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AMINO ACID ACCUMULATION IN FROG MUSCLE

II. ARE CYCLOLEUCINE FLUXES CONSISTENT WITH AN ADSORPTION MODEL FOR CONCENTRATIVE UPTAKE OF AMINO ACID?

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SUMMARY

Cycloleucine accumulation by frog muscle was studied at 0 °C and 25 °C. At external concentrations less than 5 mM the distribution ratio of cycloleucine is higher at 0 °C than at 25 °C. At concentrations greater than 5 mM the converse is true due to apparent exclusion of cycloleucine from a larger portion of the cell water at 0 °C.

The steady state data are consistent with an adsorption model for amino acid accumulation. Flux studies provide a means to rule out this model if all the possible rate-limiting steps in the movement of amino acid into and out of the cell are considered. These steps include intra-cytoplasmic diffusion, desorption from cytoplasmic or membrane sites and passage through the cell membrane. The assumption is made that the rate-limiting step for influx and efflux is the same, allowing the use of either influx or efflux data to examine the model.

Diffusion-limited flux is ruled out on the basis of "influx profile analysis" of the time course of cycloleucine entry at both 0 °C and 25 °C.

At least 95 % of all intracellular cycloleucine leaves frog muscle cells with a single exponential time course at both 0 °C and 25 °C. The rate constant of efflux does not vary with cellular concentration.

These findings are shown to be incompatible with desorption-limited efflux. They are compatible with membrane-limited efflux only if (i) adsorption sites are located on membranes with direct access to the extracellular space and (ii) the rate constant for desorption is equal to the rate constant of membrane-limited efflux of free amino acid. It is considered unlikely that such a coincidence would occur at both 0 °C and 25 °C. Therefore, an adsorption model for cycloleucine accumulation in frog muscle appears to be untenable.

INTRODUCTION

Ling [1, 2] proposed that concentrative uptake of solutes by cells results from reversible adsorption to specific protoplasmic sites rather than active transport pro-

cesses. The following observations make this hypothesis an attractive one for the accumulation of amino acids: (i) glycine is accumulated against a substantial gradient by frog muscle at 0 °C, a temperature where its metabolism is insignificant [3, 4], (ii) at low external concentrations the distribution ratio of cycloleucine is higher at 0 °C than at 25 °C [5], and (iii) at 0 °C the cycloleucine influx rate constant appears to be 10 times faster than the efflux rate constant (Neville, M. C., unpublished).

The present author has shown [4] that the arguments which have been raised against substantial adsorption of accumulated amino acid do not exclude a model for reversible adsorption of the type proposed by Ling [1, 2]. For example, the swelling which accompanies the entry of amino acids into many cells has been offered as evidence that accumulated amino acids are free and osmotically active [6, 7]. However, Cope [8] has suggested that adsorbed solutes may cause swelling by dissociating the cytoplasmic matrix. Udenfriend et al [9] observed that the fluorescent properties of aminonaphthylalanine were only minimally altered on accumulation by ascites cells whereas the fluorescent properties of tightly bound substances such as eosin and atabrine were markedly altered inside cells. However, eosin and atabrine are so tightly bound to cellular elements thay they are not released on lysis of cell and, therefore, may not serve as proper controls for the fluorescent properties of substances postulated to be reversibly bound such as aminonaphthylalanine [4].

Heinz [10] suggested that the kinetics of glycine flux in ascites cells were probably inconsistent with an adsorption model. However, because his data did not allow accumulated amino acid to be partitioned between presumptive bound and free fractions, he was unable to completely exclude glycine adsorption on the basis of a kinetic analysis. In the present study both the steady state accumulation of cycloleucine and the kinetics of cycloleucine movement into and out of frog muscle cells have been examined at 0 °C and 25 °C. These data make possible a quantitative comparison between the kinetic predictions of an adsorption model and the experimental findings. It will be shown that this comparison makes an adsorption mechanism for amino acid accumulation extremely unlikely. A preliminary report of some of these data has geen given [11].

THE ADSORPTION MODEL

Let us consider a cell, bounded by a plasma membrane, which contains a substantial number of specific amino acid binding sites. Accumulated amino acids may exist in two states; bound to these sites, or free in the cell water. At equilibrium the apparent concentration of amino acid in the cell is given by:

$$[S]_{cw}^{ss} = q[S]_e + \frac{[S]_{max}}{1 + K_m/[S]_e} \tag{1}$$

where $[S]_{cw}^{ss}$ is the steady state concentration of amino acid in μmol/ml cell water; q is the distribution coefficient of free amino acid between the cell water and the external solution. $[S]_{max}$ is the number of binding sites per ml of cell water and K_m their apparent dissociation constant. The first term on the right hand side of Eqn (1) gives the free amino acid, the second term is bound amino acid. Use of Eqn (1) with steady state data allows the cellular amino acid to be partitioned into presumptive bound and free fractions.

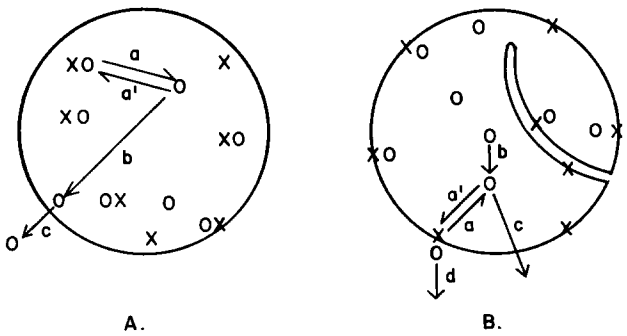

Fig 1. Models of cells with solute adsorption sites. Cell bounded by membrane represented as continuous line separating cytoplasm from extracellular phase. Specific solute adsorption sites represented by X; adsorbed solute, XO; free solute, O. (A) Adsorption sites located mainly in the cytoplasm. Adsorbed solute must leave the cell by passing through steps a (desorption), b (diffusion), and c (passage through the cell membrane). (B) All adsorption sites are located on membranes with direct access to the extracellular space. Free amino acid passes either through these sites (Steps a′ and d) or through a leakage pathway (Step c) to reach the cell exterior.

In order to rule out amino acid adsorption on the basis of kinetic data it is necessary to develop a series of inclusive models which specify: (i) the location of the binding sites, (ii) the pathway for the movement of solute into and out of the cell and (iii) the rate-limiting step in this pathway. If the adsorption sites are located within the cytoplasm (Fig. 1A), adsorbed solute must pass through the cell water prior to movement out through the cell membrane. The rate-limiting step can be either desorption (Step a, Fig. 1A), passage through cell water (Step b, diffusion-limited flux) or passage through the cell membrane (membrane-limited flux, Step c). In frog muscle, amino acid fluxes are not sufficiently rapid that a limitation imposed by an extracellular unstirred layer need be considered.

If adsorption sites are located only on cell membranes with direct access to the extracellular space (Fig. 1B), adsorbed solute leaves the cell only by desorbing into the extracellular phase (Step d). Free solute may leave the cell either by passing directly through the cell membrane (Step c) or by complexing with a membrane site (Step a′) and then desorbing into the extracellular space. Cycloleucine fluxes will be analyzed on the basis of the above models.

If we make the reasonable assumption that the rate-limiting step for entry into the cell is the same as the rate-limiting step for loss of amino acid from the cell, it should be possible to use either influx or efflux data to determine whether the kinetics of amino acid fluxes are consistent with an adsorption model. On this basis, data from influx studies will be used to examine diffusion-limited flux. Efflux experiments will be used to evaluate desorption and membrane-limited models. It will be shown that data on cycloleucine fluxes in frog muscle are not consistent with a reasonable model for substantial intracellular adsorption.

MATERIALS AND METHODS

The procedure for incubation of muscles was the same as described previously

[3] except that 20 to 25 ml of incubation solution was used for the 25 °C incubations. Detailed balance studies at both 0 °C and 25 °C showed that more than 97 % of the radioactivity lost from the bathing solution was recovered from the trichloroacetic acid extracts of the muscles. Thin-layer radiochromatograms of extracts of incubated muscles using the solvent system *n*-butanol/acetone/diethylamine/water (10 : 10 : 2 : 5, by vol.) showed that 99 % of the extracted radioactivity moved as a single peak with an R_F value corresponding to cycloleucine. These results indicate that trichloroacetic acid extraction is sufficient to recover all accumulated cycloleucine and that cycloleucine is not significantly broken down by the tissues during the prolonged incubations necessary to achieve a steady state. Efflux studies were also carried out as described previously [3] except 5 ml polyethylene scintillation vials were used for the washout solutions, to prevent a loss of radioactive amino acid observed with glass vials. Cyclo[^{14}C]leucine was obtained from Amersham-Searle and New England Nuclear.

Data analysis

For steady state and time course of entry data a correction for the extracellular space was made as described fully in ref. 3. This correction takes into account the connective tissue space in the muscle (5 %) and amounts to about 12 % of the total muscle volume. A cell water content of 69 % was assumed in converting data to μmol/ml cell water.

The efflux curves presented here consist of an initial concave portion which flattens out to a straight line on a plot of log amino acid remaining in the cell vs time. The rate constant of the exponential portion of the curve was calculated from the equation:

$$\ln([AA]_{cw}^{t_1}/[AA]_{cw}^{t_2}) = -K_A(t_2 - t_1) \quad (2)$$

where K_A is the apparent efflux coefficient in min^{-1} and $[AA]_{cw}^{t_1}$ and $[AA]_{cw}^{t_2}$ are the concentrations of amino acid in the cell water at times t_1 and t_2, respectively. For 25 °C studies the curves were corrected for the contribution of a small slow fraction (see Results). For efflux experiments the muscles were not blotted; total extracellular space amounts to about 19 % in such muscles (see Table II) so that the cell water content is 61 % of the tissue weight.

RESULTS

Steady state cycloleucine accumulation at 0 °C and 25 °C

At external concentrations ranging between 0.1 and 10 mM about 4 h at 25 °C and 5 days at 0 °C were required to achieve a steady level which was maintained up to 22 h at 25 °C and to 9 days at 0 °C. The time to reach a steady level appeared to be the same at all external concentrations. It has been shown previously that this prolonged incubation at 0 °C has no deleterious effects on frog muscle [3, 5].

Fig. 2 shows the steady levels achieved at the two temperatures as a function of external concentration, $[S]_e$. For $[S]_e < 5$ mM significantly more cycloleucine is accumulated at 0 °C; at higher $[S]_e$, the converse is true. Both curves can be fit by Eqn (1) using the constants given in the legend.

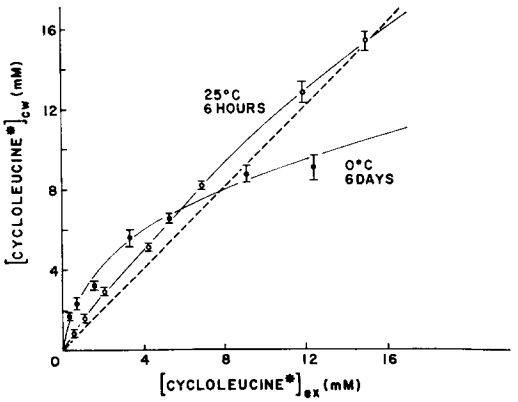

Fig. 2. Steady state cycloleucine accumulation at 0 °C and 25 °C by washed frog muscle. Points are the means of 6–8 muscles. Distance between bars represents 2 S.E. Lines theoretical according to Eqn (1), with $q = 0.27$, $[S]_{max} = 6.65$ mM and $K_m = 1.57$ mM at 0 °C and $q = 0.83$, $[S]_{max} = 4.25$ mM and $K_m = 5.26$ mM at 25 °C. Dotted line represents equal distribution between cell water and external solution.

TABLE I

VALUES OF STEADY STATE PARAMETERS

Average values for coefficients in Eqn 1 obtained from data on steady state accumulation of cycloleucine by frog muscle. Values were obtained by computer analysis of experiments similar to those depicted in Fig. 2 using the non-linear least squares analysis of Marquardt [18]. Values are expressed as mean±S.E. (number of experiments).

Parameter	Units	0 °C	25 °C
q		0.33±0.03 (4)	0.80±0.03 (5)
$[S]_{max}$	μmol/ml cell water	8.59±1.13 (4)	4.92±1.11 (5)
K_m	mM	2.00±0.34 (4)	5.00±1.5 (5)

Table I gives average values of q, $[S]_{max}$ and K_m obtained by nonlinear least squares analysis of several experiments similar to that depicted in Fig. 2. From these values it is possible to partition the intracellular amino acid into presumptive bound and free fractions. The values for $[S]_{max}$ and K_m suggest that the enhanced accumulation of cycloleucine at 0 °C could result from an increase in the presumptive bound fraction for a given external concentration.

At high concentrations the greater observed accumulation at 25 °C results from the much larger distribution coefficient, i.e. q is 0.8 at 25 °C and 0.33 at 0 °C. However, at both temperatures q is less than unity suggesting that cycloleucine is excluded from a portion of the cell water.

Time course of cycloleucine uptake

Ling [12, 13] has used a technique called "influx profile analysis" to identify diffusion-limited entry of solutes into cells. He has shown that a plot of the time course of solute entry as a function of the square root of time yields a sigmoid curve for membrane-limited influx (solid line, Fig. 3). Diffusion-limited influx yields a curve

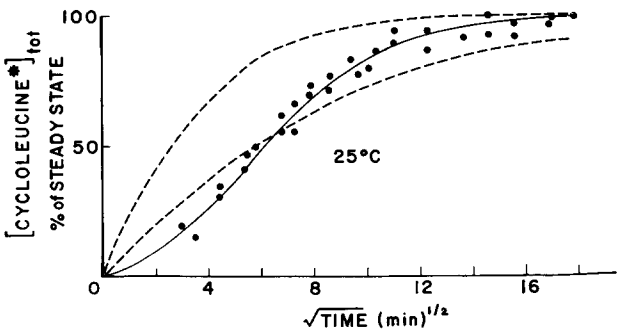

Fig. 3. Time course of cycloleucine entry into frog muscle at 25 °C plotted as a function of the square root of time. Points experimental (average of 4–6 muscles). Solid curve, theoretical surface-limited influx profile from the relation: percent remaining = $100\,(1-e^{-K_A t})$ with K_A equal to $0.9 \cdot 10^{-2}$ min^{-1}. Dotted curves, theoretical bulk-phase-limited influx profiles extrapolated from Ling [13] using half-times of uptake as 1.25 and 32 min, respectively, Initial external cycloleucine concentration, 5 mM.

which is concave downward (dashed lines, Fig. 3). If, in addition to membrane- or diffusion-limited flux there is a rate-limiting adsorption step, the curve will appear to be multicomponent. However, the initial portion of the curve will still be concave upward if the membrane is rate-limiting and downward if the process is diffusion-limited.

The time course of cycloleucine uptake in frog muscle at 25 °C is shown as a function of the square root of time in Fig. 3. The sigmoid character of the experimental curve is obvious. Two theoretical diffusion-limited curves are shown for comparison (dashed curves). These indicate that in no way can entry at 25 °C be construed as diffusion-limited.

The time course of entry at 0 °C (Fig. 4) is also sigmoid in shape. However, the entire curve fits neither a theoretical surface-limited profile (solid line) nor a diffusion-limited profile (dashed curves). It is possible to obtain an approximate fit at early times by assuming that a diffusion-limited fraction makes up 75 % of the total cellular

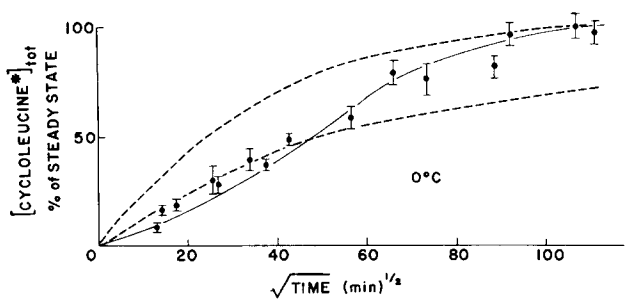

Fig. 4. Time course of cycloleucine entry into frog muscle at 0 °C plotted as a function of the square root of time. Points experimental (average of 4–6 muscles, distance betwenn horizontal bars is 2 S.E.). Solid curve, theoretical surface-limited influx profile from the equation given in legend to Fig. 3 with K_A equal to $2.5 \cdot 10^{-4}$ min^{-1}. Dotted curves, theoretical bulk-phase-limited profiles extrapolated from Ling [13] using half times of uptake as 625 and 900 min. For the lower curve an uptake into the bulk-phase-limited fraction of 75 % of the total cellular amino acid was assumed. Initial external cycloleucine concentration, 5 mM.

amino acid (lower dashed curve) and that a secondary process continues at later times. However, the half-time of uptake by the diffusive process would be 900 min.

In order to obtain diffusion-limited influx of cycloleucine into muscle fibers (radius about 30 μm) on this time scale [14] a diffusion coefficient of about 10^{-11} cm^2/s would be necessary. This reduction of 6 orders of magnitude from the diffusion coefficient of neutral amino acids in free solution (10^{-5} cm^2/s, [15]) is not compatible with the fact that most solutes diffuse within cells at a rate about one-half that in free solution [16, 17]. Diffusion-limited flux can therefore be ruled out at both 0 °C and 25 °C.

Time course of efflux

Fig. 5 is a typical curve for the loss of previously equilibrated cycloleucine from frog sartorius muscle at 25 °C. The curve flattens out gradually towards the end of the washout period. If a straight line (A) is drawn through the final portion of the curve

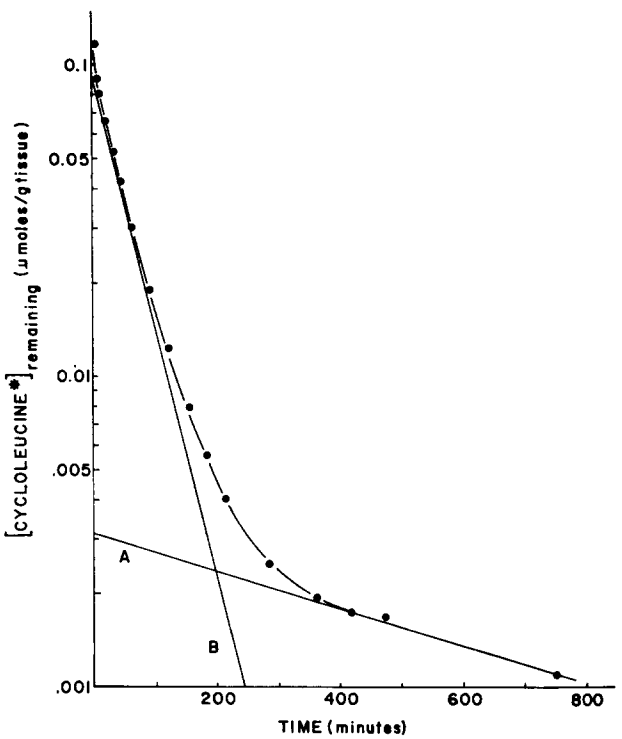

Fig. 5. Time course of cyclo[^{14}C]leucine efflux from a sartorius muscle at 25 °C. Muscle incubated 4 h with 0.1 ml cyclo[^{14}C]leucine at 25 °C. For washout, muscle was shaken in successive polyethylene scintillation vials containing 1 ml aliquots of Ringer solution with no amino acid for the time periods indicated. At the end of the experiment the muscle was extracted with 5 % trichloroacetic acid and the radioactivity in the extracts as well as in the washout solution determined by liquid scintillation counting. The curve was obtained by successively summing the counts in the washout solution at time t with all succeeding counts. Similar curves were obtained when the initial incubation solution contained 1 and 10 mM cycloleucine and in the presence or absence of up to 10 mM cycloleucine in the washout solution. For analysis, see text.

to the ordinate a slow fraction can be delineated. Subtraction of this slow fraction gives a second curve which becomes exponential after the first 20 min and encompasses the bulk of the accumulated amino acid. Extrapolation (B) of the straight portion of this curve to the ordinate leaves a small rapid fraction.

The slow fraction (curve A) amounted to less than 5 % of the total accumulated amino acid at all concentrations tested (0.1–10.0 mM). It may represent a small, tightly bound fraction of cycloleucine. The possibility that it represents a radiologic impurity is unlikely, since 99 % of the extractable radioactivity in these muscles after prolonged incubation can be chromatographically identified as cycloleucine. The muscle used for the experiment depicted in Fig. 5 was incubated with 0.1 mM cyclo-[^{14}C]leucine. At this external concentration we can calculate from Eqn (1) and the parameter values in Table I that 55 % of the cellular cycloleucine should be bound and 45 % free. Since the slow fraction is too small to represent either the presumptive bound or free fractions in the adsorption models under consideration, it will be disregarded in further discussion. This fraction was subtracted in order to obtain the rate constant of the intermediate fraction, B.

Fig. 6 shows the loss of cycloleucine from sartorius muscles at 0 °C following equilibration with varying concentrations of labelled amino acid. An initial rapid loss is followed by a very slow, apparently exponential release from the tissue. In the case of both 0 °C and 25 °C efflux we wish to know whether the exponential portion of the curve represents all the intracellular amino acid and whether the apparent rate constant, K_A (Eqn 2), varies with concentration.

If the exponential portion of the efflux curve does represent all the cellular amino acid the rapid fraction must arise entirely from the extracellular space. Table II shows the average percent of total tissue space occupied by the rapid fraction. To avoid damage, the muscles used in these experiments were neither closely trimmed nor blotted as in the steady state and entry experiments. Probably for this reason the space occupied by the fast fraction was larger than the 12 % value used for extracellular space in previous calculations and varied considerably. The data in Table II

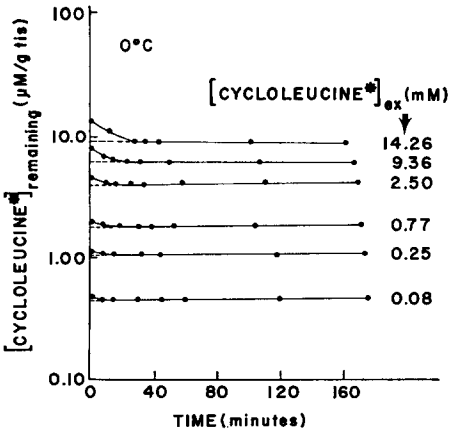

Fig. 6. Time course of cycloleucine efflux from frog muscle at 0 °C. Sartorius muscles equilibrated 6 days at 0 °C with cycloleucine and cyclo[^{14}C]leucine at the concentration given to the right of each curve prior to washing as described in legend to Fig. 5. Each line represents efflux from a single muscle.

TABLE II

TISSUE SPACE OCCUPIED BY RAPID FRACTION

Average percent of total tissue space occupied by rapid fraction obtained from the expression:

$$\text{Tissue Space} = \frac{[S]_{tis} - [S]_{int}}{[S]_{ex}} \cdot 100$$

where $[S]_{tis}$, $[S]_{int}$ are the initial total tissue concentration of cycloleucine and the ordinate intercept of the exponential fraction, respectively. Values expressed as mean for the concentration range ± S.E. (number of experiments). Both efflux and exchange experiments (Neville, M. C., unpublished) are included in this data accounting for the larger number of experiments reported here than in Table III. To obtain RISA space sartorius muscles were incubated 10 h at 25 °C or 29 h at 0 °C with [^{125}I]-Albumotope (^{125}I-labelled human serum albumen, Squibb). They were then drained on filter paper or trimmed and blotted hard followed by weighing and counting in the well of a Packard Tri-carb γ-counter. Results were obtained from the expression:

$$\text{Percent RISA space} = \frac{\text{cpm in muscle}}{\text{Muscle weight} \times \text{cpm/ml solution}} \cdot 100.$$

$[\text{Cycloleucine}]_{ex}$ range (mM)	25 °C Tissue space (%)	0 °C Tissue space (%)
0.08–0.10	25.3±3.3 (5)	25.1±5.0 (8)
0.105–0.50	17.6±1.5 (4)	22.6±2.6 (10)
0.51–1.00	17.7±1.9 (8)	23.8±1.8 (24)
1.05–5.0	14.7±3.4 (5)	21.1±2.4 (7)
5.1–10.0	18.6±1.9 (5)	16.7±1.4 (15)
10.5–50.0	13.5±1.2 (4)	14.0±3.0 (2)
51.0–100.0	23.0±7.7 (4)	20.8±5.5 (6)
Average	18.3±1.4 (35)	21.6±1.0 (72)
RISA space		
Blotted muscles	11.5±1.0 (4)	12.8±1.5 (4)
Unblotted muscles	19.5±1.3 (4)	18.3±0.7 (4)

show, however, that it is comparable to the radio-iodine serum albumin (RISA) space obtained from similar unblotted, untrimmed muscles. Therefore the rapid fraction represents extracellular amino acid and the slower, apparently exponential fraction must represent all intracellular amino acid.

Theoretically it should be possible to determine whether the apparent rate constant, K_A, varies with concentration by examining the entire efflux curve as a relatively high initial cellular concentration of amino acid falls to zero. In practice, this is not possible in this tissue. At 25 °C the small, slow fraction interferes with analysis of the terminal portion of the curve. Efflux at 0 °C is so slow that nearly a month would be required for all amino acid to leave the cell. Since we have not been able to preserve muscles at 0 °C much longer than 12 days this approach is again not practical. For this reason efflux curves containing various initial cycloleucine concentrations were analyzed.

The apparent efflux rate constants, K_A, calculated from experiments similar to Figs 4 and 5, are plotted as a function of the cellular concentration of cycloleucine in Fig. 7. Within the limits of the variance of the data the observed rate coefficients do not vary with concentration. Therefore, all the cellular amino acid leaves the cell with

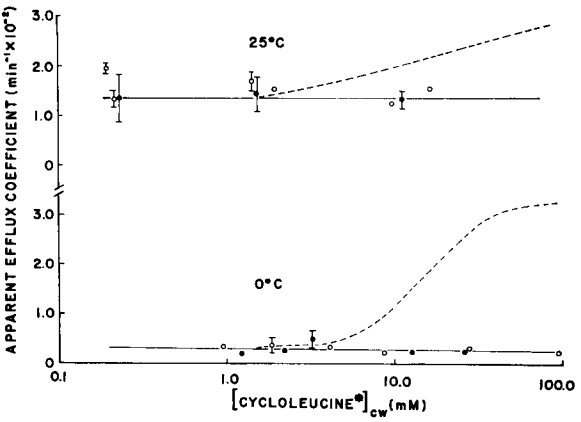

Fig. 7. Apparent rate constants for loss of cycloleucine from frog muscle at 25 °C and 0 °C. Points represent means of apparent rate coefficients calculated as described in Methods for four muscles. Distances between bars is 2 S.E. Where bars are not shown the S.E. is smaller than the diameter of the symbol: (●) muscles loaded with cycloleucine at 0 °C, (○), muscles loaded at 25 °C. Dashed lines represent expected apparent efflux coefficient calculated (see Appendix) for surface-limited efflux of bound and free amino with binding sites located in the cytoplasm, using values of steady state parameters given in Table I.

a single rate constant which is not affected by concentration within the ranges examined. Similar results were obtained by Heinz for ascites cells [10].

DISCUSSION

Apparent exclusion of cycloleucine from a portion of the cell water

Amino acid exclusion has not often been reported in biological systems. Parrish and Kipnis [19] found a steady state distribution coefficient of less than unity for uptake of α-aminoisobutyric acid by rat diaphragm incubated in Na-free Ringer solution. In the preceding paper of this series [3] the distribution coefficient for glycine in frog muscle water at 0 °C was shown to be very much less than unity.

The possible mechanisms for the apparent exclusion include:

(i) An outwardly-directed active transport mechanism for removing excess amino acid from the cell. While not ruled out by the present data, this mechanism would require that active transport systems work in opposition to each other in this tissue. From an energetic standpoint this would seem inefficient.

(ii) Organization of the cell water in such a way that its solvent properties for amino acid are decreased relative to the extracellular medium. Ling [1, 2] has postulated an entropic mechanism for such exclusion of solutes from the cell water. On this basis one would expect a decrease in the distribution coefficient with an increase in temperature [20] rather than the observed increase.

(iii) A compartment or compartments of the cell water inaccessible to the amino acid in question. The possibility that the observed exclusion arises from intracellular compartmentation is currently under active investigation in this laboratory.

Are cycloleucine fluxes compatible with amino acid adsorption?

The rate-limiting process for the movement of amino acid into and out of cells must be specified when adsorption models are considered. The possible rate-limiting steps are diffusion through the cell water, desorption from binding sites and passage through the cell membrane. We have ruled out diffusion-limited influx on the basis of "influx profile analysis" in the results section. Although it can be shown that the observed pattern of efflux is not compatible with diffusion-limited efflux (unpublished), this analysis would be redundant here, since it is difficult to imagine a cell in which influx would be diffusion-limited and efflux, at least of the free amino acid, would not be. Therefore, diffusion-limited cycloleucine flux can be ruled out in frog muscle. The question we must now ask is whether the pattern of amino acid loss from the cells is compatible with either a desorption or a membrane-limited process.

Desorption-limited efflux

If desorption is the rate-limiting step, free amino acid should leave the cell more rapidly than bound. If the free fraction leaves very rapidly it would mingle with the extracellular fraction increasing the size of the "fast" fraction. In this case the rapid fraction would occupy a space larger than the RISA space. As Table II shows, this is not the case.

If free amino acid left the cell somewhat more slowly it should be possible to identify three efflux fractions. While this is the case with cycloleucine loss at 25 °C, the second fraction is much too large to represent free cellular amino acid and the very slow fraction much too small to represent the presumptive bound fraction.

It might be argued, in the case of efflux at 0 °C, that loss from the cell is so slow that the adsorbed fraction is never revealed. However, in this case we would be considering for all practical purposes, a membrane-limited process. Therefore the data are inconsistent with desorption-limited efflux.

Membrane-limited flux

Here we must consider the location of the adsorption sites. If the sites are located within the cytoplasm, the pathway must be from adsorbed to free and thence through the membrane (Fig. 1A). In this case the efflux rate constant, k', should be proportional to the concentration of the free fraction, i.e.,

$$J_{out} = -k'[S]_{fr} \tag{3}$$

where J_{out} is the unidirectional efflux and $[S]_{fr}$ is the concentration of free amino acid within the cell. Because the adsorption sites saturate at a finite solute concentration, the free amino acid would not be a constant fraction of the total cellular amino acid and k' would not be proportional to K_A. That is to say, K_A, the observed rate constant, will vary with solute concentration in a manner dependent on the number of adsorption sites and their dissociation constant. A quantitative prediction of K_A based on an iterative computer method (Appendix) shows that K_A should increase with $[S]_{cw}$ at concentrations where $[S]_{cw} > K_m \cdot q$.

Fig. 7 shows the apparent efflux coefficients, K_A, calculated from all efflux curves at 0 °C and 25 °C as a function of concentration. The dotted line shows the variation in the apparent rate constant expected for surface-limited flux of bound

and free fractions, calculated as described in the Appendix. In the higher concentration ranges an increase in K_A is expected but not observed.

If the adsorption sites are located on the surface of the cell (Fig. 1B), the pattern of loss of amino acid from the cell would depend on the rate constants of the two pathways by which amino acid leaves the cell; e.g. desorption from surface sites and migration of free amino acid through the membrane. The following cases are possible, defining k'_d as the desorption rate constant and k'_c as the rate constant for migration through the membrane:

(i) $k'_c > k'_d$. This case is essentially desorption-limited efflux, and has been ruled out above.

(ii) $k'_c < k'_d$, Step a' (Fig. 1B) prohibited or slow. Adsorbed amino acid would leave the cell prior to free amino acid. At 25 °C there would be three components in the curve and the final exponential portion of the curve would intercept the ordinate at a value equal to the free cellular amino acid. At 0 °C K_A would vary with concentration as the proportion between free and bound fractions varied. Neither possibility is consistent with the data.

(iii) $k'_c < k'_d$, Step a' rapid. If equilibration between adsorption sites and intracellular amino acid is rapid the rate of efflux would be proportional to the concentration of adsorbed amino acid. A computer model for this case is also given in the Appendix. It shows that the apparent rate constant of efflux decreases with increasing cellular concentration of solute at cellular concentrations of free amino acid greater than $K_m \cdot q$. The dashed line in Fig. 8 shows the expected apparent efflux coefficient

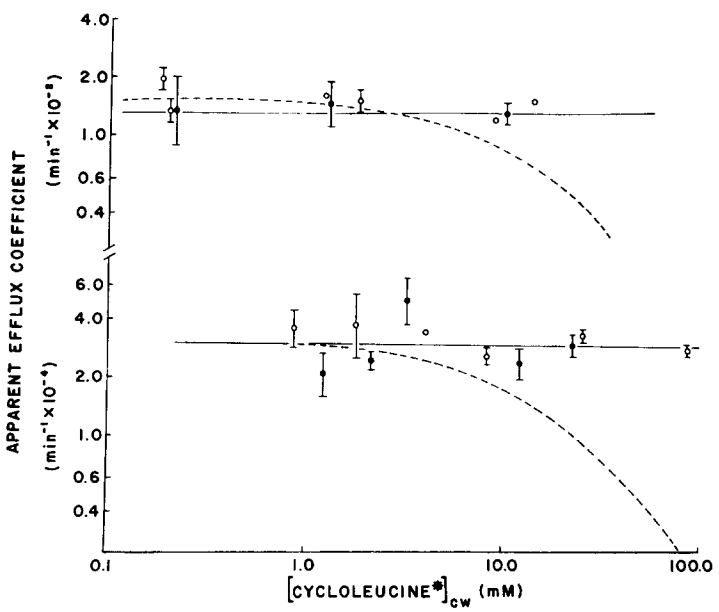

Fig. 8. Apparent rate constants for loss of cycloleucine from frog muscle at 25 °C and 0 °C. Data the same as Fig. 7. Dashed line represents expected apparent coefficient calculated (see Appendix) for surface-limited efflux of bound and free amino acid with binding sites located on the cell membrane using values of steady state parameters given in Table I. Free amino acid must associate with the sites in order to leave the cell.

for this case. There is a significant difference between the predicted and the observed efflux coefficients at higher concentrations.

(iv) $k'_c \approx k'_d$. In this case all cellular solute would leave the cell with a single rate constant. The ordinate intercept would be equal to the total cellular concentration of solute and the rate constant would not vary with cellular solute concentration. This variant of the model is consistent with experimental data.

The above analysis infers that the only variation of an adsorption model which is consistent with the data is one in which (i) the adsorption sites are located on membranes with direct access to the extracellular space and (ii) the rate constant for desorption from these sites is equal to the rate constant for passage of free amino acid across the cell membrane. It would seem to be an unlikely coincidence that the rate constants for desorption and diffusion through the membrane would be equal at both 0 °C and 25 °C. Therefore the fact that all intracellular amino acid leaves the cell with a single rate constant at both 0 °C and 25 °C suggests that accumulation of amino acid is not accomplished by adsorption to specific sites but rather by active transport systems located in the cell membrane.

APPENDIX

COMPUTER SIMULATION OF EFFLUX FROM A SYSTEM CONTAINING BOUND AND FREE SOLUTE

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We consider a cell, bound by a cell membrane, containing F μmol of sites per ml of cell water which reversibly absorb solute, S. We will consider surface-limited efflux in two situations; the adsorption sites are located within the cytoplasm (Model A, Fig. 1) and the adsorption sites are located on cell membranes with direct access to the extracellular space (Model B, Fig. 1).

A. Cytoplasmic adsorption sites

The rate of efflux is given by text Eqn 3 repeated here for convenience:

$$\frac{d[S]_{tot}}{dt} = -k'[S]_{fr} \tag{A1}$$

where k' is the rate constant for passage of solute through the membrane. The total concentration of S, $[S]_{tot}$, in the cell at any given time, t, is the sum of the free, $[S]_{fr}$, and bound, $[S]_{ads}$ fractions:

$$[S]_{tot} = [S]_{fr} + [S]_{ads} = [S]_{fr} + \frac{F}{1 + K_d/[S]_{fr}} \tag{A2}$$

where K_d is the equilibrium constant for the reaction of free cellular solute with the

sites, F. $[S]_{fr} = q[S]_{ex}$ and $K_d = K_m \cdot q$ (see text Eqn 1). We assume that reaction equilibrium is attained rapidly so that for all times during an efflux experiment Eqn A2 is obeyed. We wish to know how the apparent rate of efflux, K_A, varies with the total concentration of solute, $[S]_{tot}$.

We have defined a parameter, K_A, the apparent rate constant for efflux of S from the cell as (see text Eqn 2):

$$K_A = \frac{\ln([S]^1/[S]^2)}{t_2 - t_1} \tag{A3}$$

where $[S]^1$ and $[S]^2$ are the total concentrations of S in the cell at times t_1 and t_2, respectively. Qualitatively it is clear from Eqns A2 and A3 that when $[S]_{ads} \ll [S]_{fr}$, $K_A \approx k'$. This will be true at very high cellular solute concentrations. At the other extreme when $[S]_{fr} \ll K_d$:

$$S_{tot} \approx [S]_{fr}(1 + F/K_d) \tag{A4}$$

and $[S]_{fr}$ is a constant fraction of $[S]_{tot}$. At this point K_A approaches a minimum and constant value.

We were unable to find a closed-form solution for the quantitative dependence of K_A on $[S]_{tot}$. However, numerical integration using an analog simulation language, MIMIC, on the CDC 6400 computer located in the computing center of the University of Colorado allows quantitative prediction of this dependence.
Differentiating Eqn A2:

$$\frac{d[S]_{tot}}{dt} = \frac{d[S]_{fr}}{dt} + \frac{F}{[S]_{fr} + K_d} \cdot \frac{d[S]_{fr}}{dt} - \frac{F \cdot [S]_{fr}}{([S]_{fr} + K_d)^2} \frac{d[S]_{fr}}{dt} \tag{A5}$$

Combining Eqn A5 with Eqn A1 and simplifying:

$$\frac{d[S]_{fr}}{dt} = \frac{k'[S]_{fr}([S]_{fr} + K_d)^2}{([S]_{fr} + K_d)^2 + F \cdot K_d} \tag{A6}$$

Eqns A1 and A6 are directly amenable to simultaneous numerical integration to obtain $[S]_{fr}$ and $[S]_{tot}$ as a function of time. K_A can then be estimated for any value of $[S]_{tot}$ from Eqn A3 using any convenient time interval.

An example of the results obtained by this method is shown in Fig. A1 in which the ratio of K_A/k' is shown as a function of $[S]_{tot}$ for various values of K_d and F. All curves reach a minimum at low values of $[S]_{tot}$. As $[S]_{tot}$ increases into the range where $[S]_{fr}$ and $[S]_{ads}$ assume similar orders of magnitude, the ratio K_A/k' increases toward the maximum, 1. The lower set of curves shows the dependence of this ratio on K_d at constant F. The upper set of curves shows the effect of varying the number of sites with K_d constant. As F increases the minimum decreases and K_A values near the minimum are maintained to higher values of $[S]_{tot}$. This suggests that, in systems where the majority of solute is bound, the efflux coefficient will be independent of cellular solute concentration regardless of whether the process is desorption of membrane-limited.

Relaxation of the assumption that equilibrium is maintained amounts to desorption-limited efflux in the extreme. In less extreme cases this would result in a

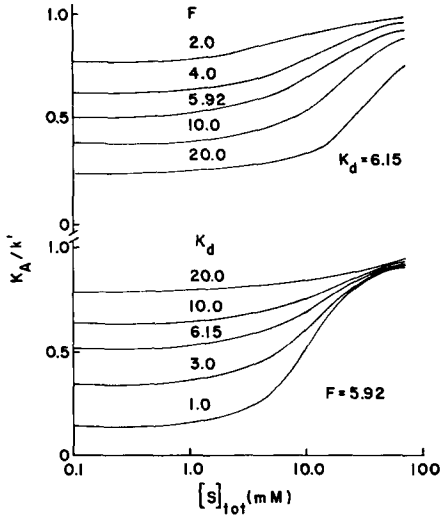

Fig. A1. Theoretical ratio of apparent rate constant K_A to true rate constant k' for surface limited efflux of adsorbed and free solutes. Adsorption sites located in the cytoplasm. Numerical integration of Eqns A1 and A8 performed as described in the text using the constants indicated and a time interval for evaluation of K_A of 10 min.

depression of K_A in the region where the bound fraction is a significant proportion of the total, i.e. at low solute concentrations.

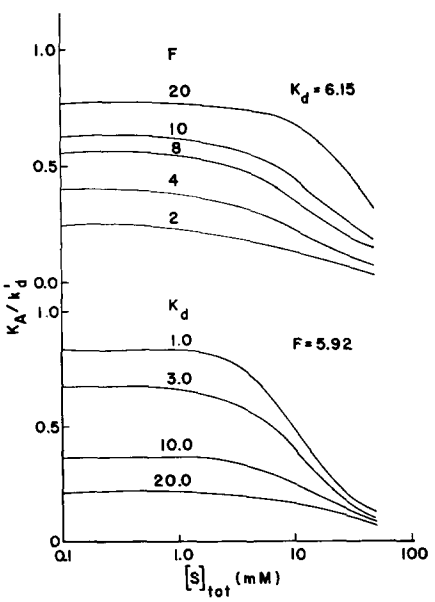

Fig. A2. Theoretical ratio of apparent rate constant, K_A, to true rate constant, k', for surface limited efflux of adsorbed and free solutes. Adsorption sites located on the cell membrane. Free solutes gains access to the extracellular space only by adsorbing to and desorbing from these sites. Numerical integration of Eqns A9 and A10 performed as described in the text using the constants indicated and a time interval for evaluation of K_A of 10 min.

B. Adsorption sites located on the cell membrane

In the case where adsorption sites are located on the cell membrane and provide the major route of efflux of free solute from the cell, the rate of efflux may be given by:

$$\frac{d[S]_{tot}}{dt} = -k'_d[S]_{ads} \tag{A7}$$

Combining Eqn A5, which is also valid for this case, with Eqn A7 and rearranging,

$$\frac{d[S]_{fr}}{dt} = \frac{k'_d[S]_{ads}([S]_{fr}+K_d)^2}{([S]_{fr}+K_d)^2+FK_d} \tag{A8}$$

Eqns A7 and A8 were solved simultaneously using the MIMIC method of numerical integration to obtain the results shown in Fig. A2. These results indicate that $K_A \simeq k'_d$ at small $[S]_{tot}$ only if F is large and k'_d small. As $[S]_{tot}$ increases K_A decreases. This situation is analogous to the saturation of carrier-mediated transport seen at high solute concentrations.

If the assumption of equilibrium is relaxed in this case, the adsorption sites would show a lower level of saturation, than expected at a given $[S]_{fr}$. This assumption amounts to an increase in K_d and would tend to push the apparent K_A to a lower line as solute concentration decreases.

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