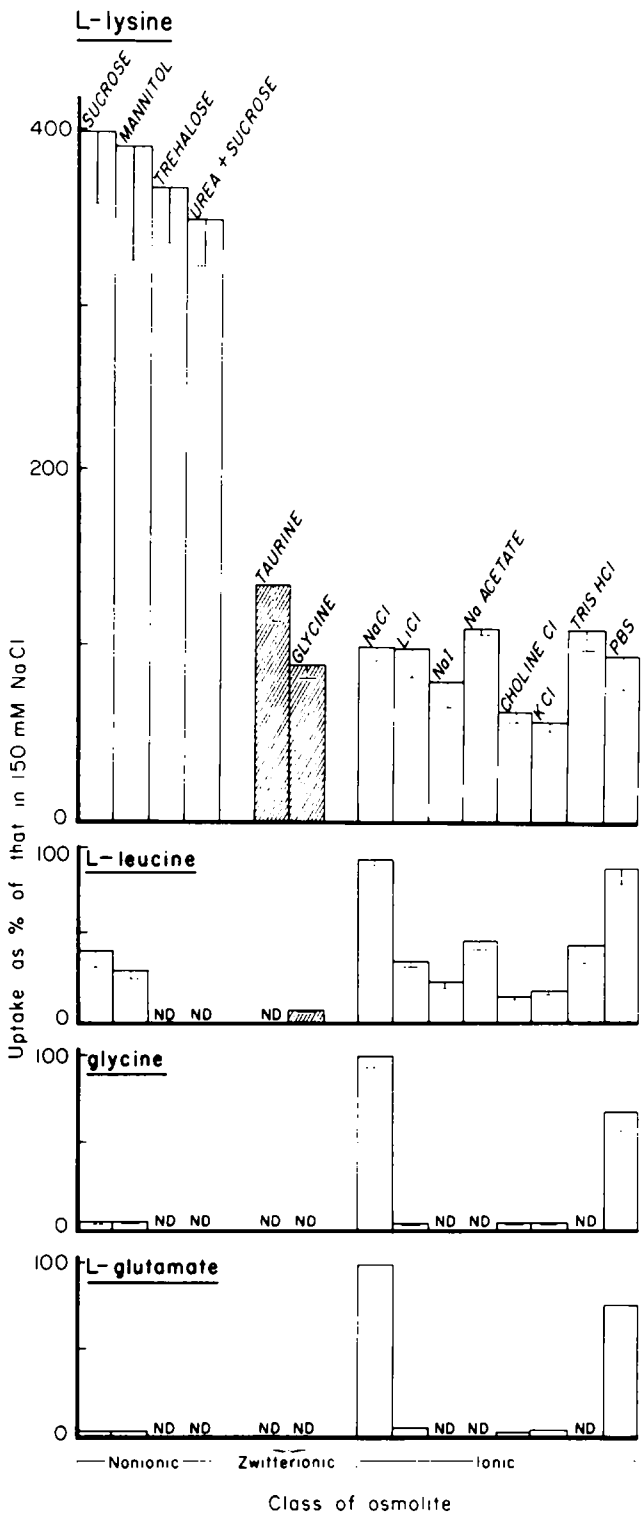

Fig. 1. Uptake of L-lysine, L-leucine, glycine and L-glutamate by blastocysts in various isotonic solutions. Blastocysts were incubated for 5 min with a ^{3}H-labeled amino acid (approx. 1 μM) in each isotonic solution (300 mosmolar of each substance). Substrate concentrations were uniformly low because the K_m value for L-lysine transport is on the order of 1 μM in isotonic sucrose (see Results). The mean ± S.E. uptake was calculated from four to 15 replicate determinations (approximately five blastocysts per determination) obtained in two to seven independent experiments (two determinations and one experiment for L-glutamate). L-Lysine uptake was more rapid in each solution of a nonionic osmolite than in each solution of a zwitterionic or ionic one ($P < 0.01$) as determined with analysis of variance. A major portion of L-leucine uptake was apparently both Na^+- and Cl^--dependent ($P < 0.05$), and glycine uptake was Na^+-dependent ($P < 0.01$). L-Leucine uptake in Na^+-free medium was the same when either ionic or nonionic substances were the major osmolites. Urea + sucrose, 100 mM urea + 200 mM sucrose; PBS, phosphate-buffered NaCl; N.D., not determined.

TABLE I

Exodus of L-lysine from blastocysts in isotonic NaCl or sucrose

Blastocysts were incubated for 5 min with 0.42 μM L-[^{3}H]lysine in one of the indicated isotonic solutions. One third of these conceptuses were processed immediately to determine the amount of L-[^{3}H]lysine they had taken up. The remaining conceptuses were washed and incubated in one of the indicated solutions for 15 min prior to being processed to determine the amount of radioactivity they had retained. The mean ± S.E. uptake was calculated from five replicate determinations (four blastocysts/determination) obtained in two independent experiments. The actual amounts of L-[^{3}H]lysine the average blastocyst had taken up in each medium before the chases are shown on the *y*-axis of Fig. 2. The amounts of radioactivity that blastocysts contained before and after the 15-min chases were statistically indistinguishable as determined with analysis of variance.

Medium for 5-min uptake	Medium for 15-min chase	Radioactivity remaining (%)
Isotonic NaCl	none	100 ± 13
	isotonic NaCl	77 ± 11
	isotonic sucrose	91 ± 17
Isotonic sucrose	none	100 ± 8
	isotonic NaCl	91 ± 12
	isotonic sucrose	89 ± 10

There are at least three reasons why some of the 92–94% confidence intervals we report for the values of kinetic parameters seem to be larger than the variability usually associated with such values. First, we report 92–94% confidence intervals, whereas standard errors of the means, which are usually smaller than 68% confidence intervals, have been reported in many other papers. Moreover, the variabilities were probably somewhat greater in some cases in the present paper than they would have been otherwise because another system was active along with system $b^{0,+}$ (see above). Perhaps most importantly, we selected the non-parametric method used to calculate confidence intervals because it does not require many of the often incorrect statistical assumptions that more commonly used parametric

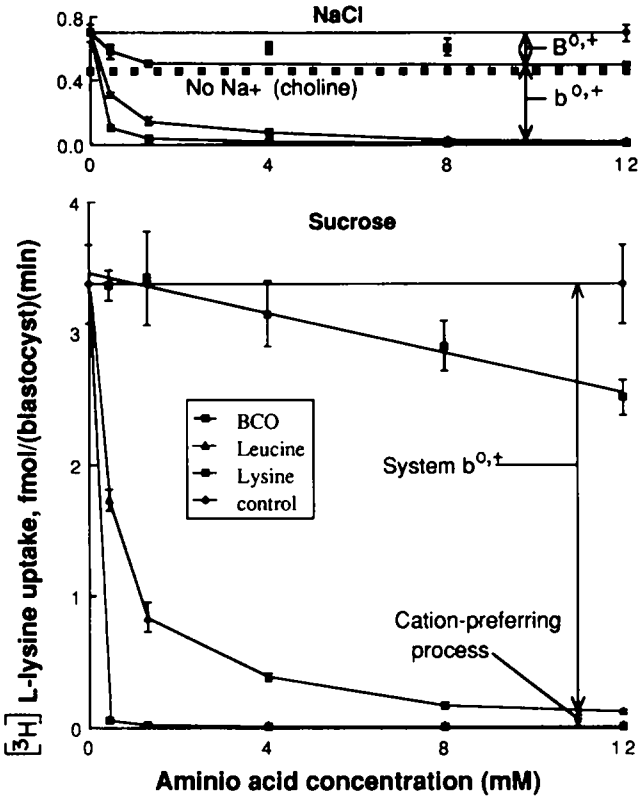

Fig. 2. Use of inhibition by nearly saturating concentrations of BCO, L-leucine and L-lysine to distinguish uptake of [^{3}H]lysine by system $B^{0,+}$, system $b^{0,+}$ and a cation-preferring transport process. Blastocysts were incubated for 5 min with 0.42 μM L-[^{3}H]lysine and the indicated concentration of a nonradioactive amino acid in isotonic NaCl (upper panel) or isotonic sucrose (lower panel). A low concentration of substrate was used in these studies because the K_m value for L-lysine uptake is near 1 μM in isotonic sucrose (see Results). The level of L-[^{3}H]lysine uptake in isotonic choline chloride (filled squares) was about 30% lower than the level in isotonic NaCl (solid horizontal line). The mean ± S.E. uptake was calculated from four to six replicate determinations (approximately six blastocysts per determination) obtained in two (NaCl and choline chloride) or three (sucrose) independent experiments. As established in prior studies (Refs. 4, 5, 9, 10), BCO inhibits the Na^+-dependent system $B^{0,+}$, L-leucine inhibits both system $B^{0,+}$ and the Na^+-independent system $b^{0,+}$, and L-lysine inhibits uptake of L-[^{3}H]lysine by system $B^{0,+}$, system $b^{0,+}$ and the cation-preferring transport process. We attribute the weak inhibition of system $b^{0,+}$ by BCO in isotonic sucrose, but apparently not in isotonic NaCl, to interaction of BCO's positively-charged amino group with the 'cation binding site' of system $b^{0,+}$ (see Discussion).

methods, such as least-squares methods, do [17–21]. The method we used to analyze 5 or 6 points in Hofstee plots produces a confidence interval roughly 2-fold larger than intervals generated by least-squares methods, however, even when least-squares assumptions are correct [21]. Nevertheless, it seems safe to conclude, based on our method of analysis, that the K_m value for L-lysine uptake was higher when uptake was measured in blastocysts in isotonic salt solutions than when uptake was measured in isotonic sucrose (Table III).

It seems unlikely that salts raise the K_m and K_i values for L-lysine transport by destabilizing the tertiary and quaternary structures of proteins [22]. An effect of salts on L-lysine uptake was observed (Fig. 3 and Table II) at salt concentrations well below those usually asociated with disruption of protein structure [22]. Moreover, urea, a nonionic protein destabilizer [22], appeared to have no effect on L-lysine transport in an isotonic solution of nonelectrolytes (e.g., compare the effects of 100 mosmolar urea in Fig. 1 to 100 mosmolar salts in Fig. 3).

In contrast to L-lysine transport, the K_m value for Na^+-independent L-leucine transport was not affected in a statistically significant manner when blastocysts were incubated in isotonic sucrose instead of isotonic LiCl or phosphate-buffered LiCl (Fig. 5; Table III). The departure from linearity of the Hofstee plots for L-leucine uptake in isotonic sucrose or LiCl (Fig. 5) is due to the presence of both the predominant system $b^{0,+}$ ($V_{max} \cong 40$ fmol · blastocyst^{-1} · min^{-1}) and a smaller amount of a variant of system L ($V_{max} \cong 6$ fmol · blastocyst^{-1} · min^{-1}) in blastocysts [5]. The latter component of transport (reflected by the lower portions of the curves in Fig. 5) can be virtually eliminated by performing experiments in the presence of 10 mM BCO

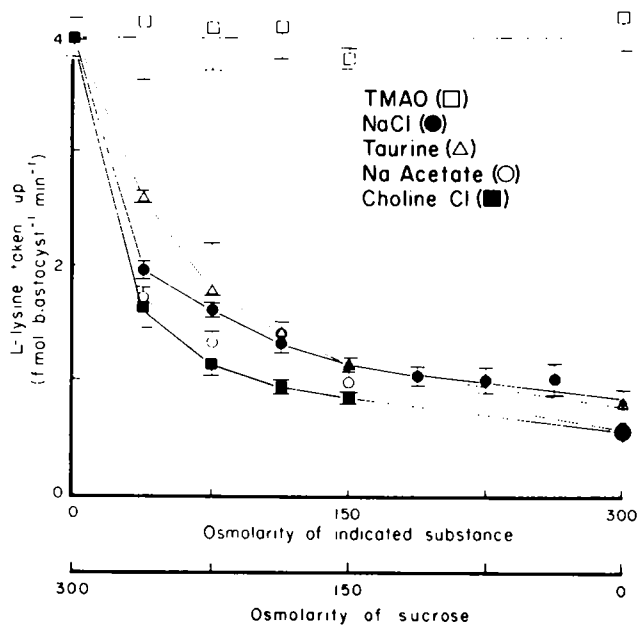

Fig. 3. Effect of varying the concentrations of electrolytes in isotonic solutions on L-lysine uptake by blastocysts. Conceptuses were incubated for 5 min with 0.42 μM L-[^{3}H]lysine in isotonic solutions. A low concentration of substrate was used in these studies because the K_m value for L-lysine uptake is near 1 μM in isotonic sucrose (see Results). When the osmolarity of the indicated substance was less than 300, sucrose was used to adjust the total osmolarity to this value. The mean ± S.E. uptake was calculated from four or five replicate determinations (approximately five blastocysts per determination) obtained in two independent experiments (one experiment and two determinations for taurine). TMAO (trimethylamine *N*-oxide) is a highly polar but undissociated solute.

TABLE II

Effect of mono-, di- and trivalent salts of Li^+ and Cl^- on L-lysine uptake by blastocysts

Blastocysts were incubated for 5 min with 1.0 μM L-[^{3}H]lysine in one of the indicated solutions made to be isotonic (300 mosmolar) with sucrose. A low concentration of substrate was used in these studies because the K_m value for L-lysine uptake is near 1 μM in isotonic sucrose (see Results). The mean ± S.E. uptake was calculated from 5 to 12 replicate determinations (approximately three blastocysts/determination) obtained in four independent experiments. Uptake was slower in each case relative to the all sucrose (i.e., 'none') group ($P < 0.01$) as determined with analysis of variance. Moreover, uptake in $MgCl_2$ or spermidine Cl_3 solutions was lower than in solutions of LiCl ($P < 0.01$), but uptake in solutions of Li_2SO_4, Li_3 citrate and LiCl could not be distinguished on statistical grounds. Thus, uptake was dependent on whether a mono-, di- or trivalent cation was present when the [Cl^-] was constant, but it was not influenced by whether the anionic species was mono-, di- or trivalent when the [Li^+] was constant.

Substance used to partially replace sucrose	% of L-[^{3}H]lysine uptake relative to uptake in isotonic sucrose
None (isotonic sucrose)	100 ± 6
Solutions containing 10 mM Cl^-	
10 mM LiCl	68 ± 5
5 mM $MgCl_2$	45 ± 5
3.3 mM spermidine Cl_3	52 ± 6
Solutions containing 20 mM Cl^-	
20 mM LiCl	62 ± 7
10 mM $MgCl_2$	39 ± 3
6.7 mM spermidine Cl_3	38 ± 6
Solutions containing 10 mM Li^+	
10 mM LiCl	68 ± 5
5 mM Li_2SO_4	69 ± 3
3.3 mM Li_3 citrate	74 ± 7
Solutions containing 20 mM Li^+	
20 mM LiCl	62 ± 7
10 mM Li_2SO_4	55 ± 5
6.7 mM Li_3 citrate	58 ± 7

(phosphate-buffered LiCl line in Fig. 5) which selectively inhibits system L [5]. However, zwitterions, such as BCO, may affect system $b^{0,+}$ activity in the same way as do salts (see above). Since our purpose was to test the effect on L-leucine transport of an environment as nearly free of exogenous ions as was possible, we did not attempt to isolate system $b^{0,+}$ for kinetic studies in isotonic solutions. Nevertheless, it was possible to conclude that the K_m value for L-leucine uptake via system $b^{0,+}$ (reflected by the slopes of the lines near the tops of the curves in Fig. 5) or for the combined activities of systems $b^{0,+}$ and L (Table III) was not lower when blastocysts were incubated in isotonic sucrose instead of isotonic LiCl.

Strong inhibition of system $b^{0,+}$ by harmaline

'Na^+-independent' L-lysine and L-leucine transport could also be distinguished on the basis of inhibition of their uptake by harmaline. This cationic substance, which inhibits systems ASC, asc_1 and asc_2 in erythrocytes [8], raised the K_m value for L-lysine transport even in phosphate-buffered LiCl, whereas it lowered the V_{max} value for L-leucine uptake (Table IV). Similar results for harmaline inhibition of L-lysine transport (not shown) were also obtained for blastocysts obtained 20 h later in development. Moreover, harmaline was an even more effective competitive inhibitor of L-lysine transport in isotonic sucrose than in phosphate-buffered

TABLE III

Kinetic parameters for L-lysine and L-leucine uptake by blastocysts in phosphate-buffered LiCl, isotonic sucrose, and isotonic NaCl or LiCl

A nonparametric statistical method was used to estimate the median values of the kinetic parameters and their 92–94% confidence intervals from the data presented in Figs. 4 and 5 (Refs. 17 and 18). Numerically adjacent median values are considered to be statistically, significantly different if their 92–94% confidence intervals do not overlap (see rationale at the end of the Materials and Methods).

Substrate	Solution in which uptake was measured	Median (92–94% confidence interval)	
		K_m value [a]	V_{max} value [b]
L-Lysine	phosphate buffered LiCl	59 (43– 72)	42 (39–51)
	isotonic NaCl	61 (48– 90)	68 (58–75) [c]
	isotonic sucrose	5 (1– 8)	44 (32–54)
L-Leucine	phosphate-buffered LiCl	138 (118–153)	39 (37–42)
	isotonic LiCl	88 (43–149)	33 (23–38)
	isotonic sucrose	98 (68–118)	33 (26–34)

[a] μM.

[b] fmol · blastocyst^{-1} · min^{-1}.

[c] About one third of this value is due to the activity of the Na^+-dependent system $B^{0,+}$, which is active in addition to mediated Na^+-independent lysine uptake when blastocysts are incubated in isotonic NaCl (see text).

TABLE IV

Effect of harmaline on kinetic parameters for L-lysine and L-leucine uptake by blastocysts in phosphate-buffered LiCl

A nonparametric statistical method was used to estimate the median values of the kinetic parameters and their 92–94% confidence intervals from data obtained in essentially the same way as described in the legends of Figs. 4 and 5 (Refs. 17 and 18). Paired values are considered to be statisticaly significantly different if their 92–94% confidence intervals do not overlap (see rationale at the end of the Materials and Methods).

Substrate	Harmaline	Median (92–94% confidence interval)	
		K_m value [a]	V_{max} value [b]
L-Lysine	None	63 (37– 96)	39 (28–46)
	8.0 mM	217 (117–226)	28 (20–28)
L-Leucine	None	121 (59–163)	31 (20–33)
	8.0 mM	178 (62–299)	16 (7–18)

[a] μM.

[b] fmol · blastocyst^{-1} · min^{-1}.

LiCl. The median and range of the K_i value for harmaline inhibition of 'Na$^+$-independent' L-lysine transport was calculated from the effect of harmaline on the median and 92% confidence interval of the K_m value for L-lysine uptake. The K_i value thus derived was 0.23 mM (0.02–0.74 mM) in isotonic sucrose (data not shown) and 3.3 mM (1.6–36 mM) in phosphate buffered LiCl (Table IV). The confidence intervals of the K_i values for harmaline have been greatly exaggerated because they were calculated from exaggerated intervals of K_m values (see above). Nevertheless, the fact that the intervals do not overlap means that the K_i value was significantly higher for harmaline in phosphate-buffered LiCl than in isotonic sucrose. The effect of harmaline on lysine uptake in blastocysts is complex, however, because it also decreased the V_{max} value for L-lysine transport in isotonic sucrose ($P < 0.01$).

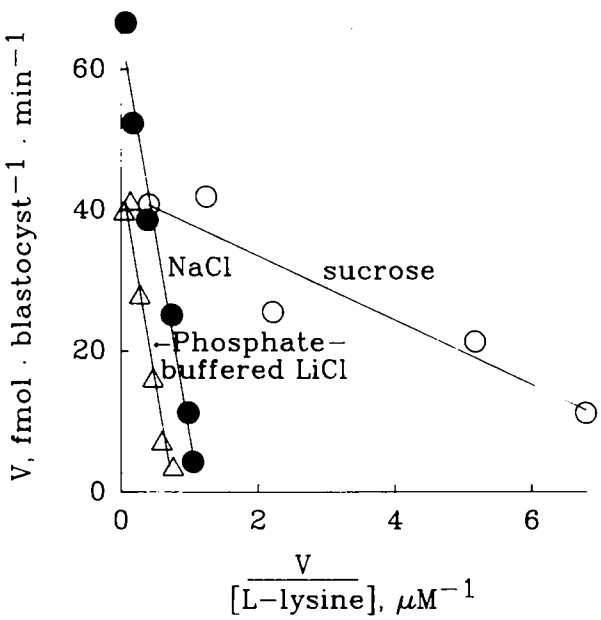

Fig. 4. Hofstee plots of mediated L-lysine uptake by blastocysts in isotonic sucrose, isotonic NaCl or phosphate-buffered LiCl. Conceptuses were incubated for 5 min with the indicated concentrations of L-lysine (0.42 μM L-[^{3}H]lysine) in one of the isotonic solutions. L-Lysine transport by the predominant system $b^{0,+}$ in blastocysts in phosphate-buffered LiCl (i.e., the L-leucine-sensitive component of transport), as determined in prior studies (Ref. 5), is also shown. 'Isotonic' solutions contain 1/100 as much phosphate as is in 'phosphate-buffered' solutions, although solutions in each category are isotonic. In general, we try to select the substrate concentrations at which uptake is measured so that most of them will not differ from the anticipated K_m value by more than a factor of about ten (Ref. 25) as determined in preliminary experiments. Each point represents the mean uptake of four replicate determinations (approximately 6 blastocysts per determination) obtained in two independent experiments. Nonsaturable uptake was subtracted from total uptake to obtain the net mediated uptake reported. The correlation coefficients were 0.94, 0.98 and 0.99 for uptake in isotonic sucrose, phosphate-buffered LiCl and isotonic NaCl, respectively.

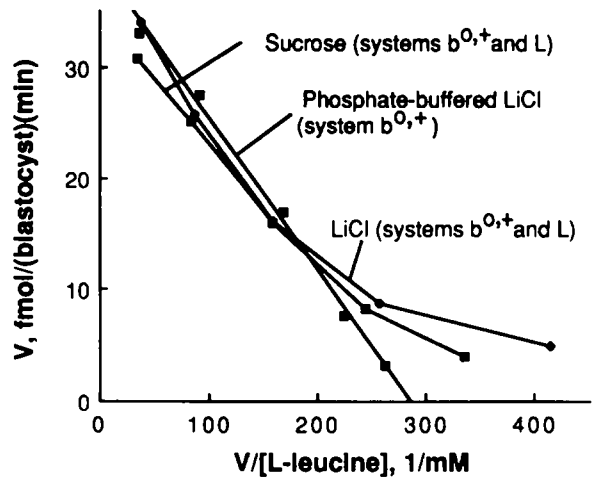

Fig. 5. Hofstee plots of mediated L-leucine uptake by blastocysts in isotonic sucrose, isotonic LiCl or phosphate-buffered LiCl. Conceptuses were incubated for 5 min with the indicated concentrations of L-leucine (1.0 μM L-[^{3}H]leucine) in one of the isotonic solutions. L-Leucine transport by the predominant Na$^+$-independent system $b^{0,+}$ in blastocysts in phosphate-buffered LiCl (i.e., the BCO-resistant component of transport), as determined in prior studies (Ref. 5), is also shown. 'Isotonic' solutions contain 1/100 as much phosphate as is in 'phosphate-buffered' solutions, although solutions in each category are isotonic. In general, we try to select the substrate concentrations at which uptake is measured so that most of them will not differ from the anticipated K_m value by more than a factor of about ten (Ref. 25) as determined in preliminary experiments. Each point represents the mean uptake of four replicate determinations (approximately five blastocysts per determination) obtained in two independent experiments. Nonsaturable uptake was subtracted from total uptake to obtain the net mediated uptake reported. The correlation coefficient was nearly 1.00 for uptake via system $b^{0,+}$ in phosphate-buffered LiCl, whereas uptake in isotonic LiCl and isotonic sucrose is attributable to the predominant Na$^+$-independent system $b^{0,+}$ (upper portions of the curves) and the less conspicuous system L (lower portions of the curves), so the relationships between v and $v/[S]$ are curved rather than straight (see text for more details).

Discussion

Expansion of the concept of ion-dependent or ion-influenced amino acid transport to include ion-inhibited transport

All of the charged osmolites we tested inhibited L-lysine uptake by the 'Na$^+$-independent' system $b^{0,+}$ in blastocysts relative to uptake in solutions of uncharged osmolites. Hence, amino acid transport systems designated 'Na$^+$-independent' because they are not stimulated by Na$^+$ may nevertheless be inhibited by this substance (Figs. 1–4). In contrast to L-lysine transport, L-leucine uptake by system $b^{0,+}$ is apparently unaffected by most of the ionic osmolites studied here (Fig. 1, upper portions of the curves in Fig. 5, and Ref. 5). Conversely, interaction of cationic amino acids with systems y^+ or (surprisingly) ASC has, so far, proved not

to be strongly influenced by Na^+, whereas transport of zwitterionic amino acids by these systems is Na^+-dependent [3,7]. Therefore, the effect of Na^+ on Na^+-inhibited as well as Na^+-dependent amino acid transport systems may depend on whether the amino acid carries a net positive charge at neutral pH. For all of these reasons, the concept of ion-influenced amino acid transport should probably be expanded to include the possibility that ions may inhibit as well as stimulate transport.

Monovalent salts of Cl^- (LiCl, choline chloride, KCl) or Na^+ (NaI, NaCl, sodium acetate) had similar effects on L-lysine uptake at concentrations of 19 mM (data not shown), so we could not attribute inhibition of system $b^{0,+}$ to cations instead of anions based on these results. When the Cl^- or Li^+ concentration was held constant while the effects of mono-, di- or trivalent cations or anions were compared, however, the di- and trivalent cations, Mg^{2+} and spermidine^{3+}, were better inhibitors of L-lysine uptake than Li^+. In contrast, no statistically significant differences were detected when the negative charges in the solutions were supplied by SO_4^{2-} or citrate^{3-} instead of Cl^- (Table II). Therefore, we attribute inhibition of system $b^{0,+}$ by these salts to cations rather than anions. Relatively strong competitive inhibition of L-lysine uptake by the cationic substance, harmaline, even in isotonic salt solutions (Table IV) also supports the conclusion that cations rather than anions inhibit system $b^{0,+}$ (see further discussion below). Since similar results were obtained with a wide variety of isotonic salt solutions (e.g., Figs. 1 and 3; Table II), several of which should have opposite effects on membrane potential, we also conclude that the effect of cations on L-lysine transport is not due to a common effect of each of the cations on membrane potential. Different osmotic effects of ionic and nonionic osmolites also cannot account for inhibition by ions of L-lysine uptake via system $b^{0,+}$ (Figs. 1–4) because a similar effect was not observed for L-leucine uptake via this system (Fig. 1 and upper portions of the curves in Fig. 5). Finally, it is unlikely that nonspecific L-lysine binding to the negatively charged plasma membrane, rather than uptake via system $b^{0,+}$, was measured under any of the conditions employed in this study because (a) virtually no detectable ^{3}H-labeled amino acid would bind to membranes after blastocysts were disrupted (see Materials and Methods), (b) relatively little radiolabel (i.e., about 4% of the 5-min level) was associated with blastocysts exposed to L-[^{3}H]lysine or L-[^{3}H]leucine for 15 s (data not shown), and (c) the K_m value for L-lysine transport and its K_i value as a competitive inhibitor of L-leucine transport were both affected in a similar way when transport was measured in isotonic sucrose instead of an isotonic salt solution, whereas the K_m value for L-leucine transport by system $b^{0,+}$ was not affected by salts (see Results).

Nature of the substrate receptor site of system $b^{0,+}$

The effects of various osmolites on amino acid transport may also lend insight into the nature of the sites that receive amino acids for transport. We sometimes refer to the latter sites as 'binding sites', although their nature may actually be more complex and could involve competition at a point in the transport process subsequent to initial binding. Competitive inhibition of uptake of the cationic substrate, lysine, by various charged osmolites is consistent with the possibility that a general cation binding site becomes a subsite of the system $b^{0,+}$ substrate-receptor site when the substrate is cationic. Binding of the ϵ-amino group of L-lysine to this subsite appears to facilitate L-lysine transport. The proposed cation binding site apparently does not need to be occupied for transport to occur, however, as evidenced by about equal transport of leucine at high or low ion concentrations. Nevertheless, the cationic portions of zwitterionic substances, such as leucine, may bind to the cation binding site. The zwitterionic substances, taurine and glycine, which are poor or non-substrates of system $b^{0,+}$ [5], inhibit L-lysine transport (Figs. 1 and 3) presumably because their amino groups bind to the cation binding site. The latter type of interaction is a second and distinct although much weaker way that L-leucine may competitively inhibit lysine uptake in addition to competition as a substrate for the rest of the system $b^{0,+}$ receptor site [5].

As for system $b^{0,-}$, cationic and zwitterionic amino acids also interact differently with transport systems ASC, asc_1 and y^+. Young and associates [8] have shown that harmaline, which appears to interact with several Na^+ transport sites in renal microvillus membranes [23], is a competitive inhibitor of L-lysine transport by the Na^+-independent system asc_1 in horse erythrocytes, whereas this substance is a noncompetitive inhibitor of alanine uptake via the same system. Similarly, the effect of harmaline on 'Na^+-independent' amino acid uptake in blastocysts is partially competitive when L-lysine is the substrate but noncompetitive when L-leucine is the substrate (Table IV). Moreover, the K_i value is higher for harmaline as a competitive inhibitor of L-lysine uptake via system $b^{0,+}$ in phosphate-buffered LiCl than it is in isotonic sucrose (see Results). Therefore, inorganic cations and harmaline appear to compete for a common subsite on the $b^{0,+}$ transporter. Unlike system $b^{0,+}$, the K_m value for L-lysine uptake by system asc_1 is larger than for uptake of its best zwitterionic substrates. Nevertheless, Young and associates argue convincingly that harmaline binds to a subsite on the system asc_1 transporter that recognizes positively charged side chain amino and guanidino groups [8], and we propose that the system $b^{0,+}$ transporter has a similar subsite. Na^+ as well as harmaline and side chain amino and guanidino groups also appear to compete for a comparable subsite on the transporter of system ASC in human erythro-

cytes [8]. The Na^+ bound to the latter subsite serves as a co-substrate for this zwitterion-preferring, Na^+-dependent amino acid transport system [7]. Interestingly, cationic amino acids competitively inhibit system ASC in a Na^+-independent manner, although they do not appear to be transported by it [3,7]. Similarly, zwitterionic amino acids are accepted by the 'Na^+-independent', cation-preferring system y^+ when Na^+ or Li^+ is also present, and a parallel has been drawn between the substrate receptor sites of systems y^+ and ASC [3,7]. It is not yet known if cationic amino acids are even better inhibitors of system ASC or substrates of system y^+ at low ionic strength, as is the case for system $b^{0,+}$ (e.g., Table III). Cations in general do not appear to affect transport of L-alanine or L-lysine by system asc_1, however [8], although 15 mM Tris-HCl was present in the medium in the latter experiments along with isotonic sucrose. This much Tris may significantly inhibit L-lysine uptake by system $b^{0,+}$ (Figs. 1 and 3). Regardless of differences in their specific characteristics, systems $b^{0,+}$, y^+, asc_1 and ASC appear to interact in similar ways with positively charged amino acid side chains, harmaline or other cations.

Evolutionary implications

The similarities among the proposed cation binding sites of systems $b^{0,+}$, y^+, asc_1 and ASC are consistent with the proposition that they have a common evolutionay origin. Since system $b^{0,+}$ appears to be the more general cation binder, it may be more like the proposed ancestral transport process than are systems y^+, asc_1 or ASC. Extending the same bias to substrate selectivity, system $b^{0,+}$ may retain more of its ancestral, broad substrate specificity, whereas systems ASC, asc_1 and y^+ may have evolved greater selectivity for zwitterionic or cationic amino acids, respectively.

A similar assessment also may apply to evolution of other types of transport processes. For example, it was suggested recently that genes for the Na^+-translocating ATPase of *P. modestum* and the H^+-translocating ATPase of *Escherichia coli* may have evolved from a common ancestral gene [24]. The authors of the latter paper apparently favor the hypothesis that the Na^+-translocating ATPase evolved from a H^+-translocating enzyme [24], but it is also conceivable that the original ancestral gene product functioned more like the Na^+-transporting one. *P. modestum* is strictly anaerobic, and, although some contemporary anaerobes may have evolved from aerobic bacteria, life on earth probably began in an anaerobic environment. Moreover, the Na^+-translocating ATPase transports both H^+ and Na^+ well when the concentrations of H^+ and Na^+ are below one mM [24]. Therefore, the increased selectivity of the H^+-translocating ATPase in *E. coli* for H^+ could have evolved from a less selective cation-translocator. This less selective cation-translocator would have been more like the less selective Na^+-translocating ATPase than like the more selective H^+-translocator. Nevertheless, it is also possible that greater substrate specificity is a more primitive characteristic of transport processes; or that similar processes, such as the H^+- and Na^+-translocators or systems $b^{0,+}$, y^+, ASC and asc_1, are the result of evolution of similarly structured transporters on more than one occasion.

Regardless of which, if any, of the preceding scenarios is correct for amino acid transport processes, the inhibition of amino acid transport systems asc_1 and ASC by harmaline [8] seems to have implications for their structures beyond the interpretation that system asc_1 is simply a Na^+-independent version of system ASC. The relationships among the structures and functions of all of these transport systems will, of course, be clearer when the detailed (i.e., primary, secondary, tertiary and perhaps quaternary) structures of their transporters have been elucidated. Nevertheless, we anticipate that the latter studies may show that the structures of the transporters of each member of this charge-altered family of systems (i.e., systems $b^{0,+}$, y^+, ASC and asc_1) are more similar to each other than to the structures of the transporters of other known amino acid transport processes (e.g., systems T, L, A, Gly and $B^{0,+}$ and the IMINO system; Refs. 1–3). For members of the 'charge-altered' family, the charges on the side chains of the amino acids they accept as substrates and competitive inhibitors helps determine other characteristics of the transport process. Although some of the other systems, such as $B^{0,+}$, also may accept both cationic and zwitterionic amino acids for transport [4], we do not anticipate that the charges on the side chains of substrates of these other systems will help determine whether or not the activities of the systems are affected by inorganic cations, and whether inhibition of the system by harmaline is competitive or noncompetitive. Moreover, members of the charge-altered family of systems appear, in general, to resist transporting substances that are α-N-substituted or whose carbon skeletons branch at the α- or β-positions, whereas each of the other systems (T, L, A, Gly and $B^{0,+}$ and the IMINO system) tolerates one or more of these structural variations relatively well [1–9]. Criteria for the provisional placement of systems in the charge-altered family may still be emerging, however (e.g., Refs. 7 and 8), and the criteria are based on data some of which have not been gathered for systems not currently placed in this category. Therefore, we urge both the detailed characterization of amino acid transport processes by well established criteria [1–3], and careful assessment of the effects of ions on these processes.

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