Regulation of Amino Acid Transport Systems by Amino Acid Depletion and Supplementation in Monolayer Cultures of Rat Hepatocytes*

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The effect of amino acid depletion and supplementation on the transport of amino acids was examined in monolayer cultures of rat hepatocytes. The activities of the amino acid transport systems were investigated by determining the uptake of either \(\text{a amino}[1-\text{H}]\text{isobutryric acid (AIB)}\) or \(\text{L-}[U-\text{H}]\text{leucine}\). The L system (sodium-independent) was distinguished from the A system (sodium-dependent) by examining the uptake of AIB in sodium-containing and sodium-free media, or by examining the uptake of \([14\text{C}]\text{leucine}\). The ASC system was separated from the A system by examining the uptake of AIB in the presence of excess N-methyl-AIB. The rate of uptake of AIB increased almost linearly from 2 to 24 h of amino acid starvation and the plateau was maintained up to 48 h of amino acid starvation. When fully induced, there was approximately 7- to 8-fold increase in the uptake of AIB. The increase in the uptake of AIB was solely due to increased activity of the A system. While amino acid depletion did not affect the uptake of leucine, preincubating the cultures with cold leucine almost doubled the uptake of \([14\text{C}]\text{leucine}\) (trans-stimulation). Amino acid-starvation-induced uptake of AIB could be completely blocked by cycloheximide, puromycin, and AIB, and partially by actinomycin D. Kinetic studies showed that the increased activity of the A system during amino acid depletion was due to increase in the \(V_{\text{max}}\) while the \(K_{M}\) was unchanged. By using a combination of cycloheximide or puromycin along with adding or removing AIB from cultures, it was found that the increased activity of the A system resulting from amino acid depletion is due to both derepression and release from transinhibition. Transinhibition was found to be saturable and dependent upon AIB concentration. Amino acid starvation induced not only the influx but also the efflux of AIB. Further amino acid depletion increased both the rate and cumulative uptake of AIB.

Three distinct systems for the transport of neutral amino acids have been characterized in the Ehrlich ascites cells (1). Two of these, \(\text{i.e.}\) the A (alanine-prefering) and the ASC (specific for alanine, serine, and cysteine), are \(Na^+\)-dependent while the third, \(\text{i.e.}\) the L system (leucine-prefering) is \(Na^+\)-independent. Similarly, the rat hepatocytes in suspensions (2) and monolayers possess three systems for the transport of neutral amino acids. Prolonged incubation of several animal tissues (3-8) and cell types (9-13) in an amino acid-free medium ("starvation") has been reported to cause an adaptive increase in the uptake of neutral amino acids. The increased transport resulting from amino acid depletion involved only the A system without any change in the transport activities of the ASC and L systems (14). This adaptive increase in the activity of the A system requires de novo RNA and protein synthesis (5, 6, 13, 15-17) and has been attributed to the derepression of synthesis of transport protein(s) in the absence of amino acids. On the other hand, in BALB/c3T3 cells this increase has been ascribed to release from transinhibition (12), which is defined as the decrease in transport resulting from high concentrations of intracellular amino acids (1).

Despite the fact that amino acid starvation has been found to increase the amino acid transport in several tissues and cell types, there are some controversial reports regarding this phenomenon in some of the tissues and cell types. Particularly contradicting are the reports on liver slices and hepatocytes in culture. Thus, an adaptive increase in the transport activity of A system was observed by some (8) and denied by others (3) in liver slices from adult rats. Similarly, suspension cultures of adult rat hepatocytes showed some increase in amino acid transport within 2 h of incubation in an amino acid-free medium (11), while monolayer cultures of the same cell type failed to show any such increase in amino acid transport up to 10 h in amino acid-free medium (18). Some workers (5, 18) have emphasized that this adaptive increase in the transport activity of A system is found only in the tissues or cells from fetal or newly born animals and not in those from adult animals.

No studies have so far been conducted on hepatocytes to demarcate the mechanisms and the systems of transport involved in the increased amino acid transport resulting from amino acid starvation. The present studies were, therefore, undertaken to investigate the above questions in monolayer cultures of adult rat hepatocytes as a prelude to our studies on hepatoma cultures. In addition, the kinetics of amino acid transport, the need for macromolecular synthesis during this adaptive increase in transport, and the reversibility of this increase have been examined. Using combinations of \([14\text{C}]\text{-AIB}\) and nonradioactive AIB it was possible to study the effect of various concentrations of AIB on its own transport in experiments not previously carried out. Since AIB does not appear to have metabolic derivatives, the results imply that free AIB (and hence free neutral amino acids) may have regulatory effects per se.

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1 Unpublished results.

2 The abbreviations used are: A system, alanine-prefering; ASC system, specific for alanine, serine, and cysteine; L system, leucine-prefering; AIB, \(\alpha\)-aminoisobutyric acid; WO/BA-M2, Waymouth's MB 752/1 modified medium; S-77, Swim's S-77 modified medium; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
**EXPERIMENTAL PROCEDURES**

**Materials**

Male albino rats were purchased from Sprague-Dawley, Waymouth's MB 752/1 medium (10) was purchased from KC Biological and was supplemented with 0.2% albumin and 0.02 mM oleate (20) and 0.5 mM serine, 0.4 mM alanine, and 5.8 mM glutamine (21). This modified medium was referred to as WO/BA-MS medium. (22) was bought from GIBCO and was modified to contain 4 mM glutamine but lacking serum, here referred to as S-77 medium. Collagen type I and II, cycloheximide, puromycin dihydrochloride, penicillin G (sodium salt), α-aminobutyric acid (AIB), and fatty acid-free serum medium were supplied by Sigma. Actinomycin D and α-methylaminoiso- butyric acid were obtained from Calbiochem, Streptomycin sulfate, U.S.P. and Gentamicin were obtained from General Biochemicals and Schering Corp., respectively. Part of the α-methylaminoisobutyric acid was a kind gift from Dr. H. N. Christensen of University of Michigan. α-Amino-β-Citrabutyric acid, -[U-14C]leucine, and -[2-14C]citruline were received from New England Nuclear. All other chemicals used were of reagent grade.

**Methods**

**Cell Isolation and Culturing**—Male rats raised on a 30% protein diet with a controlled lighting (12 + 12) and feeding (2 + 22) schedule (23) were used for isolation of liver cells. The animals weighed 125 to 250 g and were without food for 22 hr prior to liver perfusion. The hepatocytes were isolated by the modified (24) collagenase perfusion technique described by Sorrentino (25). The cells were suspended to a final concentration of 1 million/ml in WO/BA-MS containing collagenase (0.5 mg/ml), insulin (0.5 μg/ml), and bovine serum albumin (1 mg/ml) was used for perfusing the liver. About 4 to 8 × 10^7 cells, with 70% or more excluding trypan blue, were obtained from a single liver. The cells after two washings with S-77 medium were then washed once in Hanks' HEPES medium containing insulin, 0.5 μg/ml and 15 milliunits/ml. Three milliliters of the cell suspension was then inoculated into 60-mm Falcon plastic dishes which had previously been coated with rat-tail collagen (21). The culture dishes were then kept in an incubator maintained at 37°C and an atmosphere of 5% CO2 + 95% air. Four hours after inoculation, the old medium was replaced with fresh WO/BA-MS containing insulin. After 28 h of culturing, cells from each dish were rinsed twice with 3 ml each time of S-77 medium and then maintained in another 3 ml of the same medium. When cultures were maintained in WB/BA M2 or S77 medium up to 120 h, the medium was either changed every 24 h or not changed after the change of 28 h of culture age. Insulin was present only in WO/BA-2 medium used at 0 and 4 h of culture feeding. Penilicillin G (100 units/ml), streptomycin sulfate (50 μM), and (26) were always included in both S-77 and WO/BA M2 media. Unless specified otherwise, the amino acid starvation started 8 h after transferring the cultures to S-77 medium (36 h after start of culturing). The starvation medium was Hanks' HEPES balanced salt solution, which was compared at the outset with both S-77 and WO/BA-MS medium. The cells in each dish were rinsed twice with 3 ml each time of this medium and then incubated with another 3 ml. After transfer to the starvation medium the cells were kept in an air incubator at 37°C and the rate of amino acid transport was examined at several different times (1 to 48 h) after the onset of amino acid starvation.

The dependence of increased transport during amino acid starvation on RNA and protein synthesis was explored by adding actinomycin D (5 μg/ml) or cycloheximide (28 or 280 μg/ml) at the onset of amino acid starvation and assaying the rate of AIB uptake 12 h later. In another series of experiments, cycloheximide (28 or 280 μg/ml) or puromycin (100 or 500 μg/ml) were added to 12-h starved cells and transport activity was measured at various times (% to 6 h) after the addition of these inhibitors. The extent of RNA and protein synthesis inhibited by actinomycin D or cycloheximide/puromycin was investigated by examining the incorporation of [3H]citruline or [14C]citruline within 30 min into acid-precipitable RNA and protein, respectively, by cells which had previously been treated with or without these inhibitors for 12 h. The Nucleoprobe II and counted in a Nuclear Chicago scop 300 liquid scintillation system. Protein was estimated by the method of Lowry et al. (26) using bovine serum albumin as a standard. The rate of AIB uptake was expressed as nanomoles of AIB/mg of protein/4 min.

The distinction between the A and ASC systems was based upon the specificity of N-methyl-AIB for the A system only. In such experiments, the uptake of [3H]AIB was examined in sodium-containing medium, with or without 30 mM N-methyl-AIB. Any increase in the uptake of AIB in the presence of excess N-methyl-AIB is due to increased activity of the ASC system. The A system was distinguished from the L system by two different methods: (a) sodium dependence of AIB (in this method, uptake of AIB was examined in sodium-containing medium, 0.1 M AIB, 4.5 h, Hanks' HEPES, N-methyl-AIB, and 2 mM glucose was added to the medium containing [3H]AIB. The uptake of AIB was linear at least up to 8 min. The dishes were kept at 4°C until the cells were removed from the incubator to a 1 ml of 0.2 M NaOH. The dishes were then rinsed with another milliliter of 0.2 M NaOH and the two scrapings were pooled. The cell suspension was thoroughly mixed and aliquots were taken for assay of radioactivity and protein content. For radioactivity, 1.0 ml of the cell solution, after neutralizing with 0.6 M of 0.4 N perchloric acid, was mixed with 15 ml of RIA Solv II and counted in a Nuclear Chicago scop 300 liquid scintillation system. Protein was estimated by the method of Lowry et al. (26) using bovine serum albumin as a standard. The rate of AIB uptake is expressed as nanomoles of AIB/mg of protein/4 min.

**Assay for AIB Transport**—Three separate culture dishes were used for each treatment in every experiment for the uptake of AIB or leucine. To examine the rate of AIB uptake, the culture dishes were washed once in a water bath at 37°C. The medium was aspirated and the culture dishes were rinsed twice with 3 ml of Hanks' HEPES minus glucose medium. Two milliliters of 1 mM AIB, containing 0.1 μCi of [1-14C]AIB/ml, dissolved in Hanks'/HEPES minus glucose medium, were then added to each culture dish and the dishes were incubated at 37°C for 4 min. At this time, medium, was aspirated, and the culture dishes were rinsed twice with 3 ml of Hanks'/HEPES minus glucose. The cumulative uptake of AIB was examined in a manner similar to the rate studies, except that the dishes were incubated in an air incubator instead of a water bath and that 5.5 mM glucose was added to the medium containing [14C]AIB. The uptake of AIB was linear at least up to 8 min. The dishes were kept at 4°C until the cells were scraped with a rubber policeman in 1 ml of 0.2 M NaOH. The dishes were then rinsed with another milliliter of 0.2 M NaOH and the two scrapings were pooled. The cell suspension was thoroughly mixed and aliquots were taken for assay of radioactivity and protein content. For radioactivity, 1.0 ml of the cell solution, after neutralizing with 0.6 M of 0.4 N perchloric acid, was mixed with 15 ml of RIA Solv II and counted in a Nuclear Chicago scop 300 liquid scintillation system. Protein was estimated by the method of Lowry et al. (26) using bovine serum albumin as a standard. The rate of AIB uptake was expressed as nanomoles of AIB/mg of protein/4 min.

**Amino Acid Starvation and AIB Uptake**—The kinetic parameters were determined by examining the 4 min uptake of 0.25, 1.0, 3.0, 7.5, 15, and 20 mM AIB both in sodium-containing and sodium-free Hanks'/HEPES media, respectively. At 12-h starved cells or cells that have been maintained in S-77 medium for 20 h. The sodium-dependent component of AIB transport was determined by subtracting the AIB uptake in the absence of sodium from the total uptake. The amino acid concentration was determined by Lineweaver-Burk and Edie-Hofstee plots.
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an amino acid-free medium on AIB uptake, from 1 to 48 h, is shown in Fig. 1 in comparison with data from cultures maintained in S-77 or WOBA-M2. These results show that up to 1 h after transferring to an amino acid-free medium, there is no increase in the rate of AIB uptake, while after that there is a linear increase up to about 24 h when the plateau is reached and maintained up to 48 h of amino acid starvation. There is a maximum of 7- to 8-fold increase in the rate of AIB uptake while after that there is a decrease.

Results from separate experiments showed that the cells maintained in WOBA-M2 medium had one-half to one-third the basal rate of AIB uptake compared to the cells maintained in S-77 medium and remained at or near the basal rate for the entire time, in contrast to cells in S-77 (Fig. 1). This difference reflects the repression of amino acid transport protein(s) by high amino acid concentration of WOBA-M2 medium compared to S-77. However, the rate of AIB uptake when maximally induced due to amino acid depletion was about the same, whether the cells were in WOBA-M2 or S77 medium, prior to the onset of amino acid starvation.

**Cumulative Uptake of AIB during Amino Acid Starvation**—The rate of AIB uptake during amino acid starvation could be increased due to new synthesis of transport proteins (derepression) or because of release from transinhibition, or both. However, if the cumulative uptake of AIB during amino acid starvation is also maintained higher, this would suggest that derepression is one of the major factors contributing to increased uptake of AIB during amino acid starvation. Results presented in Fig. 2 show that the cells previously starved for amino acids maintained a greater accumulation of AIB than nonstarved cells, up to 5 h after AIB addition, thus suggesting that more AIB transport protein was actually synthesized and made available for AIB transport during amino acid starvation.

**Efflux of AIB during Amino Acid Starvation**—The rate of

Fig. 1. Time course of amino acid starvation and AIB uptake. Thirty-six hours after culturing (8 h after transfer to S-77 medium), the cells were rinsed twice with Hanks'/Hepes medium and then incubated in the same medium (○). In all the subsequent figures, amino acid starvation of cultures started at the same age of cultures as in this figure. The 4 min uptake of AIB was determined at various times (1 to 48 h) after transfer to the Hanks'/Hepes medium. The data presented are the mean ± S.E. from nine different experiments, excepting the 36 and 48 h of starvation, which represent only four and two experiments, respectively. Each experiment utilized three separate culture dishes for each time point. The figure includes data (three to five experiments) for cells incubated in S 77 without change (●) or with changes shown by arrows (○) and in WOBA-M2 without change (□) or with change (▲). When the medium is changed, transport was examined 20 h after the medium change. For the first 28 h, WOBA-M2 medium contained insulin (0.5 μg/ml), while at later times none of the media contained insulin.

Fig. 2. Cumulative uptake of AIB. The amount of AIB accumulated in 4, 10, 30, 60, 120, 180, 240, and 300 min was determined in both 12 h starved and nonstarved cultures. Each data point is the mean of triplicate determinations, while the standard errors are within the symbols.

AIB efflux was determined for two reasons: (a) the decreased rate of efflux during amino acid starvation could misleadingly give greater rate of influx, and (b) if both the rates of influx and efflux are increased during amino acid starvation, the same transport protein could be involved in both these processes. To determine the rate of efflux, cells previously starved for 12 h or those continuously maintained in amino acid-containing medium were incubated 1 h with 1 μM [3H]AIB and then transferred to AIB-free medium. Medium from the cultures was replaced and saved after 5, 15, 30, and 60 min of efflux. Radioactivity was determined in the media recovered as well as that remaining in the cells. The total AIB concentration was determined by adding the radioactivity obtained in the media and that remaining in the cells. The per cent efflux was calculated based upon the total AIB concentration. Each data point is the mean of the determinations made on three separate culture dishes.

Fig. 3. Efflux of AIB. Cultures starved 12 h for amino acids or those maintained in S77 medium were incubated 1 h with 1 μM [3H]AIB and then transferred to AIB-free medium. Medium from the cultures was replaced and saved after 5, 15, 30, and 60 min of efflux. Radioactivity was determined in the media recovered as well as that remaining in the cells. The total AIB concentration was determined by adding the radioactivity obtained in the media and that remaining in the cells. The per cent efflux was calculated based upon the total AIB concentration. Each data point is the mean of the determinations made on three separate culture dishes.

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cells, although the absolute amount of AIB retained was slightly higher in the starved than nonstarved cells. From an increase in both the influx and efflux of AIB during amino acid starvation, one is tempted to conclude that the same system of transport is involved both in the influx and efflux of AIB. The above inference may be true but should be taken with caution since the rate of efflux will also depend on the intracellular AIB concentration and the starved cells had about 3 times more intracellular AIB than the nonstarved cells at the onset of efflux. Thus, the energy-independent diffusion of AIB should also be higher in the starved than in the nonstarved cells. It would therefore be desirable to determine the rates of efflux either when the intracellular AIB concentration is the same in both the starved and nonstarved cells or to determine the rate of efflux both in the presence and absence of sodium.

Amino Acid Starvation and Activities of A, ASC, and L Systems—When the rate of uptake was examined, using 1 mM AIB at pH 7.4 and 37°C, in monolayer hepatocyte cultures, about 85% of the AIB transport was through the A system, about 5% through the ASC system, and the remaining 10% was through the L system and simple diffusion. When the uptake of AIB was examined in a sodium-free medium, no increase in AIB uptake was found up to 12 h of amino acid starvation, suggesting thereby that the increase in AIB transport represented only the sodium-dependent systems. In another series of experiments, the uptake of leucine, which is preferably transported through the L system, was examined. As shown in Fig. 4, there was no increase in the uptake of leucine after 19 h of amino acid starvation. These results suggest that amino acid starvation did not regulate the L system of transport. A decline in the uptake of leucine due to amino acid depletion could not be observed even when the cultures were provided with fresh S-77 medium for 1 h prior to the onset of amino acid starvation. Although the activity of the L system did not change with amino acid depletion, preloading the 12-h starved cultures for 1 h with 10 mM cold leucine caused about 2-fold increase in the uptake of radioactive leucine. This increase in the activity of the L system is due to exchange-diffusion between the radioactive and nonradioactive leucine and has been termed trans-stimulation by earlier workers (1). When the uptake of [14C]AIB was examined in the presence of 30 mM N-methyl-AIB (which is specific for the A system) in sodium-containing medium, no increase in the uptake of AIB could be detected at 12 h of amino acid starvation, suggesting that the starvation-induced increase in the uptake of amino acids did not involve the ASC system of amino acid transport either. From these results it is concluded that in hepatocyte cultures amino acid starvation increased the uptake of AIB only through the A system.

Kinetics of AIB Transport during Amino Acid Starvation—After having established that the increased transport of AIB resulting from amino acid depletion involved only the A system, experiments were designed to investigate if the increase in AIB uptake was due to an increase in V_{\text{max}} or due to lowering of K_{m}. Because of only a minor contribution of ASC system in the uptake of AIB, we have not separated it from the A system while determining the kinetic parameters. In these studies we have separated only the sodium-dependent and sodium-independent systems for AIB transport. In these experiments, the rate of uptake of AIB was examined, employing AIB concentrations of 0.25, 1.0, 3.0, 7.5, 15.0, and 20.0 mM, both in sodium-free and sodium-containing media. Results presented in Fig. 5A show that the uptake of AIB at all these AIB concentrations in sodium-free medium was approximately the same in cultures which had been starved for amino acids for 12 h or continuously maintained in S-77 medium for 20 h. The same figure also shows a marked increase in the uptake of AIB from sodium-containing medium at all these concentrations of AIB by those cultures which had been starved for amino acids. By subtracting the sodium-independent component from the values obtained in the presence of sodium, the sodium-dependent component was determined and found to be markedly higher in cultures depleted of amino acids. The Lineweaver-Burk and Eadie-Hofstee plots for the sodium-dependent component of AIB uptake are shown in Fig. 5, B and C. From both of these plots, the V_{\text{max}} values of 2.6 and 15.1 nmol of AIB/mg of protein/4 min were obtained for the cultures maintained in amino acid-containing and amino acid-free media, respectively. From the same figures, K_{m} values of 1.1 and 1.3 mM AIB were obtained for the cultures provided with or without amino acids. These results show that the increase in AIB uptake caused by amino acid depletion is due to an increase in the V_{\text{max}} while there is no change in the K_{m}.

Need for RNA and Protein Synthesis to Elicit the Starvation Effect—To examine the role of RNA and protein synthesis for the induction of AIB transport due to amino acid depletion, cycloheximide (1.0 or 0.1 mM) or actinomycin D (4 \mu M) were added to separate cultures at the onset of amino acid starvation and the rate of AIB uptake examined 12 h after their addition. Fig. 6A shows that cycloheximide, at both the concentrations tested, completely blocked the increase in the AIB uptake due to amino acid depletion while actinomycin D partially blocked the increase. In another series of experiments, cycloheximide (1.0 mM) or puromycin (500 \mu g/ml) were added to cultures that had already been starved 12 h for amino acids and the rate of AIB uptake was examined 3, 6, 9, and 12 h thereafter. Results from such experiments shown in Fig. 6B again demonstrate that both puromycin and cycloheximide could block any further increase in the rate of AIB uptake after their addition. Up to 12 h after the addition of inhibitors to cultures maintained in amino acid-free medium, there was no decline in AIB uptake. This lack of decline in the transport activity suggests a rather long half-life for the transport pump protein(s) under these conditions. The complete inhibition of increase in AIB uptake by both cycloheximide and puromycin leads one to think that the increase in the rate of AIB uptake due to amino acid starvation is entirely due to derepression and that there is no contribution made by the release from trans-inhibition. Such an interpretation is not
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FIG. 5. A, uptake of AIB as a function of its concentration in the presence and absence of sodium by nonstarved and 12-h starved cultures. The 4-min uptake of [¹⁴C]AIB using AIB concentrations ranging from 0.25 to 20 mM was examined in the absence of sodium by cultures maintained in S-77 medium (△) or those starved for amino acids (○). Similar determinations on AIB uptake were made on separate cultures (in the same experiments) in the presence of sodium by starved (○) and by nonstarved (●) cultures. The sodium-dependent component was determined by subtracting the AIB uptake in the absence of sodium from the uptake in the presence of sodium and is shown by □ for starved and by ■ for nonstarved cultures. The data presented are the mean from nine determinations (three experiments) while standard errors are within the symbols. It should be noted that the sodium-independent component is nonsaturable and approximately the same in both starved or nonstarved cultures. B, Lineweaver-Burk plot of the sodium-dependent component for AIB uptake. The data used are from A. Nanomoles of AIB taken up in 4 min/mg of protein are abbreviated as V. C, Eadie-Hofstee plot of the sodium-dependent component for AIB uptake. The data used are from A. Nanomoles of AIB taken up in 4 min/mg of protein are abbreviated as V.

FIG. 6. A, effect of cycloheximide and actinomycin D on the starvation-induced uptake of AIB. Cycloheximide or actinomycin D were added to the cultures at the onset of amino acid starvation and the uptake of [¹⁴C]AIB was examined 12 h later. The results presented are the mean ± S.E. from nine determinations (three experiments). B, effect of cycloheximide and puromycin added 12 h after the start of amino acid starvation on the uptake of AIB. Cycloheximide, puromycin, or no inhibitor were added to 12-h starved cultures and made because in the subsequent experiments we will demonstrate that both these phenomena are operative in hepatocyte cultures. We feel that when these inhibitors are added to the culture medium the utilization of the intracellular pool of amino acids is completely arrested, and thus the amino acid depletion is slowed. An analysis of the intracellular amino acids is desirable to clearly understand the interaction of these inhibitors and amino acid depletion.

Amino Acid Starvation in the Presence of AIB—Since AIB cannot be metabolized by the cell, it was desirable to examine if it will inhibit the induction of its transport due to amino acid depletion, as in the case of natural amino acids (Ala, Ser, Gly, Prol, Thr, etc.). Any of these neutral amino acids, when added to the amino acid-deficient medium to a 10 μM concentration at the beginning of amino acid starvation, partially blocked the increase in AIB uptake (results not presented). The incomplete inhibition of increase in AIB uptake by these natural amino acids is due to their continued use by the cultures. However, when the amino acid-deficient medium was supplemented with 10 mM AIB, there was a complete inhibition of the increase in AIB uptake (Fig. 7). The fact that AIB could completely block the induction of transport pump due to amino acid depletion suggests either (a) the signal for the repression of amino acid transport induction is provided by the free amino acids and not by any of their derivatives or (b) AIB may be able to make some derivative other than aminoacyl-tRNA which may be the final signal for shutting off the synthesis of transport pump proteins. The latter possibility is inferred from the fact that AIB cannot be incorporated into proteins and is under study.

Derepression versus Release from Transinhibition—Analogous to the approach taken by several other workers, our initial experiments using inhibitors of RNA and protein synthesis suggested that the increase in AIB transport during amino acid depletion was all due to derepression. However, we felt that these inhibitors will arrest the cellular metabolic activities and thus interfere with amino acid depletion. In the uptake of [¹⁴C]AIB was examined at 0, 3, 6, 9, and 12 h after the addition of these inhibitors. Results are the mean ± S.E. from six determinations (two experiments).
order to distinguish derepression and release from transinhibition, we starved the cultures for amino acids for 12 h and then provided them with 10 mM AIB in amino acid-free medium for 2, 4, 16, 32, 70, 120, 180, and 240 min. Parallel experiments were conducted using \(^{15}\)C]AIB to determine the intracellular concentration of AIB at all these times. Results shown in Fig. 8 demonstrate that, within 4 min of adding AIB, the pump activity was reduced by 25% of the induced level and, by 32 min, the pump activity dropped to about one-half of the induced level. After 32 min, the decrease in pump activity was gradual and only small. This marked decrease in the pump activity within the first 32 min of adding AIB suggested that the immediate decline after adding AIB was due to transinhibition, while the subsequent gradual decrease was due to repression, caused by the intracellular AIB. To further support this hypothesis, 12-h-starved cultures were incubated with 10 mM AIB for 4 h, after which the AIB-containing medium was aspirated and the cultures were rinsed once with 3 ml of amino acid-free medium and then maintained in another 3 ml of the amino acid-free medium, from 0 to 9 h. After transferring to AIB-free medium, parallel cultures were provided with cycloheximide (1.0 or 0.1 mM) or puromycin (100 or 500 \(\mu\)g/ml) or maintained without any inhibitors. Initial experiments (results not presented), without using any inhibitors, showed that up to 60 min after transferring to AIB-free medium, there was no increase in the pump activity, when the intracellular AIB concentration had already dropped from 160 to 80 nmol/mg of protein. The pump activity started increasing about 90 min after transferring to AIB-free medium and there was a linear increase up to 6 h, when it reached a level found in the 12-h-starved cultures, prior to the addition of AIB (Fig. 9A). Results presented in Fig. 9B show that when cycloheximide, 0.1 mM, or puromycin, 100 \(\mu\)g/ml, were added to cultures that had just been freed of AIB, the pump activity could be partially recovered even in the presence of these inhibitors, although to a lesser extent when compared to cultures without these inhibitors. Results obtained by using higher concentrations of cycloheximide (1.0 mM) and puromycin (500 \(\mu\)g/ml) were comparable to those acquired with the lower concentrations of these inhibitors, as presented in Fig. 9B. The increase in pump activity seen in the presence of these inhibitors represents the release from transinhibition while the activity regained in the absence of these inhibitors represents the sum of both derepression and release from transinhibition. Results presented in Fig. 9B also demonstrate that, within 6 h after transferring to AIB-free medium, maximum pump activity is attained and is maintained at least another 2 to 3 h.

About 8 to 10 h after transferring to AIB-free medium, the pump activity rapidly declined within the next few hours, which may be due to cell death resulting from AIB toxicity or amino acid starvation. This rapid decline in pump activity does not seem to be due to amino acid starvation per se but the presence of AIB may be limiting the utilization of whatever amino acids are available. Such a conclusion is based on the following findings. Cells starved for amino acids maintain their induced pump activity up to 48 h of amino acid starvation, but when 10 mM AIB is added to the amino acid-free medium there is no increase up to 12 h of amino acid starvation; rather, at 24 h of starvation in the presence of 10 mM AIB, the pump activity is reduced to about one-half to one-third the starting uninduced level. If the cells were transferred to AIB-free medium after 12 h in the presence of AIB, the pump activity was induced to a maximum within 8 to 10 h and then within the next 8 to 10 h the pump activity rapidly declined, even when the cultures which were never exposed to AIB were still maintaining their maximally induced pump activity. Similar findings were made when cultures from Fig. 9B were continued in AIB-free medium longer than 8 to 10 h after their transfer to AIB-free medium.

**Dependence of Transinhibition on AIB Concentration**—The maximum transinhibition of AIB pump was attained by an AIB concentration of 60 to 80 nmol/mg of protein (Fig. 8). Similarly in Fig. 9A, the recovery of pump activity did not start until the AIB concentrations fell below 60 nmol/mg of protein. These results suggested that AIB up to 60 nmol/mg of protein may cause dose-dependent transinhibition, while AIB concentrations higher than 80 nmol/mg of protein may have no further transinhibitory effect. To investigate the dependence of transinhibition on AIB concentration, 12-h starved cultures were incubated with 0.1, 1.0, or 10.0 mM radioactive or nonradioactive AIB for 4, 30, and 150
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Fig. 9. A, derepression and release from transinhibition by efflux of intracellular AIB. Twelve-hour-starved cultures were incubated with 10 mM cold AIB or [3H]AIB for 4 h, when the AIB-containing medium was aspirated and cultures were incubated from 0 to 9 h in Hanks'/Hepes medium after once being rinsed with the same medium. Four-minute uptake of [3H]AIB was examined on those cultures previously incubated with cold 10 mM AIB and cultures previously incubated with 10 mM [3H]AIB were used to find the intracellular AIB concentration. Results presented are the mean ± S.E. of nine determinations for 90-min and 9-h time points and of 12 determinations for all other time points. 

B, separation of the contributions made by derepression and release from transinhibition. Twelve-hour-starved cultures were incubated with 10 mM cold AIB for 4 h, at which time the AIB-containing medium was aspirated and cultures were incubated from 0 to 9 h. Four-minute uptake of AIB was examined at 0, 2, 6, 8, and 9 h after such incubations. The increase in the uptake of AIB by cultures incubated without cycloheximide or puromycin represents the sum of increase due to derepression and due to release from transinhibition. Increase in the uptake of AIB shown by cultures incubated with cycloheximide or puromycin is all due to release from transinhibition. Results presented are the mean ± S.E. of nine determinations (three experiments).

Fig. 10. A, dependence of transinhibition on AIB concentration. Twelve-hour-starved cultures were incubated with 0.1 mM AIB (○), 1.0 mM AIB (△), or 10.0 mM AIB (□) (cold or [3H]-labeled) for 4, 30, or 150 min. At the end of each of these time periods, cultures incubated with cold AIB were used to determine the 4-min uptake of 1 mM [3H]AIB and those incubated with [3H]AIB were used to determine the intracellular AIB concentration. Transport activity is expressed as a function of intracellular AIB. Each point is the mean of three determinations; the standard errors are within the symbols.

B, dependence of release from transinhibition on AIB concentration. Twelve-hour-starved cultures were incubated 4 h with 2 mM AIB (○) or 10 mM AIB (□) (cold or [3H]-labeled). At the end of 4 h the AIB-containing medium was aspirated, and cultures were rinsed once with Hanks'/Hepes medium and then incubated in the same medium for 0, 1, 2, 5, 8, 11, or 22 h before the transport activity or the intracellular AIB concentration was determined. Results presented are from a single experiment. Results obtained from another experiment supported the results presented here.

min. Results from such an experiment presented in Fig. 10A show that AIB concentrations up to about 60 nmol/mg of protein caused an increased transinhibition of AIB pump activity. Any further increase in AIB concentration was without any further lowering of the pump activity. In another two experiments, the 12-h starved cultures were incubated 4 h with either 2 or 10 mM radioactive or nonradioactive AIB, after which the recovery of pump activity was monitored by transferring to an AIB-free Hanks'/Hepes medium. Fig. 10B shows that cultures incubated with 2 mM AIB started an increase in the pump activity within 1 h of their transfer to AIB-free medium, while those maintained in 10 mM AIB did not show any increase until after 90 min of transferring to AIB-free medium. In cultures incubated either with 2 or 10 mM AIB and subsequently transferred to AIB-free medium, the recovery of pump activity did not start until the AIB concentration fell below 40 to 50 nmol/mg of protein. The subsequent recovery, after the intracellular concentration of AIB dropped below 50 nmol/mg of protein, was at about the same rate in cultures previously maintained in either 2 or 10 mM AIB. Even the delayed decline in AIB pump activity was found to be at approximately the same rate in both these cultures, although the absolute AIB pump activity at any time remained higher in cultures preincubated with 2 mM than in
those preincubated with 10 mM AIB. Taken together these results show that the transinhibition caused by AIB is a saturable phenomenon and maximum transinhibition is attained by AIB concentration of about 50 nmol/mg of protein.

**DISCUSSION**

Our results, showing increased uptake of AIB caused by amino acid depletion, in hepatocyte monolayer cultures are in agreement with the earlier observations in liver slices (8) placenta (4, 7) uterus (6), and several other tissue and cell types (5). Similar observations were made in HTC cells (9), Ehrlich ascites cells (10), BALB/3T3 cells (12), thymic lymphocytes (13), and liver cell suspensions (11). The failure to detect this starvation-induced increase in the uptake of AIB in liver slices (3) and kidney slices (5) from adult animals may perhaps be due to the fact that these investigators kept these tissues in amino acid-free medium only up to 2 h, which may not be long enough to demonstrate the starvation effect in these tissues under the experimental conditions employed. In our case, no increase in AIB uptake could be observed until after 1 h of transferring the hepatocyte cultures to amino acid-free medium. Our results do not agree with the earlier comments from our laboratory (18) that hepatocyte cultures prepared from adult rats failed to show any increase in AIB uptake up to 10 h of maintaining in amino acid-free medium. We here report that in the hepatocyte cultures there was a linear increase in the AIB pump activity from 2 to 24 h after their transfer to an amino acid-free medium. We are not in a position to point out the exact reason for the failure to detect this starvation-induced uptake of AIB by Kletzien et al. (18), but our preliminary results have shown that this amino acid starvation-induced uptake of AIB is very sensitive to the pH of the amino-deficient medium. We were unable to see any increase in the uptake of AIB when the cultures were maintained for 6 h at pH 6.0 or 6.5 in Hanks'/Hepes amino acid-free medium and the uptake of [14C]AIB was examined at the same pH. However, 6-h incubation in amino-free media at pH 7.0, 7.4, and 8.0 demonstrated the starvation effect in the order pH 7.0 < 7.4 < 8.0 (results not presented). Our preliminary experiments have shown that the pH-dependent increase in the rate of AIB uptake was also sodium-dependent. This enhancement of the starvation effect by increasing the pH from 7 to 8 may not be the result of starvation effect per se. In thymic lymphocytes, increasing pH from 6.5 to 8.0 increased the uptake of AIB, proline, and alanine but not that of leucine (13). These workers concluded that the pH effect was distinct from the amino acid starvation effect. These possible interactions of pH changes and amino acid depletion will be examined in future studies. If the pH of the amino acid-free medium was not carefully monitored, this may explain our discrepancy with these earlier reports (18). In view of our findings along with those of earlier reports, we do not think that amino acid starvation-induced uptake of AIB is a property only of the fetal or neonatal tissues, as suggested by some workers (3, 18).

Our results showing that amino acid depletion causes an increase in the activity of only the A system agree with such findings in HTC (9), BALB/3T3 cells (12), thymic lymphocytes (13), and chick embryo heart cells (14). Amino acid depletion was reported to decrease the L system activity in HTC (9) and in BALB/3T3 cells (12). We have not detected any decrease in the activity of the L system up to 12 h of amino acid starvation, examined either by the uptake of leucine or by the uptake of AIB in sodium-free medium. However, incubating cultures with 10 mM nonradioactive leucine for 1 h, caused a 2-fold increase in the uptake of [14C]leucine, an illustration of the trans-stimulation phenomena due to exchange diffusion (1).

Our results showing that inhibitors of both RNA and protein synthesis blocked the amino acid depletion-induced uptake of AIB are in agreement with results on other types of cells (5, 6, 13, 15–17). However, from these results we do not conclude that the increase in the rate of AIB uptake due to amino acid depletion is all due to derepression as concluded by the workers cited above. We think that the above workers failed to recognize the fact that in the presence of these inhibitors the utilization of amino acids by the cell is almost completely arrested and the only loss of amino acids is due to diffusion into the culture medium. To answer this question, the intracellular amino acid concentrations need to be examined when the cultures are incubated with or without the inhibitors.

By using a different approach of amino acid starvation followed by incubation with 10 mM AIB (which is not metabolized) and then transferring to AIB-free medium supplemented with or without cycloheximide or puromycin, our results have clearly demonstrated that at least in hepatocyte cultures, amino acid depletion causes an increase in AIB uptake both due to derepression and due to release from transinhibition. We feel following such a protocol may show the release from transinhibition in some of the tissues where such a phenomenon has been reported to be nonexistent. To our knowledge, this is the first report where the regulation of the A system is subject to both derepression and release from transinhibition along with the trans-stimulation of the L system. The increased activity of the A system is reflected not only in increased influx but in increased efflux as well. Our K values of 1.1 or 1.3 mM AIB are markedly lower than the earlier reports of 6.6 mM AIB for hepatocyte monolayer cultures (18) or of 30 mM AIB for hepatocyte suspension cultures (2).

Our data show that AIB acts on at least two sites in relation to amino acid transport: it can exert transinhibition and thus decrease transport activity, and it can decrease the synthesis of the transport system. Since AIB appears not to have quantitatively significant metabolic derivatives, the results imply that free AIB (and hence free amino acids) exerts both effects. It is not surprising that free AIB should exert transinhibition, but it is less plausible that free AIB should repress synthesis. Hence, although no derivatives are presently known, a search for AIB derivatives such as tRNA . AIB or an AIB-protein complex will be attempted.

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