Efflux of amino acids from the lens

**Harold L. Kern**

Estimation of efflux of amino acids from the mammalian lens indicates it is of small magnitude compared to influx at equivalent nonsaturating levels of substrate. This finding is a necessary part of the explanation of the complex problem of the maintenance of elevated, intracellular levels of these substances. Accelerative countertransport was observed when appropriate amino acids were present on the trans side of the plasma membrane; this phenomenon provides evidence of a mediated path for exodus. The two neutral amino acids examined had rate coefficients for efflux which were similar, and approximately equal to rate coefficients for the nonsaturable component of entry of this category of substrates. On the other hand, the rate coefficient for efflux of L-lysine exceeded that which could be attributed to a nonsaturable process; the difference may be due to lower levels of endogenous, competing diamino acids compared with the neutral amino acids.

**Key words:** Amino acids, efflux, kinetics, mammalian lens, exchange diffusion.

Considerable information is available concerning the uptake and steady state levels of amino acids in the mammalian lens. This work indicates that certain amino acids are accumulated by a process that requires metabolic energy, and is dependent on external Na+. There is also evidence for considerable complexity in the mechanism of transport, with at least three classes of sites for neutral amino acids in the plasma membrane of the epithelial cells of bovine lens. The epithelium is a single layer of cells on the anterior aspect of the lens; this structural asymmetry apparently produces an asymmetry in the transport of metabolites through the tissue, with the result that a number of them accumulate anteriorly and leak posteriorly. Preliminary findings indicated that the efflux of amino acids from the lens was only a small fraction of influx under comparable conditions, and that the former process did not depend on external Na+. A more detailed analysis of efflux was undertaken since the steady-state levels of amino acids depend to a large extent on the relative size of the rate coefficients describing the fluxes into and out of the lens, and since a good deal can be learned about the nature and specificity of transport from analysis of exchange diffusion. In experiments on exchange diffusion, the initial effect of a substrate on one side of the plasma membrane on the flux of the same or a similar substrate on the other (trans) side of the membrane is evaluated. A finding of equimolar replacement of internal with external substrate is evidence that exchange diffusion occurs, and favors the concept of

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This work was supported by United States Public Health Service Grant NB-05033.

involvement of mobile carriers in the transport. Finally, the carrier-substrate complex may cross the membrane at a greater rate than the free carrier does, resulting in an increased, transmembranous flux in the direction of the added substrate.

Materials and methods

Preparation of tissues. Lenses (950 to 1350 mg.) were removed from calf eyes within two hours after the animals were put to death. An equatorial cut was made ¾ of the way through the globe with the blade from a Stadie-Riggs tissue slicer (A. H. Thomas Co., Philadelphia); the anterior segment was inverted; the vitreous humor was deflected from the posterior side of the lens with a wooden applicator without touching that organ; the zonules were broken with the thumb and forefinger, and the remaining zonules were broken by a rolling motion. Male, Sprague-Dawley rats, weighing about 400 grams, were killed by being held in a solution containing 140 mM. Na+, 5 mM. K+, C1-. Radioisotopes were added, and the remaining 2- (4-terf-butylphenyl) -5-(4-biphenylyl)-1,3,4-oxidiazole (40 mg.), and BBS-3 (Beckman Instruments) (2 ml.) were transferred on the metal mesh. Measurements on efflux were taken for determination of influx. Measurement of radioactivity. Equal volumes of the TCA extract and of the medium in 10 per cent TCA were counted in a scintillating solvent having the following composition: toluene (10 ml.), 2-(4-terf-butylphenyl)-5-(4-biphenylyl)-1,3,4-oxidiazole (40 mg.), and BBS-3 (Beckman Instruments) (2 ml.). Influx and efflux were determined on the same lens by measuring the radioactivity in both lens and medium after reincubation. In experiments on efflux, radioactive D-mannitol, plus 0.5 mM. carrier, was used to measure the extracellular space of each lens. With this indicator, the extracellular space, expressed as the ratio of the concentration of mannitol in the lens water to mannitol in the medium, ranged from 0.10 to 0.15. The radioactive amino acid and mannitol were counted simultaneously in two channels. Corrections were made as required for extraneous counts in each channel.

Digestion with collagenase. Rat lenses were digested, during preincubation, with purified collagenase from Clostridium histolyticum having a specific activity of 2000 units per milligram. The enzyme, at a concentration of 1,200 units per milliliter, effectively removed the capsule and, as judged from photomicrographs, had no discernible effect on the underlying epithelium.

Lysine and arginine in the lens. Ten fresh rat lenses were dispersed in 0.5 ml. each of cold 10 per cent TCA in 1.0 ml. polystyrene centrifuge tubes (Clay-Adams, Inc., New York, N. Y.), allowed to stand for 72 hr. at 4° C., centrifuged, the supernatants combined, and extracted six times with 10 ml. each of ether. The neutral, aqueous solution was lyophilized, and subsequently redissolved in 0.10 ml. of 10 per cent isopropanol. Electrophoresis at 2.5 K. for 1½ hr. was performed in

*The collagenase was furnished by Dr. S. Takahashi.
1 per cent pyridine containing 0.4 per cent acetic acid (pH 5.3) on Whatman 3mm. paper, taking 5 μl of the lenticular extract or 5 μl of 10 mM. arginine or lysine. The paper was stained with Cd-ninhydrin and stored overnight over H 2SO4 in the dark. Arginine and lysine were almost completely resolved. The spots were cut out, eluted into methanol, and the solutions read at 505 nm. The absorbance of the standards was proportional to concentration over the range encountered.

Four rat lenses were incubated in the presence of L-lysine-H 3(G) (10 μM per milliliter) plus 0.5 mM. carrier for two hours to determine whether the amino acid was metabolized under the conditions used to evaluate transport of this amino acid. An extract was prepared as above, which was then desalted electrolytically. The solution was lyophilized, and finally redissolved in 20 μl of 10 per cent isopropanol. Chromatography on Whatman No. 1 paper was done in n-butanol-acetic acid-water (12:3:5) and in 75 per cent phenol-ethanol-NH.OH (160:40:1). Strips were stained with 0.25 per cent ninhydrin in acetone, and the distribution of radioactivity on unstained strips was determined with a radiochromatogram scanner. The L-lysine-H4 from the lens filtrates was found to be at least 90 per cent pure; no alteration of the amino acid by the lens was detected on comparison with tracer put through the same procedure, but not incubated with the tissue.

**Expression of data.** Fluxes are expressed on a wet weight basis, and have been corrected for extracellular space. Efflux is based on the average level of amino acid in the lens, assuming a linear decline in concentration with time. A correction of 6 per cent or less was applied to the rate coefficient for the nonsaturable component5 of influx to account for the average lenticular level of the entering amino acid:

\[
\text{rate coefficient} = \frac{\text{mM}_{\text{in}}}{(\text{mM}_{\text{station}} - \text{Avg. mM}_{\text{in}}) \text{hr}}.
\]

In calculating flux coefficients, the water content of the lenses was taken as 60 per cent for the rat and 68 per cent for the calf. The standard error of the mean of 3 to 6 replicates is given.

**Results**

The purpose of this investigation was to make a kinetic comparison of efflux and influx of amino acids. Entry of amino acids into the lens as into other tissues is primarily a mediated process, may be Na+ dependent, and is often complex in that it can occur simultaneously at a number of classes of sites in the plasma membrane.\(^4\)\(^5\) Efflux could be mediated, but nonsaturable in nature, as is a part of the flux in Ehrlich ascites carcinoma cells,\(^17\)\(^18\) and apparently about 10 per cent of the total influx of a given amino acid in the lens.\(^5\) The nonsaturable flux, besides exhibiting a linear dependence on concentration of substrate, is unaffected by the presence of analogues. Most of the studies were done with the amino acids which cannot be metabolized, α-aminoisobutyric acid (AIB) and 1-amino cyclopentane-1-carboxylic acid (cycloleucine). It is pertinent to analyze influx of these amino acids, as efflux may take place by related paths. Table I gives a characterization of the uptake by the rat lens of AIB and cycloleucine from a 0.1 mM. solution. Most of the methods have been described previously,\(^5\) and depend on selective, inhibitory effects of competing amino acids as outlined in the footnote to Table I. Ten to fifteen per cent of the total uptake of the amino acids examined was presumed to be nonsaturable\(^5\) and is not shown in Table I. The nonsaturable component was calculated as the difference between the total uptake and the sum of the saturable components. It can be seen that uptake of cycloleucine occurred at about three times the rate of AIB, and the cyclic amino acid appeared to utilize the L- or leucine-preferring sites. However, an L*\(^\circ\) component,\(^*\) amounting to about 20 per cent of the total uptake, may be included in the L-component as determined.\(^19\) In evaluating entry of cycloleucine, excess sarcosine was used to block both the A-sites and the X-sites. Entry of AIB was more complex, and the L-site, A- or alanine-preferring site, and possibly the X- or glycine site were all utilized.\(^+\) Uptake of 0.1 mM. AIB by the calf lens followed a pattern similar to that in the rat, but was much lower (10 mMoles/g • hr.) owing

\(^{\circ}\)The L*\(^\circ\) sites are common areas of entry for several neutral amino acids and the diaminoo acids.

\(^{\circ}\)Uptake of 0.1 mM. AIB at the glycine site of 1.1 ± 1.1 mMole/g/hr. was obtained as the sarcosine-inhibitable component in the presence of excess L-methionine.\(^+\) This is probably a more reliable value than that in Table I, as paired lenses were used. The Strozier method was used to prepare N-methyl-a-aminoisobutyric acid, which was found to selectively block the A-site (see Table I).\(^\circ\)
Table I. Characterization of the cellular uptake of 0.1 mM cycloleucine and AIB by the rat lens*

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Total uptake</th>
<th>Component</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>L</td>
</tr>
<tr>
<td>AIB</td>
<td>69 ± 6</td>
<td>24 ± 6 (35)</td>
</tr>
</tbody>
</table>

*The lenses were incubated for 1 hr. at 37° C. in the presence of AIB, and for ½ hr. in the presence of cycloleucine. Values in parentheses give uptake as a percent of the total. The conditions for determination of the components were: for cycloleucine, L = 10 mM. D,L-tarteleucine-inhibitable and A,X = 10 mM. sarcosine-inhibitable; for AIB, L = 20 mM. D,L-tarteleucine-inhibitable, A = 10 mM. N-methyl-D,L-aminoisobutyrate (MeAIB)-inhibitable; X = 10 mM. sarcosine-inhibitable – 10 mM. MeAIB-inhibitable, and ASC = Na⁺ dependent in presence of 10 mM. sarcosine.²

Table II. Efflux of AIB from the calf lens*

<table>
<thead>
<tr>
<th>Na⁺ in medium (mM.)</th>
<th>Cellular efflux (μMoles/g⋅hr.)</th>
<th>10 mM. AIB present in medium</th>
<th>No AIB</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.80 ± 0.18</td>
<td>−0.11 ± 0.13</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>1.05 ± 0.17</td>
<td>+0.01 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>115</td>
<td>0.81 ± 0.13</td>
<td>−0.08 ± 0.09</td>
<td></td>
</tr>
</tbody>
</table>

*Lenses (six in each group) preincubated 3 hr. at 37° C. in the presence of 0.121 mM. AIB, 1 μC per milliliter AIB-1-C¹4, and 4.0 mM. D-mannitol, 3 μC per milliliter D-mannitol-1-H³. They were rinsed with 0.15M NaCl, and reincubated for 3 hr. at 37° C. in fresh medium. Values in parentheses give uptake as a percent of the total. The conditions for determination of the components were: for cycloleucine, L = 10 mM. D,L-tarteleucine-inhibitable and A,X = 10 mM. sarcosine-inhibitable; for AIB, L = 20 mM. D,L-tarteleucine-inhibitable, A = 10 mM. N-methyl-D,L-aminoisobutyrate (MeAIB)-inhibitable; X = 10 mM. sarcosine-inhibitable – 10 mM. MeAIB-inhibitable, and ASC = Na⁺ dependent in presence of 10 mM. sarcosine.²

Fig. 1. The capsule of the lens as a potential barrier to transport of amino acids.

to the lower ratio of surface to volume in the large bovine lens. Fifty-eight percent of the total uptake from 0.1 mM solution was at the L-site, 25 percent at the A-site, and 8 percent at the X-site.⁵

An examination of the efflux of AIB from the calf lens is given in Table II. The data indicate that efflux was negligible in the absence of external AIB, but that a significant loss occurred when 10 mM. AIB was present in the medium. Exit of AIB was independent of Na⁺ on isosmotic replacement of NaCl with tetraethylammonium chloride.⁶ Efflux, even in the presence of external AIB, was only two percent of influx. Because of the small magnitude of efflux of this amino acid, and for reasons of economy, most further studies were done on the rat lens.

One of the first problems we faced was that the capsule of the lens might be a significant barrier to diffusion of amino acids. This effect, illustrated in Fig. 1, would be more predominant in exit than in entry. For an amino acid entering the lens, the gradient in concentration across the capsule would tend to decline on proceeding inward toward the epithelium because of the presence of mechanisms for uphill transport in the cellular membranes; for exit, the gradient would be oppositely directed with a resultant tendency for amino acid to be reaccumulated by the epithelium. Re-entry of amino acids could also be a complicating factor if the volume of medium was not sufficiently large in comparison with the volume of water in the lens. The influence of these factors was evaluated by determining the effect on efflux when the capsule was removed with collagenase, and when the volume of the medium was var-
Figs. 2 and 3 show that the volume of the medium was not a factor which significantly influenced efflux of AIB or cycloleucine. Digestion with collagenase, Fig. 3, appeared to enhance efflux slightly. Both Figs. 2 and 3 show that there was a stimulation of efflux in the presence of external L-methionine, with the effect being much more marked for cycloleucine than for AIB. The greater scatter of the data in Fig. 2 may be due to the facts that results from six experiments are plotted, and the amount of AIB exiting was less than that of cycloleucine.

A comparison is made of influx and efflux of cycloleucine and AIB in Figs. 4 and 5. These data illustrate that efflux was relatively small in the absence of an external amino acid, but significantly increased when L-methionine was present in the medium. Again, it may be noted that digestion with collagenase had a rather small effect on influx and efflux of cycloleucine. Finally, the cellular influx appeared to be saturable, but cellular efflux increased in a manner which was proportional to the average, lenticular level of amino acid during this period. Efflux of cycloleucine was determined over a wider range of concentration of substrate with the results plotted in Fig. 6. The highest level of amino acid present during preincubation was 30 mM., and the $(Na^+)_e$, ranged from 98 to 112 mM. The rate of loss of cycloleucine by exchange with external methionine remained a linear function of the average internal concentration. Fig. 6 shows that the magnitude of the two fluxes was comparable at 5 mM. substrate. This may be attributed to a relatively low affinity of the amino acid for the carrier during exit. The resultant high value of $K_t$, the coefficient representing half saturation (assuming the process is saturable), would facilitate transport at high levels of substrate.

Evidence was also obtained for stimulation of efflux of L-leucine in the presence of external L-methionine. Lenses were preincubated for 1 hr. in the presence of L-leucine-4,5-H$^3$ (0.1 mM.), and reincubated for 1 hr. with or without L-methio-
Volume 9
Number 9

Amino acids from the lens 697

![Graph](image)

**Fig. 3.** Effect of volume of medium on efflux of cycloleucine from the lens. Preincubation, in the presence of 0.2 mM amino acid, and reincubation were for 1 hr. each in media containing 137 to 140 mM Na⁺.

![Graph](image)

**Fig. 4.** Comparison of influx and efflux of AIB in the rat lens. Preincubation (1 ml.) and reincubation (5 ml.) were for 1 hr. each in media containing 137 to 140 mM Na⁺. The solid symbols give the results of two experiments in which influx was determined.

nine in the medium. The following increments in efflux, expressed as per cent of total influx, were found when compared to paired control lenses: L-methionine (1 mM.), 8 per cent; L-methionine (5 mM.), 25.5 per cent; and L-methionine (20 mM.), 28.5 per cent.

An attempt was made to learn whether there was any relation between the nonsaturable influx and efflux in the absence of an external amino acid. Data for cycloleucine are summarized in Fig. 7. The nonsaturable influx was determined as the uptake in the presence of a fiftyfold, molar
excess of L-methionine. Although the data for influx are rather scattered and cover a narrower range than the data for efflux, comparison of the least square lines indicates that they occupy similar positions. Fig. 8 depicts a related analysis for AIB, with the influx and exchange-flux lines shown for purposes of reference. The line labeled “nonsaturable flux” was obtained from the limiting slope of the curve representing the total influx of AIB in a kinetic study in which the concentration ranged from 1.5 to 30 mM. In this experiment, the slope was almost linear from 5 to 30 mM. AIB, indicating that most of the increment in total uptake over this range was nonsaturable in nature. Data are shown in Fig. 8 for efflux in the absence of an external amino acid and for nonsaturable influx in the presence of a fiftyfold, molar excess each of L-methionine and glycine. The concentration of Na⁺ was maintained at 110 mM, in the latter experiment. It can be seen that the points are a good fit for the line. Thus, by these criteria, efflux in the absence of exchange, a portion of the influx of AIB, and, possibly, cycloleucine occur by a kinetically equivalent mechanism.

A comparison was also made of the fluxes of L-lysine; the findings are presented in Fig. 9. The relative positions of the curves are similar to those for the synthetic amino acids. No evidence was found for saturation of efflux of lysine with or without

*The data for efflux were not corrected for endogenous L-lysine. Values of 0.48 mM lysine and 0.39 mM arginine were found by high-voltage electrophoresis of an extract from unincubated lenses. The slope of the lines in Fig. 9 should not be altered by such correction, since both efflux and average lenticular concentration of lysine would be increased proportionately.
L-arginine present in the medium. On the other hand, the cellular influx was saturable, as was the case for AIB and cycloleucine. Lysine differed from the synthetic amino acids in that the slope of the line representing efflux in the absence of arginine was about three times the nonsaturable influx (50 times molar excess of L-arginine present). Therefore, it appears that lysine has another pathway for exit besides the nonsaturable route.

Apparent rate coefficients were calculated as the ratio of the flux (μMole/ml·hr.) to the average gradient in concentration of amino acid (μMole per milliliter) producing the flux. Coefficients for efflux and for the nonsaturable component of influx are listed in Table III. The rate coefficient for the nonsaturable component of influx is fairly constant, varying over a 2.5-fold range for the amino acids characterized in Table III (extreme limits are given for the influx coefficient of cycloleucine; the correct value probably lies well within the limits). The coefficients for efflux in the absence of exchange, and for nonsaturable influx, are in reasonable agreement for AIB and cycloleucine (refer to Figs. 7 and 8). However, these coefficients for lysine differ by a factor of 3.7, indicating that exit of lysine occurs by another route in addition to the nonsaturable, since efflux has the higher rate coefficient. A comparison of the two types of efflux of L-lysine, AIB, and cycloleucine indicates that AIB exchanged less readily than the other two amino acids, and that cycloleucine was the best exchanger of the three under the experimental conditions.
Discussion

It is apparent from the data presented in Table I that cycloleucine is taken up by the rat lens more readily than is AIB. The cyclic amino acid appears to use the L-site primarily, whereas entry of AIB is distributed between a number of sites. Other investigations have demonstrated that the L-site serves mainly for exchange in several tissues including Ehrlich carcinoma cells, reticulocytes of rabbits, mature mammalian erythrocytes, and the pancreas of the mouse; and that cycloleucine is a better exchanger than AIB. The rate coefficient found for efflux by exchange of lenticular cycloleucine was 2.7 times that of AIB, and probably reflects the greater affinity of the former amino acid for the L-site. This accelerated flux, due to counterflow, is not only indicative of mediation, but may be regarded as evidence for mobile carriers. However, it was not possible to approach saturation of the mechanism for exchange of cycloleucine at the highest, intralenticular concentration attained. This situation may arise because the apparent constant for half saturation of efflux is much higher than that of influx (0.69 mM., corrected for the non-

Table III. Comparison of efflux and the nonsaturable component of influx of amino acids in the rat lens

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>$K_{\text{efflux}}$ (h$^{-1}$)</th>
<th>$K_{\text{non-exchange}}$ (h$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Lysine</td>
<td>0.67 (10mM L-Arg)</td>
<td>0.275</td>
</tr>
<tr>
<td>AIB</td>
<td>0.272 (5mM L-Met)</td>
<td>0.083</td>
</tr>
<tr>
<td>Cycloleucine</td>
<td>0.74 (5mM L-Met)</td>
<td>0.153</td>
</tr>
<tr>
<td>L-Phenylalanine</td>
<td>0.464†</td>
<td></td>
</tr>
<tr>
<td>L-Serine</td>
<td>0.142§</td>
<td></td>
</tr>
<tr>
<td>L-Glutamic acid</td>
<td>0.205§</td>
<td></td>
</tr>
</tbody>
</table>

*Incubation in the presence of L-phenylalanine, L-serine, and L-glutamic acid was for 1/2 hr. at 37° C. Levels of Na$^+$ ranged from 85 to 140 mM. When it was necessary to keep a constant level of Na$^+$, tonicity was maintained with tetraethylammonium chloride. The conditions of incubation for L-lysine, AIB, and cycloleucine have been given in the legends of Figs. 4 to 9.
†The rate coefficients were calculated on the basis of the average level of amino acid in the lens during the period of efflux. The coefficient for exchange has not been corrected for a nonexchange component. The concentration and nature of the external exchanging amino acids are given in brackets under Exchange.
§Determined graphically from the slope of the asymptote to the curve representing total, cellular influx at saturating levels of substrate. A value of 0.14 was found for the rate coefficient of influx of L-lysine in the presence of a 50 times molar excess each of L-phenylalanine and L-threonine in good agreement with that in the table.
saturable component), as has been found in the Ehrlich cell for amino acids utilizing the A-, L-, and diamino acid sites, and for cycloleucine in pancreas.

The neutral amino acids appear to use the nonsaturable pathway for exit in the absence of an external exchanger, since the coefficient for this mode of efflux is in agreement with the rate coefficient for the nonsaturable component of influx. Restriction of efflux to the nonsaturable path may be a consequence of competition for exodus by the endogenous, lenticular amino acids, many of which are present at greater than 1 mM levels. In this connection, similar nonsaturable rate constants for entry and exit have been reported for the tumor cell. The greater facility of efflux of L-lysine into a solution free of amino acid might be due to increased availability of mediating sites, hence less competition, for the diamino acid.

The nonsaturable component seems to have a broader specificity than other systems of transport. Most amino acids have similar rate coefficients for this pathway. In the calf lens, these ranged from 0.011 h⁻¹ to 0.023 h⁻¹ for eight amino acids (L-phenylalanine, L-leucine, glycine, sarcosine, AIB, L-arginine, taurine, and β-alanine). The data for the rat lens indicate that it is a symmetrical system since similar rate coefficients were found for influx and for efflux (Table III).

The author wishes to thank Mrs. Diane Brassil and Mr. David Fox for capable technical assistance, and Mr. Jack Siegel for help in the synthesis of selective competing amino acids.

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