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Simultaneous Regulation of Amino Acid Influx and Efflux by System A in the Hepatoma Cell HTC

OUABAIN SIMULATES THE STARVATION-INDUCED DEREPRESSION OF SYSTEM A AMINO ACID TRANSPORT*

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In the cultured hepatoma cell HTC, amino acid starvation stimulated both influx and efflux of 2-(methylamino)isobutyric acid (MeAIB) across the plasma membrane with little effect on the ultimate cellular accumulation of this amino acid. In agreement, prior amino acid starvation had little effect on the cellular steady state levels reached for various natural amino acids during subsequent incubation in an amino acid-rich medium containing cycloheximide. Furthermore, efflux of [^{14}C]MeAIB was markedly increased by amino acid starvation. These findings do not mean that adaptive regulation of neutral amino acid transport is pointless. If membrane transport rather than metabolism is the rate-limiting step for net amino acid production or consumption, or becomes so during times of elevated formation or catabolism of an amino acid, then proportionate changes of both the opposed fluxes should enhance its net generation or consumption.

Amino acid starvation enhances MeAIB-dependent Na^+ influx. Alteration of the external [Na^+] changes the K_m, not the V_{max}, for MeAIB influx when the degree of System A derepression is stabilized with cycloheximide. In both starved and unstarved cells, K_m/V_{max} for MeAIB entry yields a linear function with the reciprocal of the external [Na^+], supporting at least for influx a rapid equilibrium-ordered kinetic model in which Na^+ binds to the carrier site before the amino acid. Elevated cellular [Na^+] obtained by ouabain treatment increased MeAIB efflux in parallel. *Trans*-inhibition of MeAIB influx by accumulated MeAIB, and as a related phenomenon by cellular Na^+, was as effective in unstarved as in starved cells, showing independence of this kinetic phenomenon from adaptive regulation. The decreased MeAIB accumulation resulting from decreased influx and increased efflux occurring at high internal [Na^+] applies both to unstarved and starved cells. We conclude that cellular Na^+ accumulations, produced by increasing levels of ouabain, reversibly reduce the ability of MeAIB to repress System A because its interior concentration is prevented from rising, although transport in both directions continues; accordingly, the repressive signal appears to come from the internal amino acid levels rather than from occupation of the carrier site for System A flux.

Adaptive regulation is a plasma membrane transport-regulatory phenomenon whereby certain amino acids repress the function of a membrane transport system for the same or similar amino acids, whereas amino acid deprivation stimulates the activity of the same transport system (see summaries in Refs. 1 and 2). This phenomenon applies to a variety of cells of mesenchymal and epithelial origins (1) including cultured hepatocytes (3, 4) and a variety of hepatoma cell lines (5, 6). Either aspect of the phenomenon requires synthesis of both mRNA and protein (2). The molecular signals that initiate the macromolecular synthesis required for expression of adaptive regulation are unknown. A model has been proposed in which the degree of occupation of the transport system receptor site by substrates provides the signal for the synthesis of mRNA (2). In the case of amino acid starvation-induced derepression, this mRNA ultimately yields a protein that activates amino acid transport, whereas in the case of repression during amino acid excess, the messenger is translated presumably into a protein that causes breakdown or inactivation of membrane transport proteins (2). For several cell types, System A has been identified as the neutral amino acid transport system under adaptive regulation, although in the rat hepatocyte maintained in primary culture another Na^+-dependent system called N is under similar regulation (7). Although the repressive amino acids have usually been substrates of the regulated system, conspicuous exceptions bearing on the question of how the repressive signal is elicited have been reported in the case of hepatocyte System N (8).

We show in this report that adaptive regulation is entirely a kinetic phenomenon, and does not enhance directly, that is without intervening metabolic reactions, the steady state cellular concentration of amino acids during nutritional changes. A substantial portion of amino acid exodus can occur through the reversibility of a transport system usually characterized for mediated entry (9, 10). In the case reported here, the reversal of System A contributes an additional site for metabolic regulation.

MATERIALS AND METHODS

The hepatoma cell line HTC[1] was cultured routinely in Medium 199 (GIBCO) supplemented with 10% dFBS[2] and antibiotics as described previously (11, 12). These cells were prepared for transport assays by dispersing confluent monolayers attached to 150-cm^2 flasks

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[1] HTC, hepatoma tissue culture cell line established by E. Brad Thompson from a Buffalo rat hepatoma, Morris 7288C, and supplied to us by Dr. Thomas Gelehrter of this University.

[2] The abbreviations used are: dFBS, dialyzed fetal bovine serum; EBS, modified Earle's balanced salts solution; FBS, fetal bovine serum; KRB, Krebs-Ringer bicarbonate; MeAIB, 2-(methylamino)isobutyric acid; PBS, phosphate-buffered saline (NaCl, 140 mM; sodium phosphate, 25 mM; pH 7.4).

(Corning) with 2.5 ml of 0.25% trypsin solution prepared in PBS. This cell suspension was diluted to 250 ml with culture medium. One milliliter of this cell suspension was distributed into each well of eight Costar cluster trays (Costar No. 3524) and the remaining 50 ml were added to a fresh Corning flask to continue the cell line. After 96 h of culture at 37 °C in an atmosphere of 5% CO_2, the confluent HTC cells were washed with sterile PBS and the medium was replaced with either Medium 199 or KRB, both supplemented with 10% dFBS. The cells were incubated in these media for 12 to 24 h before the transport assay was carried out. Specific details of this general protocol are explained in the corresponding figure or table legend.

Prior to each MeAIB flux experiment, the monolayers in Costar cluster trays were washed and incubated for 3 h in serum-free and amino acid-free EBS, pH 7.4, 37 °C, and usually containing 20 μM cycloheximide. The use of cycloheximide in human fibroblasts has been shown to halt adaptive regulation in System A activity resulting from changes of the amino acid concentration in the culture medium (2). If amino acid loading was required in an experimental protocol, the desired amino acid was added to this culture medium. After quickly washing the cells at 37 °C, influx of MeAIB was estimated by 20- to 60-s incubations of the prepared cells with various concentrations of [1-^{14}C]MeAIB (960 mCi/mmol, New England Nuclear) in EBS. These time intervals proved adequate to measure the initial rate of uptake of MeAIB in starved and nonstarved HTC cells, respectively, as will be illustrated below. Efflux of MeAIB from HTC cells was approximated by loading the monolayers cultured in Costar trays with [^{14}C]MeAIB and then measuring the initial rate of solute excape into 2 ml of EBS. Initial amino acid exit was estimated in each case during time intervals yielding less than 20% loss of the initial cellular amount of the [^{14}C]MeAIB. This method was used successfully to measure arginine efflux from HTC cells (10). Whenever necessary, choline chloride was used as an equimolar replacement for NaCl in the incubation buffers. All experimental incubations were terminated by washing the cells twice with ice-cold PBS, pH 7.4, extracting the monolayers with 220 μl of 5% trichloroacetic acid, and counting the radioactivity in 200-μl aliquots of this extract in 2 ml of the 3a70B complete counting mixture (Research Products International Corp.). The protein precipitated in each well of the cluster tray was measured with a modified Lowry assay (11). Complete details of all of these manipulations have been published previously (10–13).

The influx and accumulation of Na^+, or loss of cellular Na^+, were studied by measuring $^{22}Na^+$ (carrier-free ^{22}NaCl, New England Nuclear) initial or net fluxes. Isosmotic mixtures of choline chloride and sodium chloride were used to establish the desired extracellular [Na_1^+]. Ouabain (Sigma) was used to alter the cellular [Na_2^+].

The cellular water volume was estimated under a variety of incubation conditions by the method of Kletzien *et al.* (14). The water volume measured after a 3-h incubation of the cell monolayers in EBS containing 20 μM cycloheximide and 1 mM 3-*O*-methyl-D-[U-^{14}C]glucose (300 mCi/mmol, New England Nuclear) was 4.5 ± 0.1 μl of H_2O/mg of protein in cells incubated previously in Medium 199 and 4.0 ± 0.3 μl of H_2O/mg of protein in cells derepressed by previous incubation in KRB. The presence of MeAIB during these measurements caused a slight concentration-dependent increase in the apparent cellular water volume as shown by the results in Fig. 1. For our purposes in this report, we take the average value of 4.9 ± 0.5 as a nominal value for the intracellular water volume in all calculations of the cellular amino acid concentrations.

Amino acid analysis of cellular extracts were obtained with a Beckman model 120C amino acid analyzer as described elsewhere (13).

Kinetic data describing [^{14}C]MeAIB or $^{22}Na^+$ influx were fitted to the Michaelis-Menten equation modified to include a first order term to account for the nonsaturable flux (15).

$$\log v = \log \left[\frac{V_{max}\cdot[S]}{K_m + [S]} + K_d\cdot[S]\right] \quad (1)$$

In this equation, V_{max} and K_m have their usual meanings, and K_d is the slope for the nonsaturable flux component measured within the substrate concentration interval studied. In some experiments involving $^{22}Na^+$ influx, a straight line was sufficient.

$$v = K_d\cdot[S] \quad (2)$$

The nomenclature adopted for the case of a single substrate described previously in detail (10) has been modified for the case of co-transport as shown in Equations 3–5. Briefly, an arabic 1 or 2 indicates arbitrarily that the kinetic parameter corresponds to the extracellular or intracellular compartment, respectively. The addition of subscript *A* or *B* to the kinetic parameter indicates an association with the first or second substrate to interact with the transport binding site, respectively.

Our kinetic data describing inward co-transport of Na_1^+ and $MeAIB_1$, $v_{1\to2}$, were fitted to an equation describing the rapid equilibrium-ordered bireactant kinetic mechanism (16).

$$v_{1\to2} = \frac{V^1_{max}\cdot[S_{1A}]\cdot[S_{1B}]}{K^1_{iA}\cdot K^1_{mB} + K^1_{mB}\cdot[S_{1A}] + [S_{1A}]\cdot[S_{1B}]} \quad (3)$$

This mechanism is based on the assumption that the first substrate to add, S_{1A}, is at equilibrium with the free carrier; the second substrate, S_{1B}, can bind only to this equilibrium complex. K^1_{iA} is an extracellular zero-*trans*[3] dissociation constant for the binding of the first substrate to the free carrier, and K^1_{mB} is an extracellular zero-*trans* Michaelis constant for influx of the second substrate. A distinctive characteristic of this mechanism is the insensitivity of the ordinate intercepts of a $1/v_{1\to2}$ *versus* $1/[S_{1B}]$ double reciprocal plot to changes in $[S_{1A}]$. V^1_{max}, measured by the influx of S_{1B}, is insensitive to $[S_{1A}]$, as long as some S_{1A} is present, whereas V^1_{max}, measured by the influx of S_{1A}, varies hyperbolically with $[S_{1B}]$. The substrate binding events presumably occur much faster than translocation of the complex across the membrane, a scheme which is a special case of the more general ordered bireactant mechanism obtained with steady state assumptions (16). Owing to the slow translocation event relative to the equilibrium-ordered binding of substrates, the true value of K^1_{mA} measured in the presence of an infinite concentration of S_{1B} reduces to zero and K^1_{iB} ($= K^1_{iA}\cdot K^1_{mB}/K^1_{mA}$) is infinite in this scheme; that is, S_{1B} cannot bind in the absence of S_{1A} but a saturating $[S_{1B}]$ can drive the binding of S_{1A} to completion. True zero-*trans* kinetic parameters are difficult to measure in the intact cell because the complete absence of cellular Na_2^+ and cellular amino acids is difficult to achieve. Therefore, we caution the reader that *apparent* values of these kinetic parameters obtained under the conditions described are reported in all cases.

Data obtained from MeAIB influx countertransport experiments were analyzed approximately by using the Iso Uni Uni velocity equation which describes exactly the flux of a single substrate by a mobile carrier (10, 16–18). Although not an exact solution to the problem of countertransport of co-substrates, Equation 4 is useful in this context under the constraint of near constant $[S_{1A}]$ and $[S_{2A}]$, that is, constant extracellular [Na_1^+] and intracellular [Na_2^+], respectively.

$$v_{1\to2} = \frac{V^1_{max}\cdot[S_{1B}]\left[1 + \frac{[S_{2B}]}{K^2_{cB}}\right]}{K^1_{mB}\left[1 + \frac{[S_{2B}]}{K^2_{mB}}\right] + [S_{1B}]\left[1 + \frac{[S_{2B}]}{K^2_{vB}}\right]} \quad (4)$$

K^2_{mB}, K^2_{vB}, *and* K^2_{cB} are apparent intracellular zero-*trans*, infinite-*trans*,[3] and countertransport Michaelis constants, respectively, which refer to cellular concentrations of the amino acid, $[S_{2B}]$. By analogy to influx, the amino acid is assumed to bind secondly at the intracellular face of the membrane following equilibrium binding of Na^+.

For the case of countertransport measured between Na_1^+ and $MeAIB_2$, Equation 4 has been rewritten empirically as follows:

$$v_{1\to2} = \frac{V^1_{max}\cdot[S_{1A}]\left[1 + \frac{[S_{2B}]}{K^2_{cB}}\right]}{K^1_{mA}\left[1 + \frac{[S_{2B}]}{K^2_{mB}}\right] + [S_{1A}]\left[1 + \frac{[S_{2B}]}{K^2_{vB}}\right]} \quad (5)$$

This expression relates the influx of Na_1^+ to a variable cellular concentration of MeAIB at constant cellular Na_2^+ and extracellular $MeAIB_1$.

FORTRAN programs described by Cleland have been used to apply the Gauss-Newton nonlinear least squares analysis to the data described by Equations 1–5 (19).

RESULTS AND DISCUSSION

Amino Acid Starvation Stimulates Amino Acid Influx and Efflux but Has No Effect on Steady State Distribution Ratios

[3] Zero-*trans* and infinite-*trans* flux are idealized experimental situations in which the initial velocity of substrate flux is measured in the absence or presence of transport-reactive compounds, respectively, on the side of the membrane opposite to the measured flux.

When Transport is Not Rate-limiting to Metabolism—Incubation of cultured mammalian cells in amino acid-free medium results in a net loss of cellular amino acids (20). The time course of this response in the HTC cell for many of the naturally occurring amino acids is shown by the data of Table I. During an 8-h time interval, the total cellular amino acid pool decreases nearly to 1/10 of its original value. Amino acid starvation in a number of cell types (21) including HTC (5, 6) and liver (3, 4), causes a 5- to 10-fold increase in neutral amino acid influx through System A. This response is reflected entirely by an increase in the maximum influx, V^1_{max}, with no significant effect on the Michaelis constant, K^1_{mB}, or on the flux constant for the nonsaturable component, K^1_{dB} (Table II). Therefore, as illustrated by the data in Table II, a change in V^1_{max}/K^1_{mB} is a valid quantitative measure of adaptive regulation in HTC cells. This observation suggests that influx of reactive amino acids is stimulated proportionally over the entire range of substrate concentrations tested. Furthermore, the measurement of amino acid efflux through System A, or more specifically, V^2_{max}/K^2_{mB}, obtained by measuring MeAIB efflux from HTC cells loaded to concentrations well below the value of the intracellular Michaelis constant, should provide an additional index for adaptive regulation of System A.

TABLE I

The effect of amino acid starvation on the cellular amino acid pools

Confluent monolayers of HTC cells grown in 75-cm² Corning flasks were washed three times at room temperature with 10-ml portions of PBS and then incubated at 37 °C with 10 ml of KRB containing 10% dialyzed fetal bovine serum. After the indicated time intervals, the cells were washed 5 times with ice-cold PBS and extracted overnight with 75% ethanol. The amino acid composition of this extract was determined with an amino acid analyzer as described under "Materials and Methods." ND, not detected.

Amino acid	Hours of incubation with KRB							
	0	0.25	0.50	1.0	2.0	4.0	6.0	8.0
	nmol·mg⁻¹ protein							
Thr, Asn, Gln	49.4	38.1	25.7	12.2	4.0	2.7	2.2	2.3
Ser	51.0	45.6	41.3	21.7	10.4	8.6	5.3	4.5
Glu	83.5	77.9	91.6	63.9	77.3	39.2	45.1	34.7
Gly	183	133	102	54.5	43.6	18.5	16.0	11.9
Ala	29.6	17.1	9.6	4.5	3.9	2.4	4.7	2.5
Val	7.3	2.4	1.4	0.7	0.3	ND	ND	ND
Met	7.3	4.0	ND	ND	ND	ND	ND	ND
Ile	9.8	7.5	5.3	3.6	2.5	1.5	ND	ND
Leu	26.1	5.6	3.2	1.5	ND	ND	ND	ND
Tyr	9.6	7.1	5.5	ND	ND	ND	ND	ND
Phe	14.2	9.8	5.7	ND	ND	ND	2.3	2.5
Lys	5.9	1.6	1.2	0.9	0.7	1.1	ND	ND
His	3.8	1.7	1.4	0.7	0.5	ND	ND	ND
Arg	2.3	0.3	ND	ND	ND	ND	ND	ND
Total	483	352	294	164	143	74	76	58

TABLE II

Stimulation of MeAIB influx following amino acid starvation

The kinetic parameters for [¹⁴C]MeAIB influx between 0.01 and 20 mM were determined in confluent HTC cells during a 30-s incubation. Prior to the kinetic measurements, the cells were incubated in KRB supplemented with 10% dialyzed fetal bovine serum for the indicated time intervals. The kinetic parameters ± S.E. were determined by fitting the data to Equation 1. The nomenclature is described under "Materials and Methods."

Incubation with KRB	K^1_{mB}	V^1_{max}	V^1_{max}/K^1_{mB}	K^1_{dB}
h	*mM*	*nmol·mg⁻¹ protein·min⁻¹*	*nmol·mg⁻¹ protein·min⁻¹·mM⁻¹*	*nmol·mg⁻¹ protein·min⁻¹·mM⁻¹*
0	0.13 ± 0.04	0.93 ± 0.16	7 ± 1	0.34 ± 0.07
0.5	0.19 ± 0.04	1.1 ± 0.2	6.1 ± 0.7	0.39 ± 0.08
1	0.09 ± 0.02	1.3 ± 0.2	15 ± 1	0.29 ± 0.06
3	0.18 ± 0.02	2.8 ± 0.2	15 ± 1	0.29 ± 0.06
5	0.24 ± 0.03	4.3 ± 0.3	18 ± 1	0.36 ± 0.07
8	0.19 ± 0.02	5.3 ± 0.3	30 ± 2	0.38 ± 0.08
12	0.23 ± 0.03	7.9 ± 0.6	34 ± 2	0.4 ± 0.1
24	0.15 ± 0.02	8.7 ± 0.7	55 ± 5	0.4 ± 0.2

Our data shown in Table III indicate that amino acid starvation has no detectable effect on the accumulation of some naturally occurring amino acids into HTC cells. Fig. 2*A* shows that amino acid starvation of HTC cells in KRB for 18 h had the expected stimulatory effect on the initial rate of MeAIB uptake but little effect on the steady state distribution of MeAIB measured during 3 h of incubation (Fig. 2*B*). In Fig. 2*A*, the steady state for the repressed cells had in 3 h reached 79% of the level attained somewhat earlier in derepressed cells, and appears headed for the same ultimate level as the latter. When we limit our attention to the four or five highest MeAIB concentrations in Fig. 2*B*, the effect of derepression on the approach to steady state appears significant.

The contradiction suggested by an accelerated influx and an unchanged steady state can be resolved only if efflux of reactive neutral amino acids from starved HTC cells increases proportionally with influx during amino acid starvation. The results presented in Fig. 3 show that loss of cellular MeAIB was indeed markedly increased following a 12-h incubation of the cells in amino acid-free KRB. Cycloheximide included during the 12-h period of amino acid starvation inhibited the adaptive stimulation of MeAIB efflux. This result is analogous to the inhibition of adaptive stimulation of influx by cycloheximide (1–8) and is consistent with the conclusion that both influx and efflux through System A are stimulated during prolonged amino acid starvation. Since the effect on influx is also seen as a difference in AIB influx between fed and fasted rats (4), both effects are probably present in freshly isolated hepatocytes. The exodus recorded in Fig. 3 obviously includes a small component of recapture, but changes in efflux dominate.

In human fibroblasts, cycloheximide inhibits the repression

TABLE III

The effect of amino acid starvation on the accumulation of some amino acids into HTC cells

HTC cells grown in 75-cm² Corning flasks were washed three times at room temperature with 10-ml portions of PBS and then incubated with 10 ml of KRB containing 10% dialyzed fetal bovine serum for the indicated time intervals. Following these treatments, the monolayers were incubated for 3 h with 10 ml of EBS containing 20 μM cycloheximide and the indicated amino acids at 0.1 mM. The cellular amino acid content was extracted with ethanol and analyzed as described under "Materials and Methods."

Amino acid	Hours of amino acid starvation					
	1	4	7	12	24	40
	nmol·mg⁻¹ protein					
Thr, Asn, Gln	10	9	9	11	9	10
Ser	8	11	11	13	12	8
Pro	4	5	5	5	5	5
Gly	60	61	57	70	60	51
Ala	7	7	7	8	7	7
Val	1	2	3	3	2	2
Met	3	3	4	4	4	3
Ile	2	1	2	2	2	2
Leu	2	2	2	3	2	2
Tyr	4	5	6	7	6	5
Phe	2	3	4	4	3	3
His	2	3	3	4	3	3
Arg	1	1	1	1	1	1
Total	106	113	114	135	116	102

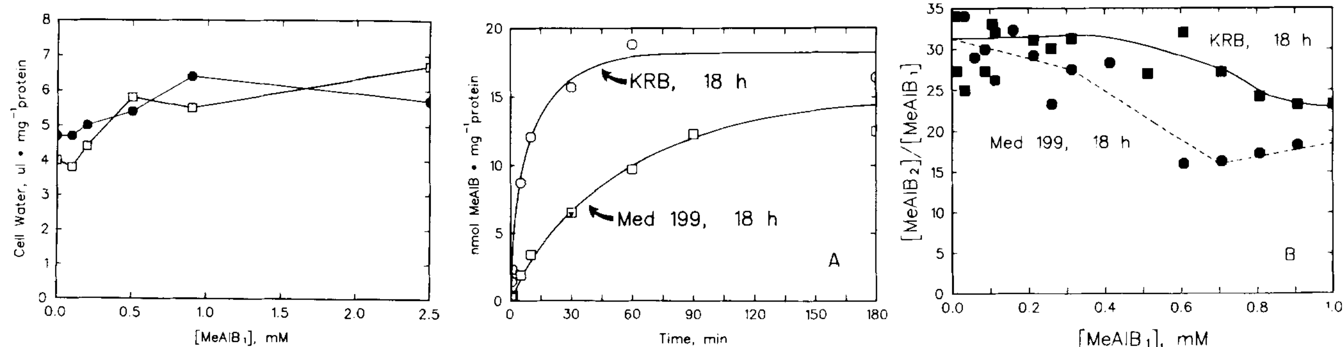

FIG. 1 (*left*). **The intracellular water volume of HTC cells estimated with 3-*O*-methyl-D-glucose.** HTC cells were washed and incubated in Medium 199 (●) or KRB (□) supplemented with 10% dFBS for 15 h. After this time interval, the monolayers were washed with EBS and incubated for 3 h in EBS containing 20 μM cycloheximide, 1.0 mM 3-*O*-methyl-D-[^{14}C]glucose, and the concentrations of MeAIB indicated on the *abscissa*. The cell-associated 3-*O*-methyl-D-glucose, nanomoles/mg of protein, was determined in triplicate, and the cell water volume, microliters/mg of protein, was calculated from these values and plotted on the *ordinate* by assuming that the concentration of 3-*O*-methyl-D-glucose at equilibrium is equal inside and outside of the cell (14).

FIG. 2 (*center and right*). **The effect of time and external amino acid concentration on the accumulation of cellular MeAIB.** In both experiments, the cells were washed and incubated for 18 h with amino acid-free KRB (O, ■) or Medium 199 (□, ●). Following this incubation, the cells in *A* were washed and incubated for 3 h in EBS containing 20 μM cycloheximide and then incubated with 0.2 mM [^{14}C]MeAIB for the time intervals indicated on the *abscissa*; each *point* represents an average of three measurements. The HTC cells in *B* were washed and incubated for 3 h in EBS containing 20 μM cycloheximide and the indicated extracellular concentrations of [^{14}C] MeAIB. After this incubation, the apparent distribution ratios of [$MeAIB_2$]/[$MeAIB_1$] in the amino acid-starved (■) and -rich (●) cells were calculated.

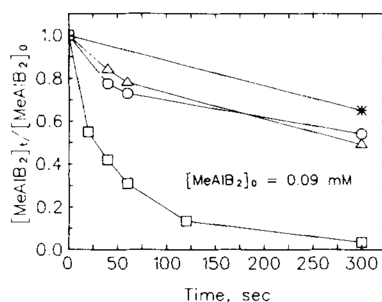

FIG. 3. **Time courses of [^{14}C]MeAIB efflux from HTC cells.** The cells were incubated for 12 h with amino acid-free KRB supplemented with 10% dFBS and 20 μM cycloheximide (O) or no cycloheximide (□) Medium 199 supplemented with 10% dFBS, and 20 μM cycloheximide (*) or no cycloheximide (△). Then all the cells were washed and incubated for 3 h with 20 μM cycloheximide and 0.008 mM [^{14}C]MeAIB. After this procedure, the cellular MeAIB level in each case was about 0.45 nmol·mg^{-1} protein (0.09 mM). Finally, these cells were quickly washed and incubated in 2 ml of cycloheximide- and MeAIB-free EBS medium for the time intervals indicated on the *abscissa*. The ratio of cellular [^{14}C]MeAIB remaining after this incubation interval to the initial cellular [^{14}C]MeAIB concentration, $[MeAIB_2]_t/[MeAIB_2]_0$, is plotted on the *ordinate*.

by amino acids of System A uptake, an observation which suggests that protein synthesis is necessary for reversal of adaptive regulation (2). We find that cycloheximide inhibits amino acid repression of System A when assayed by MeAIB exodus. Fig. 4 shows that starvation of HTC cells by incubation in KRB for 12 h, followed by incubation of these cells for 10 h in Medium 199 with or without cycloheximide, and finally loading all the cells with [^{14}C]MeAIB yields an enhanced rate of MeAIB exodus from cells treated for the 10-h time interval with Medium 199 containing cycloheximide. Therefore, as measured by the difference of the curves, cycloheximide antagonizes the repression of System A in HTC cells exposed to a normal *in vitro* extracellular amino acid nutrition. These results obtained by measuring MeAIB release establish for the HTC cell that cycloheximide inhibits, as in fibroblasts (2), both the derepression of System A (Fig. 3) and the repression of System A (Fig. 4).

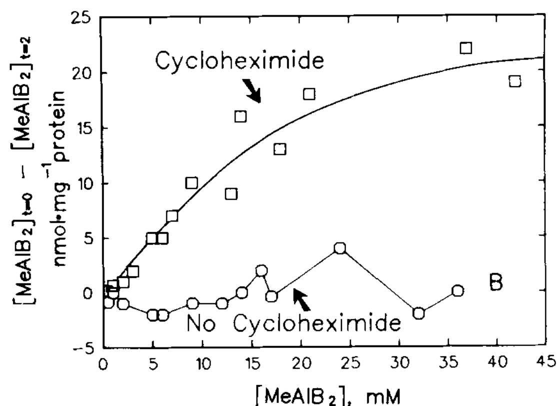

FIG. 4. **The effect of cycloheximide on the repression of MeAIB exodus.** Confluent HTC cells were incubated in KRB supplemented with 10% dFBS for 12 h. Then the cells were incubated for 10 h with Medium 199 containing 10% dFBS and 20 μM cycloheximide (□) or no cycloheximide (O). After this time interval, the monolayers were incubated for 3 h in EBS containing 20 μM cycloheximide and various concentrations of [^{14}C]MeAIB ranging from 0.025 to 10 mM. Finally, the cells were washed with EBS at 37 °C and incubated in amino acid-free EBS for 2 min. The MeAIB lost from the HTC cells during this final 2-min incubation, $[MeAIB_2]_{t=0} - [MeAIB_2]_{t=2}$, is plotted on the *ordinate* as a function of the initial cellular [$MeAIB_2$].

This section of the paper has shown that System A activity in HTC cells as measured by the unidirectional flux of MeAIB is stimulated in both directions after amino acid starvation. The net fluxes of amino acids appear scarcely affected by adaptive regulation as inferred from the insensitivity of the steady state distribution ratios observed following amino acid starvation (Fig. 2 and Table III). Note the use here of cycloheximide for "freezing" the activity of System A in HTC cells during experimental changes in culture conditions. This technique for controlling the level of repression will be used often in the remaining experiments.

Sodium Ion Dependence of System A Flux—The data of Fig. 5 show that extracellular [Na_1^+] alters exclusively the observed value of the Michaelis constant, $K^1_{m_B}$, for MeAIB

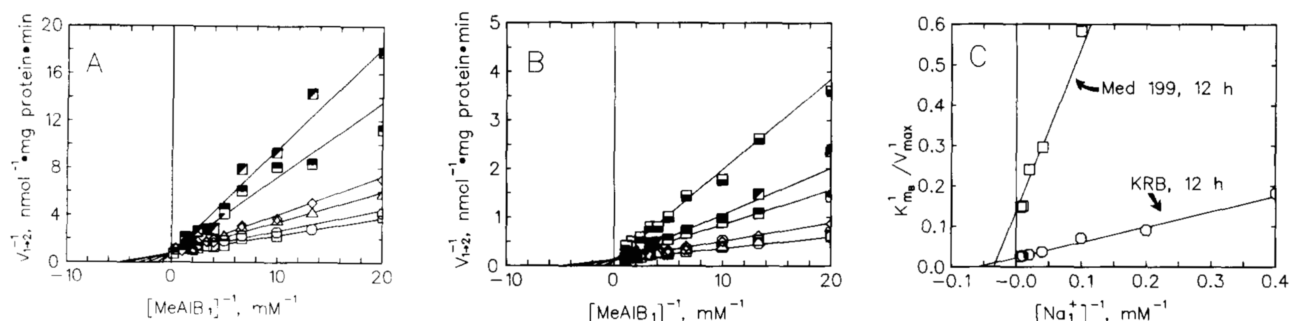

FIG. 5. The effect of $[Na_1^+]$ on the influx of [^{14}C]MeAIB into amino acid-starved and -nonstarved HTC cells. HTC cells were incubated for 12 h in Medium 199 (*A*) or KRB (*B*), both supplemented with 10% dFBS. After this time interval, the monolayers were incubated in fresh EBS containing 20 μM cycloheximide for 2 h and then in Na^+-free EBS containing 20 μM cycloheximide for 1 h. The influx ($v_{1\to2}$) of [^{14}C]MeAIB was estimated from 1-min (*A*) or 20-s (*B*) incubations between 0.025 and 20 mM MeAIB at the following millimolar concentrations of extracellular $[Na_1^+]$: 150 (□), 100 (○), 50 (△), 25 (◇), 10 (■), 5.0 (■), and 2.5 (■). The nonsaturable component (nanomoles/mg of protein·min^{-1}·mM^{-1}), had a value of 1.0 in *A* and 2.70 in *B* and has been subtracted from the plotted data in each case. A replot of the slopes of the *lines* shown in *A* (□) and *B* (○) against the $[Na_1^+]^{-1}$ is provided in *C*. See Table IV for a numerical evaluation of the data in *B*.

influx into both repressed (Fig. 5*A*) and derepressed (Fig. 5*B*) HTC cells. The data in Fig. 5*B* are presented numerically in Table IV and show that V^1_{max} and K^1_{dB} for MeAIB influx are independent of the extracellular $[Na_1^+]$. A replot of these data presented in Fig. 5*C* shows that K^1_{mB}/V^1_{max} is a linear function of $1/[Na_1^+]$. The kinetic data for co-transport of MeAIB shown in Fig. 5, *A* and *B*, were well described by Equation 3 and the derived kinetic parameters are listed in Table V. In repressed and derepressed HTC cells, the measured dissociation constant for Na_1^+, K^1_{iA}, and the Michaelis constant for $MeAIB_1$ influx, K^1_{mB}, were not significantly altered. The maximum influx, V^1_{max}, and the ratios, V^1_{max}/K^1_{iA} and V^1_{max}/K^1_{mA}, displayed 8- to 10-fold increases after starvation. These results indicate provisionally that a rapid equilibrium-ordered bireactant kinetic mechanism (16) operates for MeAIB co-transport into starved and unstarved HTC cells. Presumably, the mechanism also occurs for efflux. Restricting our attention to System A of the HTC cell, Na^+ and MeAIB combine with the transport receptor in an obligate order; that is, MeAIB adds only to the carrier·Na^+ complex. Na_1^+ is the first substrate (S_{1A}) and $MeAIB_1$ is the second substrate (S_{1B}). Na_1^+ affects only the V^1_{max} for AIB influx into freshly isolated hepatocytes (4), and both V^1_{max} and K^1_m for AIB influx into the Ehrlich cell (22, 23); however, the flux of AIB is not necessarily restricted to System A (24). In the Ehrlich cell, only the values of K^1_m and K^2_m for glycine flux (25) which is restricted largely to System A in that cell (26, 27) were altered by Na^+.

The kinetic plots of Na_1^+ influx in the presence of 20 mM extracellular $MeAIB_1$ shown in Fig. 6, *A* and *B*, and the kinetic parameters listed in Table VI obtained by fitting these data to Equation 1, indicate that amino acid starvation caused a significant stimulation of V^1_{max}, but no change in the apparent value of K^1_{mA}. In the absence of $MeAIB_1$ and under the conditions used in our assay, the amino acid-independent flux of Na_1^+ into HTC cells was not saturable between 0 and 50 mM and well described by a straight line (Equation 2). The value of K^1_{dA}, the parameter describing the linear and amino acid-independent portion of Na_1^+-influx, was only increased slightly after amino acid starvation whether or not $MeAIB_1$ was present in the incubation solution (Table VI).

MeAIB exodus is sodium-dependent as expected for the reverse operation of System A (9). The time courses in Fig. 7 show that $MeAIB_2$ is lost faster from HTC cells with relatively high cellular $[Na_2^+]$ than from cells containing low $[Na_2^+]$. This difference is striking in derepressed cells. Apparently, as

TABLE IV

The effect of extracellular Na^+ on the observed kinetic parameters for MeAIB influx into starved HTC cells

HTC cells were incubated in amino acid-free KRB containing 10% dialyzed fetal bovine serum for 12 h and then incubated for an additional 3 h in EBS containing 20 μM cycloheximide. The monolayers were washed twice with choline-EBS and the influx of [^{14}C] MeAIB was estimated from 20-s incubations between 0.025 and 20 mM MeAIB at the indicated extracellular concentration of Na^+. Choline was used as an equimolar replacement for Na^+. The kinetic parameters ± S.E. were obtained by fitting the influx curves measured at each $[Na_1^+]$ to Equation 1.

$[Na_1^+]$	K^1_{mB}	V^1_{max}	V^1_{max}/K^1_{mB}	K^1_{dB}
mM	*mM*	*nmol·mg^{-1} protein·min^{-1}*	*nmol·mg^{-1} protein·min^{-1}·mM^{-1}*	*nmol·mg^{-1} protein·min^{-1}·mM^{-1}*
150	0.19 ± 0.02	7.3 ± 0.5	39 ± 2	3.2 ± 0.2
100	0.21 ± 0.02	7.7 ± 0.5	36 ± 2	3.1 ± 0.2
50	0.26 ± 0.03	8.7 ± 0.6	33 ± 6	2.8 ± 0.2
25	0.31 ± 0.08	8.2 ± 1.4	27 ± 3	2.4 ± 0.4
10	0.44 ± 0.12	6.4 ± 1.3	14 ± 2	2.6 ± 0.3
5.0	0.72 ± 0.12	7.8 ± 1.1	11 ± 1	2.1 ± 0.2
2.5	1.4 ± 0.3	7.9 ± 1.7	5.5 ± 0.3	2.7 ± 0.2

in other cells, ouabain has no direct effect on the operation of System A; rather, the alterations it produces in the Na^+ gradient increase efflux via System A, presumably by lowering the apparent value of K^2_{mB} in a way analogous to that shown for influx in Table IV.

The Effect of Amino Acid Starvation, Sodium Ion Concentration, and Cellular MeAIB on the Countertransport Properties of System A—Accelerative countertransport is observed for a number of transport systems in most mammalian cells (10, 26). Cellular System A substrates, however, tend to inhibit influx, a phenomena known as decelerative countertransport or *trans*-inhibition (26). Our results presented in this section show that *trans*-inhibition and adaptive regulation of System A are entirely independent regulatory phenomena. Line-weaver-Burk plots can be constructed for the influx of [^{14}C]MeAIB into HTC cells that have been loaded with various concentrations of unlabeled MeAIB. These primary kinetic plots (not shown) are similar to those constructed in Fig. 5. The apparent values of the kinetic parameters V^1_{max} and V^1_{max}/K^1_{mB}, obtained for [^{14}C]MeAIB influx by fitting the primary influx data to Equation 1 are, however, plotted in Fig. 8, *A* and *B*, respectively. These results show that cellular $[MeAIB_2]$ has a clear *trans*-inhibitory effect on [^{14}C]MeAIB

TABLE V

Kinetic parameters describing the equilibrium-ordered influx of MeAIB into HTC cells

HTC cells were prepared for assay as described in Fig. 5. The kinetic parameters ± S.E. were determined by fitting the influx data to Equation 3. K^1_{iA} represents the observed dissociation constant and K^1_{mB} represents the observed Michaelis constant for the binding of Na^+ and the influx of MeAIB, respectively. The average nonsaturable component (K^1_d, nanomoles/mg of protein·min·mM) 1.11 ± 0.23 (Medium 199) or 2.7 ± 0.4 (KRB) determined by fitting the data obtained at each $[Na_1^+]$ concentration to Equation 1, was subtracted from original data prior to the numerical analysis with Equation 3.

Kinetic parameter	Description	Medium 199, 12 h	KRB, 12 h
V^1_{max}	Maximum influx	1.1 ± 0.03	7.50 ± 0.08
K^1_{iA}	Dissociation constant for Na_1^+	27 ± 6	19 ± 1
K^1_{mB}	Michaelis constant for $MeAIB_1$	0.179 ± 0.02	0.165 ± 0.008
V^1_{max}/K^1_{iA}		0.045 ± 0.009	0.40 ± 0.02
V^1_{max}/K^1_{mB}		6.7 ± 0.7	45 ± 2

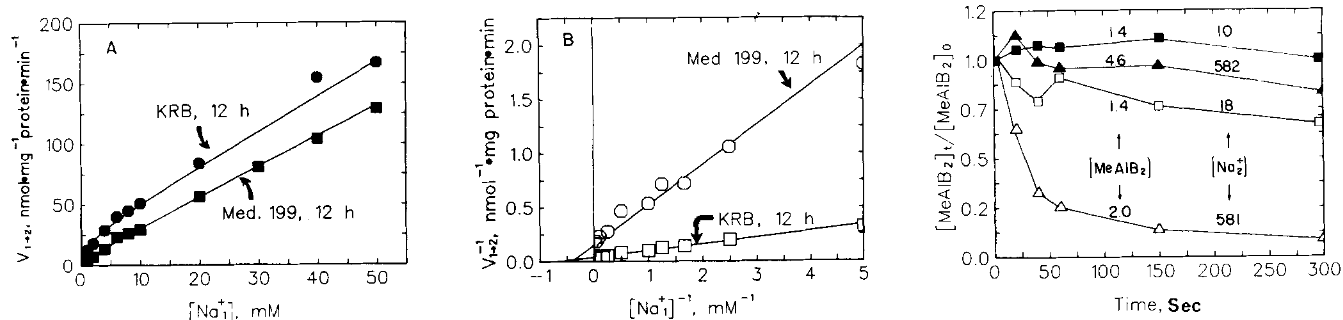

FIG. 6 (*left and center*). **MeAIB-stimulated influx of $^{22}Na^+$ into amino acid-starved or -nonstarved HTC cells.** HTC cells in *A* were incubated in Medium 199 (■) or KRB (●) supplemented with 10% dFBS for 12 h. After this time interval, the monolayers were incubated in fresh EBS containing 20 μM cycloheximide for 2 h and then in Na^+-free EBS containing cycloheximide for 1 h. The influx of Na^+, $v_{1\to2}$, was estimated from 20-s incubations of these monolayers with the indicated concentrations of $[^{22}Na_1^+]$. After subtraction of the nonsaturable components, about 2.7 nmol·mg^{-1} protein·min^{-1}·mM^{-1} in both experiments, the corresponding Lineweaver-Burk plots for derepressed cells (□) or repressed (○) cells were constructed in *B*.

FIG. 7 (*right*). **The effect of intracellular $[Na_2^+]$ on the release of [^{14}C]MeAIB from HTC cells.** The cells were incubated for 20 h with amino acid-free KRB (*open symbols*) or with Medium 199 (*closed symbols*). Then the cells were incubated for 3 h with 0.1 mM [^{14}C]MeAIB, 1 mM ouabain, 20 μM cycloheximide, and the following concentrations of extracellular Na_2^+: 1 mM (■, □), 80 mM (▲), and 160 mM (△). The initial cellular concentration (nanomoles/mg of protein) of MeAIB and Na^+ corresponding to each time course are indicated. After this incubation, the monolayers were incubated in amino acid-free EBS for the time intervals indicated on the *abscissa*. The ratio of cellular MeAIB remaining after this time interval (t) to the initial (0) cellular MeAIB concentration, $[MeAIB_2]_t/[MeAIB_2]_0$, is plotted on the *ordinate*.

TABLE VI

Na^+ influx into HTC cells incubated with Medium 199 or KRB

HTC cells were incubated in Medium 199 or KRB both containing 10% dialyzed fetal bovine serum followed by a 3-h incubation in EBS containing 20 μM cycloheximide. During the final hour, some of the monolayers were incubated with 10 mM MeAIB in EBS with cycloheximide to obtain a high cellular concentration of this amino acid. The monolayers were quickly (<5 s) washed with choline-EBS followed immediately by a 20-s incubation of these monolayers with $^{22}Na^+$ between 0.2 and 160 mM. The kinetic parameters ± S.E. shown here were obtained by fitting the $^{22}Na^+$ influx measured between 0.2 and 50 mM to Equation 2 in the absence of external MeAIB or to Equation 1 in the presence of 10 mM external $[MeAIB_1]$.

$[MeAIB_1]$	$[MeAIB_2]$	K^1_{mA}	V^1_{max}	K^1_{dA}
mM	*mM*	*mM*	*nmol·mg^{-1} protein·min^{-1}*	*nmol·mg^{-1} protein·min^{-1}·mM^{-1}*
Incubation with Medium 199				
0	0			2.0 ± 0.1
0	>10			2.8 ± 0.1
10	0	2.4 ± 0.9	6.5 ± 2.2	2.5 ± 0.1
Incubation with KRB				
0	0			3.6 ± 0.1
0	>10			3.3 ± 0.1
10	0	1.4 ± 0.3	24 ± 4	2.9 ± 0.2

influx. The observed values of V^1_{max} and V^1_{max}/K^1_{mB} measured in the presence of 143 mM Na^+ decreased hyperbolically, whereas the apparent values of K^1_{mB} increased slightly. *Trans*-inhibition was observed to the same relative extent in both repressed and derepressed HTC cells, although as expected the absolute values of the apparent maximum velocity for [^{14}C]MeAIB influx measured at all intracellular MeAIB concentrations were about one-eighth as high in repressed cells.

The apparent kinetic parameters describing MeAIB countertransport experiments were obtained by fitting the primary influx data to Equation 4 which describes the unidirectional influx of a single substrate by a mobile carrier. We have described the parameters for this Iso Uni Uni velocity equation elsewhere (10). In the present experiments, unlike those for cationic amino acid transport (10), each kinetic parameter for MeAIB countertransport is a complex function of the $[Na_1^+]$ and $[Na_2^+]$. Therefore, these parameters are apparent values applying for a fixed extracellular $[Na_1^+]$, namely 143 mM, and a somewhat lower cellular $[Na_2^+]$ fixed mostly by the action of the ouabain-sensitive (Na^+-K^+)-ATPase. MeAIB countertransport behaves like a single-substrate transport system under these experimental conditions. This result occurs presumably because the presence of inhibitory countertransport is due to the slow translocation of the loaded carrier across the membrane and is entirely independent of the rapid equilibrium-ordered binding of the Na^+ followed by MeAIB

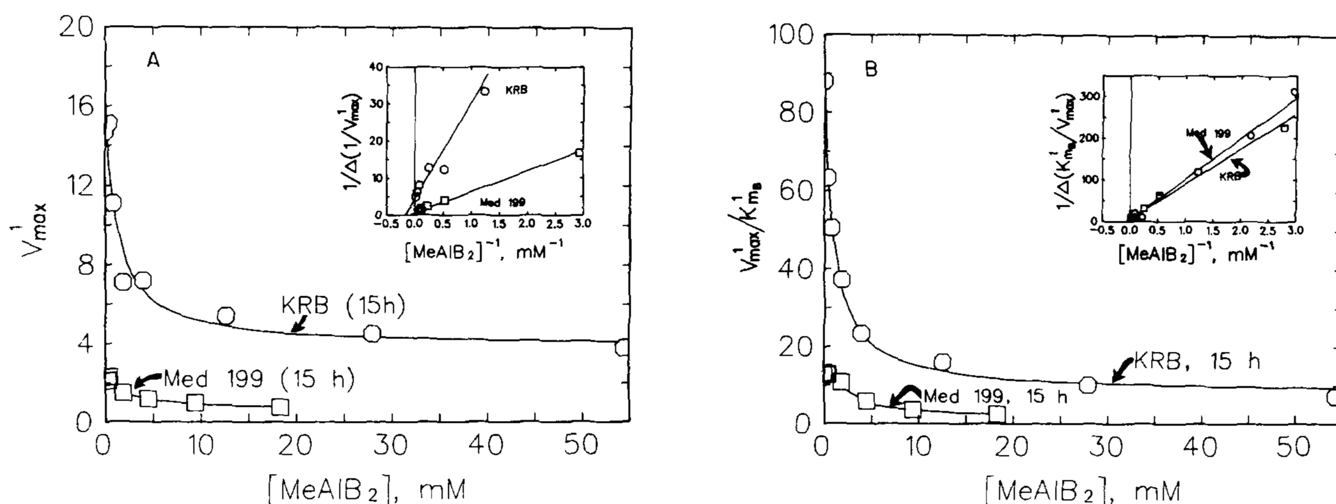

FIG. 8. **The effect of [$MeAIB_2$] on the kinetic parameters for [^{14}C]MeAIB influx countertransport into HTC cells.** The cells were incubated in Medium 199 (□) or KRB (○) for 12 h. Then the cells were incubated for 3 h in EBS containing 20 μM cycloheximide and various concentrations (0, 0.02, 0.04, 0.10, 0.20, 0.50, 1.0, or 2.5 mM) of unlabeled MeAIB. The apparent internal [$MeAIB_2$] plotted on the *abscissa* was estimated radiochemically from parallel experiments. Prior to the influx experiments, the monolayers were quickly washed (<5 s) with EBS at 37 °C and then incubated for 1 min (□) or 20 s (○) with [^{14}C]MeAIB within a concentration range of 0.025–20 mM. The apparent values of V^1_{max} and V^1_{max}/K^1_{mB} plotted on the *ordinate* were determined by fitting the influx data to Equation 1. The *insets* in *A* and *B* are linear transformations of these hyperbolic curves (10). A quantitative representation of the data shown is provided in Table VII.

which occurs at the receptor site before translocation.

The zero-*trans* Michaelis constant for cellular $MeAIB_2$ obtained by these countertransport measurements, K^2_{mB}, is about 10-fold larger than the corresponding extracellular value, K^1_{mB}, in both repressed and derepressed cells. This difference of measured kinetic parameters is similar to the findings for several neutral amino acids in the Ehrlich cell (9, 25), cationic amino acids in the HTC cell and human fibroblast (10), and 2-aminobicyclo(2,2,1)heptane-2-carboxylic acid (BCH) in human fibroblasts where the value for K^1_m/K^2_m is about 60 (28). The high absolute values for all the intracellular Michaelis constants measured in starved cells (Table VII) relative to unstarved cells may result from the roughly 77% higher cellular [Na_2^+] found in these cells after amino acid starvation (data not shown); compare, for example, K^2_{mB}, K^2_{vB}, and K^2_{cB} in repressed and derepressed cells. The ratios of these intracellular Michaelis constants provide an index of *trans*-stimulation if the value is greater than 1 and an index of *trans*-inhibition if the value is less than 1 (16). The ratios K^2_{vB}/K^2_{cB} and K^2_{mB}/K^2_{cB} indicate that the observed *trans*-inhibitions of the values of V^1_{max} and V^1_{max}/K^1_{mB} for MeAIB, respectively, in both repressed and derepressed HTC cells are the same, about 10-fold. This result establishes clearly that adaptive regulation is independent of *trans*-inhibition, and that at least kinetically, System A has similar countertransport properties before derepression and when derepressed. The apparent zero-*trans*, K^1_{mB} and infinite-*trans*, K^1_{vB} Michaelis constants for MeAIB influx countertransport are similar in repressed and derepressed cells, that is, $K^2_{vB}/K^2_{mB} \approx 1$. The values of V^1_{max} measured in parallel experiments with derepressed cells by MeAIB influx and by Na_1^+ influx are very similar, the result expected for a 1:1 cotransport stoichiometry.

Recalling that the subscripts *A* and *B* identify parameters for Na^+ and MeAIB, respectively, the final column of data in Table VII shows that MeAIB-stimulated Na_1^+ influx into starved HTC cells is also *trans*-inhibited by cellular [$MeAIB_2$]. Unlike the case for MeAIB, the apparent infinite-*trans* Michaelis constant for Na^+ influx, K^1_{vA}, is 7-fold larger than the apparent zero-*trans* parameter, K^1_{mA}, that is, $K^2_{vB}/K^2_{mB} \approx 7$ when [$MeAIB_2$] was raised to high levels. Cellular [$MeAIB_2$] decreases, however, the apparent V^1_{max}/K^1_{mA} for Na_1^+ influx to 1/10 of the uninhibited value, that is, $K^2_{mB}/K^2_{cB} \approx 0.10$. This latter finding parallels exactly the result for *trans*-inhibition of [^{14}C]MeAIB influx by cellular MeAIB.

Contrary to our finding for influx, MeAIB efflux is not *trans*-inhibited significantly by extracellular MeAIB in either repressed or derepressed HTC cells (Fig. 9). The values of $v_{2\to1}$/[$MeAIB_2$] shown on the *ordinate* of Fig. 9 are approx-

TABLE VII

Kinetic parameters for MeAIB and Na^+ countertransport in HTC cells

The HTC cells were prepared as described in Fig. 8. The internal [^{14}C]MeAIB concentration, [$MeAIB_2$], was estimated radiochemically in parallel experiments. Prior to the influx experiment, the monolayers were quickly washed (<5 s) with EBS (for MeAIB influx) or with Na^+-free EBS (for $^{22}Na^+$ influx) at 37 °C and then incubated for 1 min (Medium 199) or 20 s (KRB) with [^{14}C]MeAIB or $^{22}Na^+$. The concentration of [^{14}C]MeAIB used ranged from 0.025 to 20.0 mM and the concentrations of $^{22}Na^+$ used ranged from 0.1 and 100 mM. These kinetic parameters ± S.E. were determined by fitting the data to Equation 4 (for MeAIB) or 5 (for Na^+). The average nonsaturable component for MeAIB influx (K^1_d, nanomoles/mg of protein·min^{-1}·mM^{-1}), 1.0 ± 0.3 (Medium 199) or 5.85 ± 1.5 (KRB), or for $^{22}Na^+$ influx 1.70 ± 0.18 was subtracted from the primary data before this numerical analysis.

Apparent kinetic parameter	Medium 199, 14 h, MeAIB influx (Substrate B)	KRB, 14 h	
		MeAIB influx (Substrate B)	Na^+ influx (Substrate A)
V^1_{max}, nmol·mg^{-1} protein·min^{-1}	2.27 ± 0.05	15.4 ± 0.5	13.7 ± 0.4
V^1_{max}/K^1_{mB} or V^1_{max}/K^1_{mA}	14.2 ± 0.9	86 ± 9	23.3 ± 1.8
K^1_{mB} or K^1_{mA}, mM	0.16 ± 0.01	0.18 ± 0.02	0.59 ± 0.07
K^2_{mB}, mM	2.9 ± 0.4	1.0 ± 0.2	0.7 ± 0.1
K^2_{vB}, mM	4.5 ± 0.5	1.4 ± 0.2	5.0 ± 1.0
K^2_{cB}, mM	38 ± 11	10 ± 1.6	8.0 ± 1.0
K^2_{vB}/K^2_{cB}	0.12 ± 0.03	0.14 ± 0.02	0.69 ± 0.08
K^2_{mB}/K^2_{cB}	0.12 ± 0.03	0.14 ± 0.02	0.10 ± 0.01
K^2_{vB}/K^2_{mB}	1.55 ± 0.27	1.40 ± 0.34	7.1 ± 1.8
K^2_{mB}/K^1_{mB} or K^2_{mB}/K^1_{mA}	17.9 ± 2.7	5.7 ± 1.3	1.2 ± 0.2

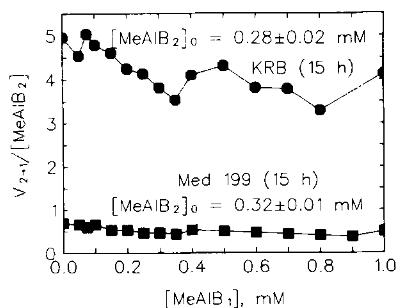

FIG. 9. **The effect of extracellular MeAIB on the efflux of cellular [14C]MeAIB.** HTC cells were incubated with KRB (●) or Medium 199 (■) for 15 h and then incubated for 3 h with EBS containing 20 μM cycloheximide and 0.1 mM [14C]MeAIB. The cells were washed and incubated for 90 (■) or 20 s (●) with EBS containing the extracellular MeAIB concentrations indicated on the *abscissa*. The *ordinate* represents the ratio of efflux, $v_{2\to1}$, to the average cellular [MeAIB$_2$]. This value approximates the magnitude of V^2_{max}/K^2_{mB} because $[MeAIB_2]_0 \ll K^2_{mB}$ (see Table VII).

imations of the apparent values of the kinetic parameter, V^2_{max}/K^2_{mB}, because the initial cellular concentration of [MeAIB$_2$] in this experiment is somewhat lower than K^2_{mB} (see Table VII). This correspondence allows a direct comparison between the data of Figs. 8*B* and 9. Recall our results in Table II which show that the ratio V^1_{max}/K^1_{mB} provides a measure of System A derepression. Therefore the quotient calculated from the value of V_{max}/K_m measured in derepressed cells to the corresponding value of this parameter measured in repressed cells provides a quantitative index of adaptive regulation. In the absence of *trans* substrates, this quotient is 6 for MeAIB influx (Fig. 8*B*) and 7 for MeAIB efflux (Fig. 9). These results suggest that the adaptive increase of System A flux by amino acid starvation occurs to an equal extent in both directions.

The Effect of Ouabain on the Flux and Steady State Distribution of MeAIB and Adaptive Regulation of System A—When ouabain and MeAIB were added simultaneously to monolayers of cultured HTC cells, no change in MeAIB influx measured during 30 s was observed (data not shown). When ouabain was added to the culture medium for 3 h prior to the transport measurements, however, an apparent *trans*-inhibition of MeAIB influx was detected (Table VIII); that is, ouabain caused the observed values of V^1_{max} to decrease and of K^1_{mB} to increase. The result of these changes was a 10-fold decrease in the apparent value of V^1_{max}/K^1_{mB}. The cellular [Na_2^+] measured by the accumulation of $^{22}Na^+$ increased significantly in response to the rising ouabain concentration. The results in Fig. 7 suggest that ouabain does not inactivate System A; rather, the cellular [Na_2^+] is the important parameter for accelerating MeAIB efflux. High cellular [Na_2^+] caused by 10 mM ouabain masks almost entirely the *trans*-inhibition of MeAIB influx induced by cellular [MeAIB$_2$] (Table IX). This result is shown best in Fig. 10 where the hyperbolic inhibition curve of the observed values of V^1_{max}/K^1_{mB} against the varying cellular [MeAIB$_2$] is perturbed by ouabain. The high [Na_2^+] caused by ouabain probably saturates the System A carrier at the inner face of the plasma membrane. As for the fully loaded carrier, the mobility of this partially loaded carrier, System A·Na^+, is lower than that of the empty carrier. Therefore, in the presence of ouabain, cellular [Na_2^+] is high and System A functions slowly as determined by influx because it is *trans*-inhibited. MeAIB$_2$ efflux shows no further *trans*-inhibition by extracellular MeAIB$_1$ when measured in medium containing 160 mM [Na_1^+] (Fig. 9). Consistent with the ouabain-induced inhibition of MeAIB$_1$ influx, high extracellular sodium slows the reorien-

TABLE VIII
Inhibition of MeAIB influx by ouabain

HTC cells were incubated in KRB containing 10% dFBS for 14 h and then incubated with EBS containing the indicated concentrations of ouabain for an additional 3 h. The intracellular sodium concentration was estimated radiochemically with $^{22}Na^+$ in parallel experiments by assuming a constant specific activity of $^{22}Na^+$ inside and outside of the cell. Finally the cells were quickly washed (<5 s) with EBS at 37 °C and incubated for 20 s with [14C]MeAIB between 0.025 and 20 mM. The kinetic parameters ± S.E. which represent the observed influx of MeAIB into HTC cells under these experimental conditions were determined by fitting the data to Equation 1.

[Ouabain$_1$]	[$^{22}Na^+$]	K^1_{mB}	V^1_{max}	V^1_{max}/K^1_{mB}	K^1_{dB}
mM	*mM*	*mM*	*nmol·mg⁻¹ protein·min⁻¹*	*nmol·mg⁻¹ protein·min⁻¹·mM⁻¹*	*nmol·mg⁻¹ protein·min⁻¹·mM⁻¹*
0.0	25	0.16 ± 0.01	15 ± 0.5	87 ± 4	5.6 ± 0.3
0.025	70	0.21 ± 0.03	10 ± 1.0	50 ± 4	3.8 ± 0.2
0.184	108	0.25 ± 0.02	7.1 ± 0.3	29 ± 2	4.8 ± 0.1
0.50	175	0.42 ± 0.2	6.4 ± 2.0	14 ± 2	6.1 ± 0.7
1.36	182	0.34 ± 0.1	3.4 ± 1.0	10 ± 1	5.8 ± 0.4
3.68	216	0.56 ± 0.2	4.4 ± 1.0	8 ± 0.8	5.5 ± 0.4

TABLE IX
The effect of ouabain and internal MeAIB on [14C]MeAIB influx into HTC cells

The HTC cells had been incubated in KRB supplemented with 10% dFBS for 12 h, washed with EBS, and incubated with various concentrations of unlabeled MeAIB in the absence of ouabain (0 mM MeAIB and 0.02, 0.04, 0.10, 0.20, 0.50, 1.0, and 2.5 mM) or in the presence of 10 mM ouabain (0 mM MeAIB and 0.025, 0.06, 0.16, 0.40, 1.0, 2.5, and 10.0 mM). The quantity of MeAIB loaded into the cells was estimated by measuring the accumulation of [14C]MeAIB in parallel experiments. These kinetic parameters ± S.E. were determined by fitting the data to Equation 1. The average nonsaturable influx component (K^1_d, nanomoles/mg of protein·min⁻¹·mM⁻¹) in the absence of ouabain was 5.8 ± 1.5 and in the presence of ouabain was 4.8 ± 1.1.

[Ouabain$_1$] = 0 mM			[Ouabain$_1$] = 10 mM		
[MeAIB$_2$]	K^1_{mB}	V^1_{max}	[MeAIB$_2$]	K^1_{mB}	V^1_{max}
mM	*mM*	*nmol·mg⁻¹ protein·min⁻¹*	*mM*	*mM*	*nmol·mg⁻¹ protein·min⁻¹*
0.0	0.17 ± 0.02	14.6 ± 0.8	0.0	0.59 ± 0.08	7.0 ± 0.7
0.36	0.24 ± 0.03	15.1 ± 1.0	0.07	0.32 ± 0.06	4.5 ± 0.7
0.81	0.22 ± 0.03	11.1 ± 1.1	0.16	0.24 ± 0.12	3.2 ± 1.1
1.9	0.19 ± 0.02	7.1 ± 0.6	0.47	0.26 ± 0.07	3.5 ± 0.7
4.0	0.31 ± 0.08	7.2 ± 1.6	1.0	0.27 ± 0.09	3.8 ± 1.0
12.6	0.22 ± 0.04	3.6 ± 0.4	4.0	0.22 ± 0.06	3.5 ± 0.6
28.0	0.36 ± 0.19	3.5 ± 1.6	15	0.32 ± 0.08	3.0 ± 0.5
54.4	0.52 ± 0.20	3.8 ± 1.3	50	0.50 ± 0.25	2.7 ± 1.2

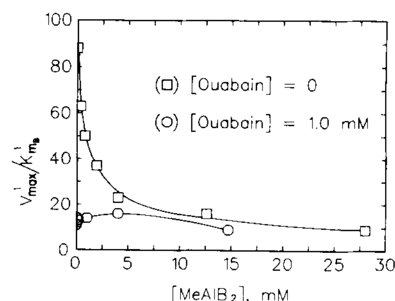

FIG. 10. **The effect of ouabain on the observed value of V^1_{max}/K^1_{mB} for [14C]MeAIB influx into starved HTC cells.** The cells were prepared for this experiment and the influx of [14C]MeAIB was measured as described in Table IX.

tation of System A to the inner face of the plasma membrane, a situation that causes *trans*-inhibition of $MeAIB_2$ efflux. Additional [MeAIB] augments *trans*-inhibition in either direction only slightly in the presence of high sodium concentrations because the relative mobilities of the fully loaded and partially loaded carrier appear similarly sluggish.

Our results in Fig. 11*A* suggest that ouabain decreases cellular accumulation of MeAIB in both starved and nonstarved HTC cells. The data in Fig. 11*B* show for starved HTC cells that ouabain collapses the MeAIB distribution ratio in parallel with an increase of the cellular $[Na_2^+]$. The *inset* to Fig. 11*B* shows that this apparent amino acid starvation caused by ouabain occurs over a wide extracellular concentration range.

At sufficient extracellular concentrations, MeAIB is known to repress the activity of System A. MeAIB is not detectably metabolized so that this effect is exerted exclusively through the presence of MeAIB in the culture system. As noted earlier, a regulatory model to explain this effect involves the occupation of the membrane-binding site by substrates (2). Our results in Fig. 12*A* show that ouabain can prevent MeAIB from repressing System A activity as measured by MeAIB influx. As the ouabain level in the culture medium was increased, the ability of increasing extracellular $[MeAIB_1]$ to repress the transport activity decreased. In another similar experiment shown in Fig. 12*B*, an increasing concentration of ouabain in Medium 199 led to derepression of System A activity measured by influx within the concentration interval chosen for ouabain. This effect was reversible by the removal of ouabain and subsequent incubation of the HTC cells in ouabain-free Medium 199 (Fig. 12*B*). The kinetic results presented in previous sections of this report suggest that

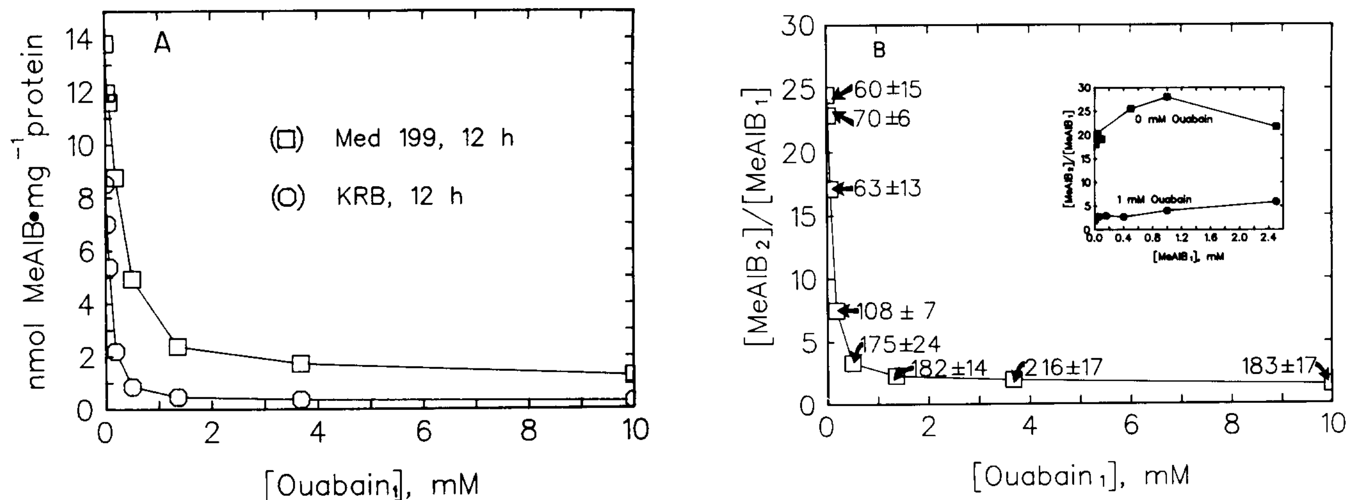

FIG. 11. **The effect of ouabain on the accumulation of MeAIB into HTC cells.** In *A*, HTC cells were incubated in Medium 199 (□) or KRB (○) for 12 h followed by a 3-h incubation in EBS containing 20 μM cycloheximide, 0.1 mM [^{14}C]MeAIB, and the concentrations of extracellular ouabain indicated on the *abscissa*. After this time interval, the cellular MeAIB was determined in triplicate and plotted against $[ouabain_1]$. In *B*, HTC cells were incubated for 12 h with KRB and then incubated for 3 h in EBS containing 20 μM cycloheximide, 160 mM $^{22}Na^+$, 0.1 mM [^{14}C]MeAIB, and the indicated concentrations of ouabain. Following this incubation, the distribution ratio, $[MeAIB_2]/[MeAIB_1]$, and the corresponding $[Na_2^+]$ ± S.D. (apparent millimolar concentration) indicated were determined in triplicate. Under identical conditions, the *inset* shows the concentration dependence of the MeAIB distribution ratio in the presence or absence of 1 mM ouabain.

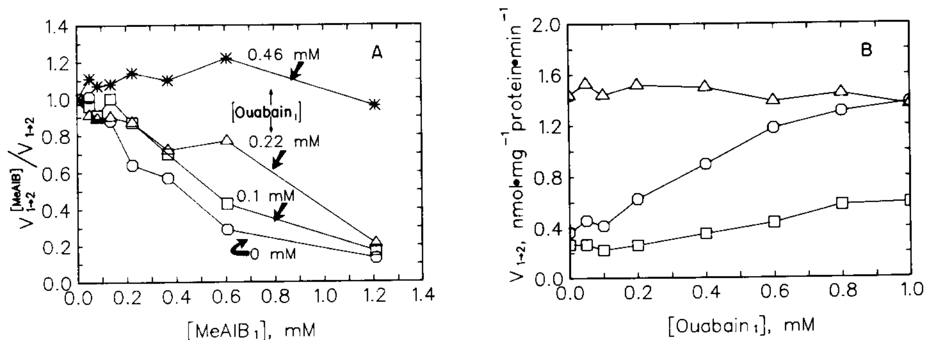

FIG. 12. **Ouabain derepresses MeAIB influx into HTC cells.** In *A*, HTC cells were incubated for 14 h in KRB containing MeAIB at the concentrations indicated on the *abscissa*. Ouabain was also included in this incubation medium at the concentrations indicated. After this 14-h interval, the cells were washed and incubated in sodium-free and amino acid-free EBS containing 20 μM cycloheximide for 3 h. Finally, the influx of 0.1 mM [^{14}C]MeAIB was measured and plotted as a ratio of the influx into repressed cells incubated with the indicated MeAIB concentrations, $v_{1\to 2}^{[MeAIB]}$, to derepressed cells incubated without MeAIB, $v_{1\to 2}$. In *B*, HTC cells were incubated for 14 h in KRB (△) or Medium 199 (○, □), both containing dFBS and the concentration of ouabain indicated on the *abscissa*. Before the influx measurements, all of the cell samples were incubated with amino acid- and Na^+-free EBS containing 20 μM cycloheximide for 3 h. The influx, $v_{1\to 2}$, of 0.1 mM [^{14}C]MeAIB was measured in triplicate during a 30-s time interval into the cells incubated with KRB (△) or half of the cell sample incubated with Medium 199 (○). The remaining HTC cells (□) incubated previously in Medium 199 containing ouabain were incubated for an additional 8 h in Medium 199 without ouabain before the influx measurements.

ouabain does not inhibit the occupation of System A by MeAIB, although it does perturb the typical balance observed between influx and efflux by altering the distribution of Na^+. We suggest that the derepression of System A activity during incubation in Medium 199 containing ouabain results from an apparent amino acid starvation of the cell owing to the collapsed Na^+ gradient (Fig. 11) and not from decrease in the level of occupation of this plasma membrane carrier. Our results show that under defined experimental conditions ouabain increases the apparent cellular $[Na_2^+]$ (Table VIII, Fig. 11), decreases the influx of MeAIB through System A (Table IX, Fig. 10), increases the efflux of MeAIB through System A (Fig. 7), and, as a result of these perturbations of the directional asymmetry between the influx and efflux, collapses the distribution ratio normally maintained by System A. Therefore, at least in the HTC cell, the signal for derepression of System A is likely to come from sensing of the intracellular amino acid levels and not from the degree of substrate saturation of the System A membrane site or from the extracellular amino acid concentration.

FURTHER DISCUSSION

Adaptive regulation of System A alone cannot maintain the cellular concentration of reactive amino acids at a constant level during fluctuations of the concentrations of these molecules in the extracellular space; that is, adaptive regulation does not seem to enhance the concentrative behavior of transport system A. The distribution ratio is determined to a large degree by the chemiosmotic forces acting on the transport system (30). However, the equal and parallel change in transmembrane influx and efflux arising presumably from a change in the number of functional membrane transport proteins does not mean that adaptive regulation is futile. Even given that the steady state distribution ratio is not changed by adaptive regulation, the steady state due exclusively to transport in many real situations will not be even approximately attained. If we look at Fig. 2*A*, we can see that after 3 h the steady state for MeAIB distribution in repressed cells is still many hours away. If the hypothetical natural amino acids to which MeAIB corresponds are at the same time consumed even at a modest rate within the cell, a different steady state constructed in part from the net metabolic flux will be observed. A high rate of cellular amino acid consumption would cause an apparent intracellular starvation of the relevant amino acids leading in turn to derepression of System A much as ouabain caused cellular amino acid starvation and derepression (Fig. 12). Therefore, we suggest that adaptive regulation may find its primary physiological role as a regulator of amino acid metabolism through a control of the inward and outward flows, rather than as a direct response to extracellular amino acid deprivation.

If instead a free amino acid were produced within the cell to feed the organism, efflux may well dominate over influx, allowing a net outward flow to the degree determined by repression and derepression. Initially, exodus may be faster than *de novo* synthesis; however, as extracellular amino acid levels rise, cellular amino acids will also accumulate to a level that represses transport activity. Repression would be expected to slow efflux. The decisive consideration will be whether membrane transport is rate-determining for metabolism. Two recent reports consider how alanine transport limits hepatic alanine consumption (31, 32). Of course, the amino acid flows are further complicated by the involvement of overlapping transport agencies which operate in the plasma membrane of all cells (2, 7, 26, 33–35) and of hepatocytes specifically (36) to provide a balanced interorgan nutrition (37).[4]

The usefulness of ouabain in manipulating the cellular $[Na_2^+]$ in the presence of cycloheximide to freeze the cells at a given degree of transport repression depends on the observation, confirming the experience of others, that the ouabain treatment does not directly influence the amino acid fluxes. Ouabain has served to separate to a degree the occupation of site A for amino acid transport and the internal levels of an amino acids as possible factors, signaling adaptive regulation of System A; this method supports the latter as the effective factor.

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[4] *Footnote Added in Proof*—What does our finding mean for the question, are interorgan amino acid flows determined by regulation of membrane transport or of cellular enzyme activity? For the particular case of alanine, flow is largely toward metabolism in the liver. Fafournoux *et al.* (Fafournoux, P., Remesy, C., and Demigne, C. (1983) *Biochem. J.* **210,** 645–652) asked this question for hepatic alanine utilization by fed rats adapted to diets containing 5 to 90% casein. Exogenous alanine delivery to and clearance by the liver were greatly increased by the 90% casein diet, although remarkably, at the same time, the hepatic alanine content *fell* with rising casein load. The following paradox arose. 1) At each alanine load tested by casein feeding, alanine transport into the liver remained rate-limiting to hepatic alanine utilization, extending earlier *in vitro* findings by Groen *et al.* (31). Our present results show that this relation is necessary to keep adaptive regulation effective. 2) What kept transport rate-limiting throughout the wide range of 5 to 90% casein was, however, a coordinate regulation of alanine transaminase activity.

Because it remains rate-limiting, transport must be the immediate target of regulation; but transamination serves as an ancillary target because this process must, we note, be modulated to keep effective the immediate target. One can only speculate whether accelerated transamination might lower cellular levels of transport-repressive amino acids enough to occasion an adaptive regulation of transport.

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