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AMINO ACID ACCUMULATION IN FROG MUSCLE

I. STEADY-STATE GLYCINE ACCUMULATION AT 0 °C

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SUMMARY

Glycine is not significantly metabolized by frog muscle maintained at 0 °C *in vitro*. Nevertheless, in this preparation steady-state levels of [^{14}C]glycine as high as 20 times the external concentration are attained after 3–6 days at 0 °C. The concentration gradient at the steady state depends on the external concentration, being highest at low external concentrations (approx. 0.1 mM) and reversed at external concentrations above 10 mM.

A plot of the steady-state cellular levels of glycine *vs* the external concentration reveals linear and saturable components. The linear fraction has an average distribution ratio of 0.54 indicating that glycine is partially excluded from the muscle water at this temperature.

Efflux of labeled glycine at 0 °C from previously loaded frog muscle follows first-order kinetics. The rate constant increases with increasing concentrations of glycine in the external medium (efflux facilitation).

The steady-state results are shown to be consistent with an adsorption model for amino acid accumulation as well as a model in which amino acid enters the cell *via* a carrier and exits *via* a bidirectional leakage pathway. A model in which efflux proceeds through the carrier does not fit the data. This indicates that an alternative to exchange diffusion is needed to explain the observed efflux facilitation.

INTRODUCTION

Early investigators of amino acid accumulation by cells postulated that protoplasmic binding was the basis of the large concentration gradients established between the cell and the external medium[1–6]. After some controversy most workers in the field came to favor a carrier-mediated active transport mechanism for reasons summarized by Christensen[7], Heinz[8,9], Cohen and Rickenberg[10], and others[11–14]. However, as discussed in detail elsewhere[15], none of these arguments conclusively rules out substantial amino acid adsorption to specific protoplasmic sites. For this reason a total reexamination of the problem is necessary at this time.

An adsorption hypothesis for solute accumulation by cells has been advanced

by Ling[16,17]. The predictions of this hypothesis for equilibrium accumulation are straightforward: solutes are postulated to exist in two states in the cell, adsorbed to membrane and/or cytoplasmic sites and free in the cell water. The size of the adsorbed fraction depends on the number of sites and their affinity for the solute under study. The size of the free fraction depends on the average solubility of the solute in the cell water which Ling[16,17] and others[18–21] have postulated to be organized in such a way that a substantial degree of solute exclusion exists.

The amount of amino acid present in the cell at the steady state in terms of the carrier hypothesis is postulated to depend on a balance between the rate of active entry and the rate of passive efflux[22,23]. Although previous workers have tended to focus on these rate processes themselves, some results have been obtained for the steady state[7,23–28], and equations are available for analysis of steady-state accumulation based on several models[22,23,29].

A logical place to begin a reexamination of the problem of adsorption *vs* active transport in the accumulation of amino acids by cells is with an examination of the steady state. Frog muscle is particularly suitable for this work. It is inexpensive and can be maintained for prolonged periods of time *in vitro*[15,30]. The metabolizable amino acid glycine is accumulated against a concentration gradient at 0 °C, a temperature at which its metabolic utilization is almost completely inhibited.

The term "steady state" is defined operationally here as the point at which influx and efflux are equal and cellular concentrations do not change with time. No prejudgment as to whether this point represents an equilibrium state or a balance of active and passive fluxes is intended by the use of this term. Portions of this work have been presented previously[15,30,31].

MATERIALS AND METHODS

Incubation procedure

The technique was modified from Ling *et al.*[32]. Four small leg muscles, the *sartorius*, *semitendinosus*, *iliofibularis* and *tibialis anticus longus* were dissected intact from healthy *Rana pipiens* and freed from the main surrounding connective tissue. The muscles were then washed in a large volume of Ringer phosphate solution for 6–8 h at 25 °C. This procedure reduces hormone pools[32], establishing a base-line condition for the tissue.

Following the washing procedure the muscles were drained and placed in prechilled vials (4–8 muscles per vial) with 2–3 ml of Ringer solution containing a measured amount of nonlabeled and ^{14}C-labeled glycine. For incubation the vials were wrapped in parafilm and immersed in a water bath at 0 ± 0.1 °C with their long axes in the direction of motion of a reciprocating shaker (100 excursions per min, amplitude 1 inch). After incubation the muscles were quickly blotted, trimmed of excess tissue and tendons and frozen in liquid nitrogen, then weighed and extracted individually with 2 ml of 5% trichloroacetic acid at 2–4 °C overnight. The balance experiment described below indicates that this procedure, without homogenization of the muscles, is sufficient to extract all the free amino acid. Aliquots (0.5 ml) of the extract were added to 5 ml of Bray's[33] scintillation fluid and counted in a liquid scintillation counter. The specific activity of the labeled amino acid and final external amino acid content of the external solution were determined by counting aliquots of

the incubation solution removed before addition of the muscles and at the end of the experiment, respectively.

The tissue level of labeled glycine, $[Gly^*]_{tis}$ in μmoles/g was calculated from the formula:

$$[Gly^*]_{tis} = \frac{(cpm)_s \cdot (c.f.) \cdot \frac{(2 + 0.8W)}{0.5} \cdot \frac{1}{SA}}{W} \quad (1)$$

where $(cpm)_s$ is the number of cpm in the aliquot; c.f. is the quench correction factor, determined by the channels ratio method; $2 + 0.8\ W$ is the total volume of extraction fluid in ml where W is the weight of the muscles in g and $0.8\ W$ is the water content of the muscle[30]; 0.5 is the size of the aliquot in ml, and SA is the specific activity of the amino acid in cpm/μmole determined as stated above. Labeled glycine includes, in the context of this paper, all exogenously added glycine, labeled and nonlabeled. It does not include endogenous glycine remaining in the tissue after washing. All calculations were carried out on a CDC 6400 computer (copies of program are available from the author).

The formulas given in Appendix I were used to correct for amino acid in the extracellular space. Identical determinations were performed on 4–8 muscles to provide a basis for statistical evaluation of the results.

Thin-layer chromatography of glycine

A 5% trichloroacetic acid extract of a muscle previously incubated with radioactive amino acid was neutralized with conc. NH_4OH and evaporated at room temperature to a suitable volume. The reduced extract was spotted on a cellulose thin layer (Brinkman polygram cell MN 200) and run in two dimensions with the following solvents[34]: Phase I, *n*-butanol–acetone–diethylamine–water (10:10:2:5, by vol.); Phase II, isopropanol–formic acid–water (40:2:10, by vol.). After drying, the chromatogram was placed face down on a sheet of X-ray film and exposed for 2–6 weeks. After development the position of the spots was compared with those of standard compounds chromatographed in an identical fashion and developed with ninhydrin reagent.

Efflux studies

Sartorius muscles were incubated with labeled glycine at 0 °C for 5 days. After blotting and trimming the muscles were weighed and washed in successive 1-ml aliquots of Ringer solution containing no labeled compound. The radioactivity leaving the muscle was assessed by counting the aliquots with Bray's[33] solution in a liquid scintillation counter. At the end of the experiment the radioactivity remaining in the muscle was extracted with 5% trichloroacetic acid and counted as above. The efflux curve was constructed by successively summing the activity in the vials with that remaining in the muscle. Time intervals were chosen so that backflux of labeled compounds would be negligible.

Materials

All chemicals used met ACS standards or better. Labeled glycine was obtained from both Amersham Searle and New England Nuclear. Frogs were obtained from

Steinhilber and Co., Oshkosh, Wisc. They were maintained in running tap water (60 °F) and force-fed liver three times per week. The composition of the Ringer solution was: NaCl, 104.7 mM; KCl, 2.5 mM; $NaHCO_3$, 6.6 mM; NaH_2PO_4, 2.0 mM; Na_2HPO_4, 1.2 mM; $CaCl_2$, 1.0 mM; $MgSO_4$, 1.2 mM. Up to 10 mM glycine could be added to this solution without producing significant changes in the volume of the muscles. When higher concentrations of glycine were used the NaCl concentration was reduced by an amount equal to 1/6 of the glycine added in order to maintain the normal water content of the tissue.

RESULTS

Time course of glycine entry at 0 °C

Fig. 1 shows the time course of glycine entry into washed frog muscles from solutions containing initial external glycine concentrations varying from 0.13 mM to 10 mM. The tissue content of labeled glycine levels off after 3–6 days and remains constant through the 9th day (216 h). Previous studies[30] have shown that the Na^+, K^+ and water contents of frog muscles do not change during incubation in Ringer phosphate at 0 °C for 8 days indicating that the muscles remain in normal condition. After the 9th day the K^+ falls slowly and the Na^+ rises. This change is often accompanied by a small secondary rise in glycine uptake.

The experiment depicted in Fig. 2 demonstrates that a true steady state is achieved after 120 h of incubation. 30 muscles were incubated at 0 °C for 24 h with Ringer solution containing 100 mM glycine at which time they contained 21.6 μmoles/g cellular glycine. They were then placed in a small volume of Ringer solution containing no glycine and further incubated at 0 °C for varying periods of time. Fig. 2 indicates that accumulated glycine leaves the cell establishing a new steady-

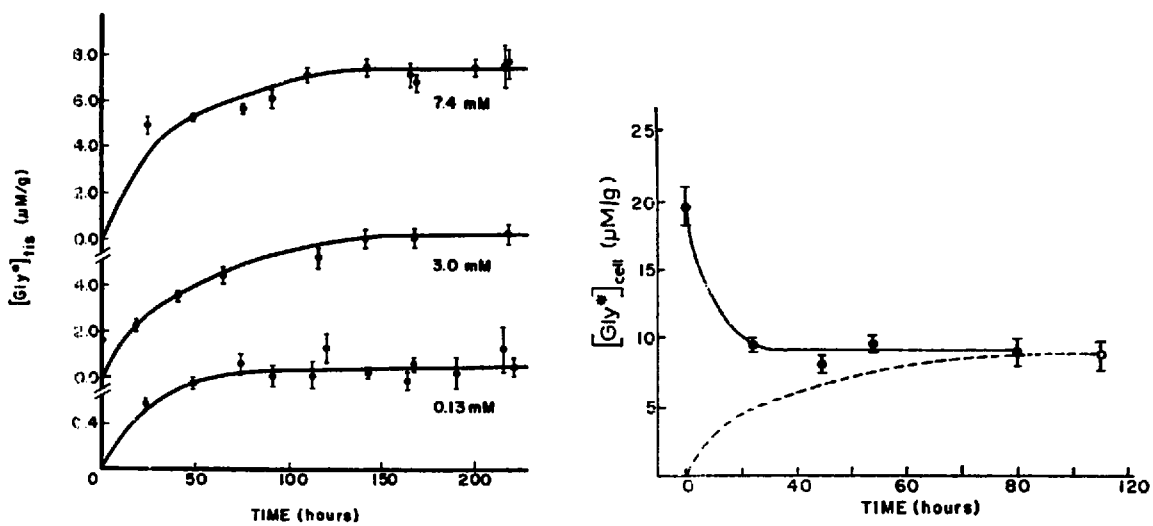

Fig. 1. Time course of glycine entry into frog muscles at 0 °C. Washed muscles incubated with labeled glycine for time indicated and analyzed as in the text. Number below each curve is the final external glycine concentration. Each point shows the mean and S.E. for 6–8 muscles.

Fig. 2. Reestablishment of steady state after incubation with high concentration of glycine. ●, Washed muscles incubated 24 h at 0 °C with 100 mM labeled glycine, then placed in a small volume of Ringer solution (6 muscles in 2.5 ml) for the time indicated. Muscle and solution glycine concentrations determined as in the text. Final external glycine concentration, 12.4 mM. O—O, Muscles incubated as described in Fig. 1. Final external glycine concentration 12.5 mM.

state level not significantly different from that achieved by muscles incubated with the same external glycine concentration (dotted line).

Steady-state distribution ratio of labeled glycine at o °C

The amount of labeled glycine accumulated by frog muscles during 6 days of incubation at o °C in solutions of varying external labeled glycine concentration was measured (Table I). The ratio of labeled glycine concentration in the cell water to the final external concentration is highest at low concentrations. Low temperature (o °C) does not prevent the establishment of large glycine concentration gradients.

TABLE I

6-DAY GLYCINE ACCUMULATION AT o °C

Muscles washed 6 h at 25 °C in Ringer phosphate prior to incubation at o °C for 6 days with varying concentrations of labeled glycine. 8 muscles per concentration. Values shown as mean ± S.E. Tissue levels are converted to glycine accumulated in the cell water by correcting for extracellular glycine and tissue water content as described in Appendix I.

Incubation solution [*Glycine**]		*Muscles* [*Glycine**]		*Distribution ratio* ($[Gly^*]_{cell\ water}/[Gly^*]_{ex}$)
Initial (*mM*)	*Final* (*mM*)	*Tissue* (*μmoles/g*)	*Cell water* (*mM*)	
0.10	0.03	0.45 ± 0.03	0.63 ± 0.04	19.9 ± 1.3
0.50	0.25	1.70 ± 0.08	2.38 ± 0.14	9.5 ± 0.4
1.0	0.45	2.14 ± 0.32	2.98 ± 0.46	6.6 ± 0.2
2.0	1.32	4.03 ± 0.36	5.54 ± 0.62	4.2 ± 0.4
5.0	3.97	4.97 ± 0.44	6.42 ± 0.63	1.6 ± 0.2
10.0	8.36	7.89 ± 0.37	9.95 ± 0.52	1.2 ± 0.1

TABLE II

R_F VALUES FROM AMINO ACID CHROMATOGRAPHY

Labeled amino acids extracted from previously loaded muscle and chromatographed as described in text. Phase I, *n*-butanol–acetone–diethylamine–water (10:10:2:5, by vol.); Phase II, isopropanol–formic acid–water (40:2:10, by vol.).

Amino acid	*Phase I*	*Phase II*
A. Control values		
Cysteine	0.28	0.05
Lysine	0.44	0.16
Serine	0.53	0.32
Glycine	0.35	0.33
Glutamate	0.20	0.46
Alanine	0.44	0.52
Isoleucine	0.63	0.69
B. Radiochromatograph		
Spot I	0.35	0.33
Spot II	0.52	0.31

Recovery of labeled glycine following prolonged incubation at 0 °C

Although a small amount of glycine is metabolized or incorporated into protein during the 0 °C incubation the following data show that this quantity is insignificant in comparison to the amount of free glycine accumulated within the cell: Part A of Table II gives R_F values obtained for a representative sample of amino acids by thin-layer chromatography on Eastman polygram cellulose MN 200. Part B gives R_F values obtained from a radiochromatogram of an extract of a muscle which had been incubated for 6 days with 1 mM glycine and 1.0 μCi/ml [^{14}C]glycine. The major portion of the radioactivity was concentrated in Spot I whose R_F values correspond to glycine. A second spot (II) had R_F values corresponding to serine. These spots were cut out of the original chromatogram and their radioactivity determined. Spot I contained 3140 counts per 500 min and Spot II, 87 counts per 500 min indicating that not less than 97%, of the radioactivity in the trichloroacetic acid extract is glycine (counts above background).

Table III shows the balance sheet of recovery of radioactivity from muscles incubated for 8 days at 0 °C with [^{14}C]glycine and 1 mM glycine. The amount of radioactivity recovered from the trichloroacetic acid extracts of the muscles corresponded within experimental error to the amount of radioactivity lost from the supernatant solution. This, along with the results of thin-layer chromatography, indicates the following: (i) trichloroacetic acid (5%) extraction without homogenization is sufficient to recover the glycine taken up by the muscle; (ii) There is no significant incorporation of glycine into protein during the experimental period (preliminary experiments indicate that 0.1–1.0% of the glycine is incorporated during this time); (iii) The technique allows the study of glycine accumulation in the absence of significant metabolic utilization of this amino acid.

The above data *plus* the fact that the solutions remained clear during the prolonged incubation indicate the lack of significant bacterial contamination of the muscles.

TABLE III

BALANCE SHEET OF LABELED GLYCINE RECOVERY AFTER INCUBATION FOR 6 DAYS AT 0 °C

Radioactivity in trichloroacetic acid extract of muscles (Column 2) compared to radioactivity lost from bathing solution (Column 3).

Sample number	*Radioactivity in muscles (cpm)*	*Radioactivity lost from bathing solution (cpm)*	*Difference (cpm)*	$\frac{\text{Muscle cpm}}{\text{Solution cpm}} \times 100$ (%)
1	$1881 \cdot 10^3$	$1851 \cdot 10^3$	$30 \cdot 10^3$	101.6
2	$1927 \cdot 10^3$	$2022 \cdot 10^3$	$-95 \cdot 10^3$	95.3
3	$2005 \cdot 10^3$	$1978 \cdot 10^3$	$26 \cdot 10^3$	101.3
4	$2057 \cdot 10^3$	$2046 \cdot 10^3$	$11 \cdot 10^3$	100.5
Average				99.7 ± 1.37

Glycine uptake by connective tissue elements

The 6-day uptake of glycine at 0 °C by small pieces of fascia dissected from around the leg muscles used in this study was determined (Fig. 3). At low external

concentrations (<3.0 mM) the distribution ratio of labeled glycine between these connective tissue elements and the external solution was 0.91; at higher concentrations this ratio fell, reaching a value of about 0.6 at 8 mM external glycine. These results were used to correct the data for glycine taken up by connective tissue elements within the muscles (Appendix I). They indicate that glycine accumulation at 0 °C is not the result of binding to connective tissue.

Analysis of steady-state accumulation at 0 °C

Fig. 4 shows the steady-state accumulation of labeled glycine in frog muscle as a function of the final external glycine concentration. The curve rises sharply at low concentrations bending off to become linear at concentrations greater than about 4 mM. This curve can be fit by an empirical equation of the form:

$$[S]_c = C_1[S]_e + \frac{C_2}{1 + \frac{C_3}{[S]_e}} \qquad (2)$$

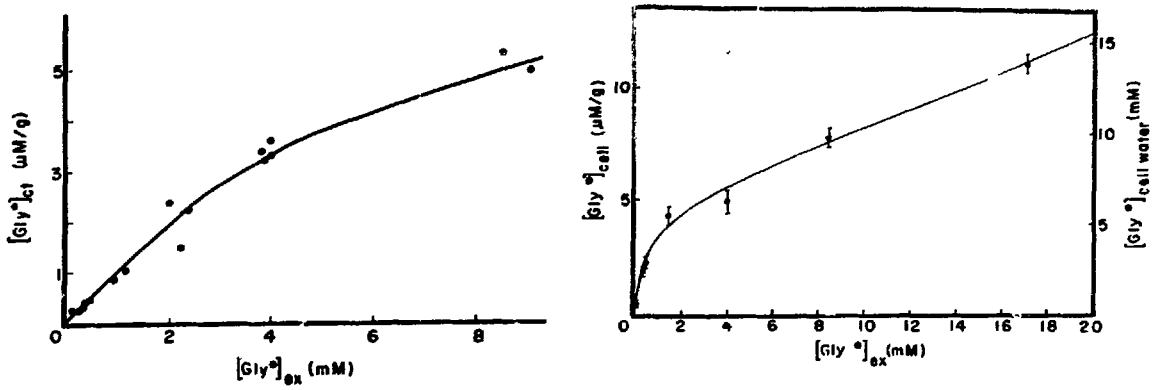

Fig. 3. Glycine uptake by connective tissue. *Fascia* from legs of *Rana pipiens* incubated 6 days at 0 °C with varying concentrations of labeled glycine.

Fig. 4. Steady-state glycine accumulation in frog muscle at 0 °C. Washed muscles incubated 6 days at 0 °C with varying concentrations of labeled glycine. Numerical data shown in Table I.

TABLE IV

COMPUTED VALUES OF CONSTANTS IN EQN 2

Data from 6–13-day accumulation of glycine by washed frog muscles at 0 °C analyzed by the method of least squares using the algorithm of Marquardt[35]. Values shown ± S.E.

Experiment	*Date*	*Incubation time (days)*	C_1 (*g/l*)	C_2 (*μmoles/g*)	C_3 (*mM*)
AAU-4	Dec. 1967	13	0.36 ± 0.03	3.47 ± 0.25	0.36 ± 0.06
AAU-6	Jan. 1968	10	0.08 ± 0.33	4.53 ± 2.2	0.43 ± 0.44
AAU-11	Mar. 1968	7	0.46 ± 0.04	4.07 ± 0.31	0.61 ± 0.09
D-31	Jan. 1970	6	0.40 ± 0.05	4.3 ± 0.60	0.35 ± 0.14
D-70	Sept. 1971	6	0.35 ± 0.03	4.3 ± 0.38	0.69 ± 0.17
D-73	Oct. 1971	6	0.31 ± 0.02	4.65 ± 3.1	0.74 ± 0.12
D-89	Apr. 1972	6	0.39 ± 0.07	3.55 ± 0.70	0.13 ± 0.16
Average			0.42 ± 0.05	4.12 ± 0.17	0.47 ± 0.08

TABLE V

NONLINEAR REGRESSION ANALYSIS OF DATA OF MANCHESTER *et al.*[28] ON STEADY-STATE AMINO ACID ACCUMULATION IN RAT DIAPHRAGM MUSCLE AND CHICK EMBRYO HEART ANALYZED ACCORDING TO EQN 2 (COLUMNS 3–5)

Values of constants obtained by Manchester *et al.*[28] using a form of Eqn 2 in which $C_1 = 1$ and a graphical analysis given in Columns 6–7. Note that this data is calculated on the basis of amino acid in the cell water rather than in the total cell protoplasm as in Table V.

Amino acid	*Tissue*	*Equation 2*			*Graphical analysis*	
		C_1	C_2 (*mM*)	C_3 (*mM*)	V/K_p (*mM*)	K_m (*mM*)
(1)	(2)	(3)	(4)	(5)	(6)	(7)
α-Aminoisobutyric acid	Rat diaphragm	0.83 ± 0.11	9.7 ± 1.6	2.20 ± 0.48	6.9	1.29
α-Aminoisobutyric acid	Chick embryo heart	1.33 ± 0.05	29.7 ± 0.8	0.62 ± 0.05	33.8	0.78
Cycloleucine	Rat diaphragm	1.31 ± 0.04	3.03 ± 0.58	2.07 ± 0.44	8.2	5.2
Cycloleucine	Chick embryo heart	1.12 ± 0.12	21.3 ± 2.8	2.78 ± 0.61	22.5	2.8

TABLE VI

GLYCINE EXTRUSION AGAINST AN APPARENT CONCENTRATION GRADIENT AT 0 °C

Washed muscles incubated as described in the text. Expected $[Gly^*]_{cell}$ derived from a control experiment (D-89) in which muscles were incubated for 6 days at 0 °C in varying concentrations of external glycine. Nonlinear regression analysis of the steady state data obtained gave $C_1 = 0.38$, $C_2 = 3.6$, $C_3 = 0.132$. Expected values were obtained by substituting these constants and $[Gly^*]_{ex}$ as given in the Table into Eqn 2. The distribution ratio is: $[Gly^*]_{cw}/[Gly^*]_{ex}$.

Incubation volume (ml)	*Final [Gly*] (mM)*	*$[Gly^*]_{cell}$ (μmoles/g)*	*Expected $[Gly^*]_{cell}$ (μmoles/g)*	*Distribution ratio*
24-h incubation with 100 μM $[Gly^*]_{ex}$				
—	87.80	21.6 ± 2.5	—	0.3
24-h incubation with 100 mM [Gly*]M *plus* 120 h incubation in indicated volume of glycine-free Ringer's				
2.5	12.45	8.70 ± 1.02	8.30	0.88
4.0	16.81	10.51 ± 0.78	10.90	0.79
5.0	24.60	13.0 ± 0.37	12.90	0.66

where $[S]_c$ is the cellular concentration of amino acid; $[S]_e$, its final external concentration and C_1, C_2 and C_3 are constants whose numerical values can be computed from the data using nonlinear regression analysis by the method of least squares (Table IV)[35]. The detailed meaning of these constants will be considered below.

A number of other tissues have been reported to show steady-state (or approximate steady-state) accumulation which can be divided into "linear" (first term, left-hand side, Eqn 2) and "saturable" (second term, left-hand side, Eqn 2) components including kidney cortex slices[24], erythrocytes[7], ascites cells[23,25], brain tissue[26] and smooth muscle[27].

Manchester *et al.*[28] determined the steady-state accumulation of α-aminoisobutyric acid by rat diaphragm muscle and chick embryo heart and analyzed their data graphically using a form of Eqn 2 in which $C_1 = 1$. The constants they obtained by this method are shown in Table V and are not greatly different from the constants obtained by nonlinear regression analysis of their data according to Eqn 2, also shown in Table V. The most significant difference between these constants and those for frog muscle at 0 °C (Table IV) is that C_1 is close to or above unity in the mammalian tissues whereas the average value of C_1 in frog muscle at 0 °C is 0.54 (in terms of the cell water content), significantly below unity.

That this finding represents the true steady-state level of glycine in the cell was shown by the data in Table VI. Following incubation for 24 h at 0 °C with 100 mM glycine, frog muscles were then placed in small volumes of Ringer solution containing no glycine and allowed to equilibrate 5 days at 0 °C, to establish a new steady state (Fig. 2). The final concentrations in the cell water (Table VI) were actually lower than the external concentration indicating that glycine is extruded against an apparent concentration gradient under these conditions. The cellular levels attained were not significantly different from those expected on the basis of a control experiment done at the same time in which muscles were allowed to equilibrate directly with the final external concentration of labeled glycine.

Glycine efflux from frog muscles at 0 °C

Sartorius muscles were incubated at 0 °C for 6 days with 1 mM labeled glycine and then washed in Ringer solution containing no labeled glycine. The results are

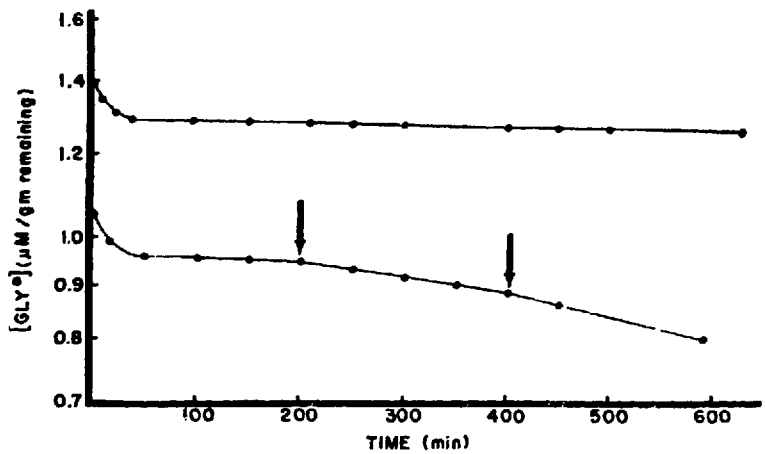

Fig. 5. Time course of labeled glycine efflux from frog muscle at 0 °C. Washed muscles incubated with 1.0 mM labeled glycine for 6 days prior to washing in Ringer phosphate containing no labeled compound as described in Methods. Muscles were transferred to solution containing 1 mM and then 5 mM nonlabeled glycine at 200 and 400 min, respectively (arrows).

plotted in Fig. 5 as the log of the amount remaining *vs* time. The upper curve shows the results obtained when no glycine was present in the washing medium. After an initial rapid loss of glycine, probably representing extracellular amino acid, the curve becomes exponential. For the lower curve, 1 and 5 mM of nonlabeled glycine were added to the washing-out solution at 200 and 400 min, respectively (arrows). The efflux rate constant was increased from $0.78 \cdot 10^{-4}$ min^{-1} to $3.5 \cdot 10^{-4}$ min^{-1} by the addition of 1 mM glycine and further increased to $5.6 \cdot 10^{-4}$ min^{-1} by the addition of 5 mM glycine. This shows that glycine exchange was much more rapid than glycine loss from the tissue. Similar findings were first reported by Riggs *et al.* in 1954[36] in ascites cells and since have been found in numerous tissues[37,38]. Such findings are often considered to be the result of "exchange diffusion" through a carrier (see, for example, ref. 37).

DISCUSSION

Comparison of pump–leak and adsorption models

Interpretations of amino acid accumulation are often[22,23,28] based on the presence of two pathways for the passage of amino acid through the plasma membrane: a passive leak and an active transport carrier. If the leak is bidirectional and carrier transport occurs only in the inward direction, at the steady state

$$[S]_c = \alpha q[S]_e + \frac{\alpha q V}{K_p\left(1 + \frac{K_m}{[S]_e}\right)} \tag{3}$$

where α is the proportion of water in the cell; q, the distribution coefficient of amino acid between the cell water and the external solution; K_p is the permeability of the leakage pathway; V, the maximum rate of entry *via* the carrier and K_m, the apparent dissociation constant of the carrier–amino acid complex. Eqn 3 has the form of empirical Eqn 2 and clearly fits the steady-state data.

Ling[16,17] has suggested that cellular solutes exist in two states: free in the cell water and adsorbed to one or more sets of specific sites. In this case, for one set of non-interactive sites:

$$[S]_c = \alpha q[S]_e + \frac{F}{1 + K_m/[S]_e} \tag{4}$$

where F is the number of adsorption sites per g of cells and K_m, their apparent dissociation constant. Again this equation has the form of Eqn 2. Thus no distinction between the carrier and the adsorption models is possible on the basis of steady-state data alone.

Substantial exclusion of amino acid from organized cell water is predicted by the Ling hypothesis[16,17,39,40]. Other possible explanations for the finding that $q < 1.0$ include compartments within the cell inaccessible to amino acid and a reverse pump in the membrane active at higher concentration ranges. Efflux of amino acid through the active-transport carrier cannot produce a reverse concentration gradient unless the direction of pumping is reversed (Eqn A4, Appendix II).

The source of energy for amino acid accumulation at 0 °C

Continuous energy expenditure is required to maintain concentration gradients between the cell water and the external solution by active transport through the cell membrane. No continuous energy expenditure is required for binding of solutes to adsorption sites. Thus, energy utilization may provide a crucial test to differentiate between active transport and adsorption mechanisms. The existing evidence, although incomplete, suggests that, at least in frog muscle at 0 °C, insufficient energy is produced to maintain observed solute concentration gradients.

We showed here that less than 1% of the glycine present was metabolized by frog muscle over a 6-day period at 0 °C; earlier work[32] indicated that no metabolism of glucose can be detected over a 24-h period under these conditions. Although residual metabolism sufficient to maintain active transport must be ruled out by direct experimental measurement, these findings suggest that metabolic processes play an indirect role in amino acid accumulation by frog muscle at this temperature.

Evidence that metabolic inhibitors do not always inhibit amino acid accumulation if monovalent cation gradients are maintained led Eddy[41] and others[42,43] to postulate that energy is provided indirectly by the Na^+ gradient between the cell interior and the external solution. If this is the case, metabolic energy must be used directly to maintain the Na^+ gradient.

Ling (ref. 16, p. 200) found that inhibition of metabolism with iodoacetic acid and a nitrogen atmosphere had no effect on the levels of Na^+ and K^+ in frog muscle over a period of 8 h at 0 °C. The possibility that endogenous stores of ATP and creatine phosphate provided sufficient energy to maintain the ionic gradients under these conditions was ruled out by the finding that the measured energy production from this source was less than 10% of that necessary to pump Na^+ out of the cell.

These data raise a serious question as to whether enough energy to maintain solute gradients by active transport is produced in frog muscle at 0 °C. Until evidence clearly answering this question in the affirmative is available, the possibility that adsorptive mechanisms are responsible for amino acid accumulation must be seriously considered.

Efflux facilitation

The finding that external glycine facilitates the loss of labeled glycine from muscle at 0 °C (Fig. 5) is not consistent with the pump–leak model described above. For this reason we examined a more generalized model in which efflux through the carrier is allowed[29]. This analysis, details of which are given in Appendix II, shows that the carrier model fits the steady-state data under conditions where binding of amino acid to carrier at the inner surface of the membrane is negligible. This suggests that exchange diffusion through the active-transport carrier is not the mechanism of efflux facilitation. Alternative mechanisms will be examined in a subsequent paper in this series.

APPENDIX I

Extracellular space calculation

A minimal value for the extracellular space was used in order to obtain the smallest possible distribution ratios between cell water and external solution. Use of

larger values (*e.g.* the 22.4% raffinose space[44]) gives a quantitative increase in distribution ratios but in no way changes the conclusions drawn from the data.

The extracellular space can be divided into two compartments[32,45]: "True" extracellular space or the solution-filled spaces between cells, and connective tissue extracellular space. These must be treated separately as the glycine distribution in the two compartments is somewhat different. A minimal estimate for the connective tissue content of the small muscles used in the present experiment has been given as 5%[45]. A minimal estimate for the "true" extracellular space can be obtained from the poly (L-glutamate) space which has an average value of 8.8%[45]. This includes not only "true" extracellular space but a portion of the connective tissue extracellular space as well. This is corrected for the distribution ratio of poly-L-glutamate in connective tissue water of 39%. Making this correction, one obtains a "true" extracellular space of 7.3%.

The concentration of glycine in the connective tissue space, $[Gly]_{C.T.}$, is calculated from the results shown in Fig. 3 and the external glycine concentration, $[Gly]_{ex}$.

For $[Gly]_{ex} \leqslant 3.0$ mM, $[Gly]_{C.T.} = 0.05 + 0.914\ [Gly]_{ex}$. For $[Gly]_{ex} > 3.0$ mM, $[Gly]_{C.T.} = 0.05 \cdot [3.0 + 0.6\ ([Gly]_{ex} - 3.0)]$.

The total extracellular glycine, $[Gly]_{ECS}$, is the sum of the glycine in the "true" extracellular space and in the connective tissue.

For $[Gly]_{ex} \leqslant 3.0$ mM, $[Gly]_{ECS} = [Gly]_{ex}\ (0.073 + 0.05 \cdot 0.914) = [Gly]_{ex} \cdot 0.119$. For $[Gly]_{ex} > 3.0$ mM, $[Gly]_{ECS} = [Gly]_{ex} \cdot 0.073 + [0.15 + 0.03\ (Gly]_{ex} - 3)] = [Gly]_{ex} \cdot 0.103 + 0.096$.

To calculate cellular glycine, $[Gly]_{cell}$:

$$[Gly]_{cell} = \frac{[Gly]_{tis} - [Gly]_{ECS}}{1 - (0.073 + 0.05)} = \frac{[Gly]_{tis} - [Gly]_{ECS}}{0.887}$$

Finally, to calculate the concentration of glycine in the cell water, $[Gly]_{cw}$:

$$[Gly]_{cw} = \frac{[Gly]_{cell}}{0.79}$$

where 0.79 is the fraction of water in the cell calculated from a tissue water content of 80.6% and a connective tissue water content of 50%.

APPENDIX II

Steady-state predictions of a generalized carrier model

We need an equation to predict the steady-state accumulation of amino acid using a model in which there are 2 bidirectional pathways for the passage of amino acid between cell and external solution, an active transport carrier and a passive leak (see Fig. A1). We first consider the case where the permeability of the leakage pathway, K_p, is approx. 0. In this case, from Jacquez[29], we find

$$F_{in} = \frac{a[S]_e}{A + B[S]_e + C[S]_{cw} + D[S]_e[S]_{cw}} \tag{A1}$$

and

$$F_{out} = \frac{b[S]_{cw}}{A + B[S]_e + C[S]_{cw} + D[S]_e[S]_{cw}} \tag{A2}$$

where F_{in}, and F_{out} are the inward and outward unidirectional flux rates and a, b, A, B, C and D are positive constants which are complex combinations of the rate constants shown in Fig. A1.

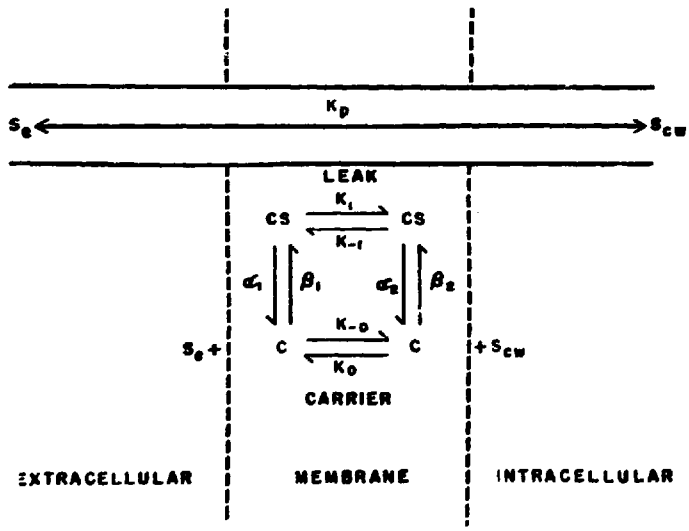

Fig. AI. Generalized carrier–leak model for amino acid transport into cells (after Jacquez[29]).

At the steady state $F_{in} = F_{out}$ and

$$\frac{[S]_{cw}}{[S]_e} = \frac{a}{b} \tag{A3}$$

In this case the distribution ratio is constant for all $[S]_e$. Clearly this model is not consistent with experimental findings in frog or mammalian muscle.

In the presence of a bidirectional leakage pathway, at the steady state[29]

$$\frac{[S]_{cw}}{[S]_e} = 1 + \frac{(a-b)}{K_p(A + b/K_p + B[S]_e + C[S]_{cw} + D[S]_{cw}[S]_e)} \tag{A4}$$

when $a > b$, amino acid is transported into the cell against a concentration gradient.

It can be shown by taking the derivative of Eqn A4 that the limiting slope as $[S]_e$ becomes large is unity. As noted previously, the limiting slope is less than one in all the experimental data from frog muscle reported here.

The assumption implicit in the derivation of Eqn A4 is that all the cell water is accessible to amino acid. If this is not the case Eqn A4 becomes

$$\frac{[S]_{cw}}{[S]_e} = q + \frac{q(a-b)}{K_p\left(A + b/K_p + B[S]_e + C\dfrac{[S]_{cw}}{q} + D[S]_e\dfrac{[S]_{cw}}{q}\right)} \tag{A5}$$

Rearranging, one obtains an equation of the form

$$\frac{[S]_e}{\dfrac{[S]_{cw}}{q} - [S]_e} = L + M[S]_e + N\frac{[S]_{cw}}{q} + P[S]_e\frac{[S]_{cw}}{q} \tag{A6}$$

where $L \equiv K_p'(A + b/K_p')/(a-b)$, $M \equiv K_p'B/(a-b)$, $N \equiv K_p'C/(a-b)$, and $P \equiv K_p'D/(a-b)$. Values for L, M, N, and P were obtained for all experimental data on

TABLE AI

VALUES OF PARAMETERS IN EQN A6

Data from frog muscle experiments analyzed by nonlinear regression analysis using the algorithm of Marquardt[35].

Expt No.	*L*	*M*	*N*	*P*
AAU-4	0.051 ± 0.011	0.164 ± 0.019	−0.00713 ± 0.0032	−0.00284 ± 0.00083
AAU-6	0.108 ± 0.024	0.457 ± 0.026	−0.0452 ± 0.0079	−0.0170 ± 0.0016
AAU-11	0.105 ± 0.011	0.190 ± 0.028	−0.0154 ± 0.0049	−0.0031 ± 0.0012
D-31	0.0336 ± 0.022	0.135 ± 0.011	−0.00254 ± 0.0042	−0.0022 ± 0.0002
D-70	0.0787 ± 0.045	0.0972 ± 0.037	−0.0057 ± 0.011	−0.00021 ± 0.0008
D-73	0.0256 ± 0.0093	0.0368 ± 0.0094	0.0058 ± 0.0021	0.00068 ± 0.00019
Average	0.0670	0.180	−0.0117	−0.0041

frog muscle again using Marquardt's[35] version of the method of least squares (Table AI). L and M were positive and significantly different from zero for all experiments. However, N and P are negative in 5 out of 6 experiments*. Since $a > b$ for transport into the cell against a gradient and K_p, C and D are positive, this is not possible in terms of the model. The values obtained for N and P are, however, close to zero and in some cases not significantly different from zero. If we assign them a value of zero we find that C and D must also be zero. Now, from Jacquez[29], we obtain the detailed expressions

$$C = \alpha_2[\beta_1(k_{-1} + k_{-0}) + k_{-0}(k_{-1} + k_1)] \tag{A7}$$

$$D = \alpha_1\alpha_2(k_{-1} + k_1) \tag{A8}$$

$$a = \beta_2 k_0 k_1 \alpha_1 C_0 \tag{A9}$$

C_0 is the concentration of carrier in the membrane. For transport to occur a must be nonzero and therefore α_1 and k_1 must be non-zero. Since all other constants must be positive or zero, it is apparent that if C and D are 0, $\alpha_2 = 0$. This is equivalent to saying that the carrier-amino acid complex does not form at the inner surface of the membrane and therefore, efflux through the carrier does not occur.

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* A variety of starting values were assigned to N and P but convergence was always obtained at the same point.

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