Modulation of Amino Acid Transport in Rat Liver Slices by Tissue Preincubation

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In rat liver slices, a prolonged preincubation resulted in a stimulation of AIB and alanine uptake. The increase of transport of both amino acids can be ascribed to a reduction of K_t without an alteration of V_t.

The enhancement of transport seems to be restricted to the A mediation as indicated by the findings that only the Na^+-dependent, MeAIB-inhibitable portion of AIB transport was increased by the prolonged preincubation, and, furthermore, that the uptake of cysteine, a specific substrate for system ASC in liver cells, was not affected.

The effect of an extended preincubation on AIB transport was abolished by the addition of high concentrations of AIB and alanine to the preincubation medium. Several other amino acids tested did not affect the increase of AIB transport.

The observed stimulation of transport seems not to be due to the derepression of the synthesis of transport proteins, as cycloheximide and 5-azacytidine did not block the increase of AIB uptake.

The enhancement of amino acid transport does not appear to be a result of a release from transinhibition since the uptake of alanine was markedly enhanced after 3 h of preincubation while the alanine concentrations in the incubated liver slices did not change significantly.

Introduction

Prolonged incubation in an amino acid – free medium has been found to increase the transport of amino acids in several tissues and cell types of animal origin. The enhanced transport resulting from amino acid depletion involved only the A system, while the transport activities of the L and ASC systems did not change. Mostly, the adaptive increase of amino acid transport into animal cells was dependent on RNA and protein synthesis and have been ascribed to a derepression of the synthesis of transport proteins. In only a few cases the observed increases of transport could be attributed to a release from transinhibition which is expressed upon decreased intracellular amino acid concentrations (reviewed by Guidotti, Borghetti, and Gazzola [1]).

With liver slices and isolated hepatocytes, contradicting results concerning the regulation of amino acid transport by the availability of amino acids have been reported: adaptive increases of AIB transport in liver slices of adult rats were found by Tews *et al.* and Ehrhardt [2, 3]. Others, however, failed to observe in the liver slices system any such increase of transport [4]. Similarly, fresh isolated hepatocytes showed some augmentation of AIB transport after 2 h of preincubation in an amino acid – free medium [5, 6]. In monolayer cultures of hepatocytes, on the other hand, an adaptive increase of AIB transport was not observed [7].

Recently, however, Potter and coworkers could demonstrate that several hours of amino acid depletion resulted in a marked augmentation of Na^+-dependent AIB transport in rat hepatocyte monolayer cultures. The results presented by these authors indicated that the increase of transport could be ascribed to both derepression and release from transinhibition of the A system [8].

In view of the controversial reports on isolated hepatocytes and liver slices regarding the regulation of amino acid transport by the availability of amino acids, the influence of a prolonged preincubation on amino acid transport in rat liver slices was reinvestigated in more detail.

Materials and Methods

Male Sprague-Dawley rats of an own inbred strain were kept at approximately 23 °C under a

Abbreviations: AIB, 2-aminoisobutyric acid; MeAIB, 2-(methylamino) isobutyric acid; A system, alanine preferring system; ASC system, alanine, serine, cysteine preferring system.

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controlled lighting schedule (12:12 h). The animals had access to a standard diet (Altromin®) and water *ad libitum*.

The radioactive compounds used ([^{3}H]inulin, [^{14}C]-AIB, [^{14}C]MeAIB, [^{14}C]alanine) were purchased from Buchler & Co., Braunschweig. Unlabelled amino acids, inulin, dithioerythritol, aminooxyacetic acid, cycloheximide, 5-azacytidine, and NADH were obtained from Sigma, Taufkirchen. L-alanine dehydrogenase was delivered by Boehringer, Mannheim. The inorganic salts and reagents for the buffer solutions were from Merck, Darmstadt.

The methods for the isolation and incubation of the liver slices as well as the calculation of the results were the same as described previously [3].

For the measurement of initial rates of AIB uptake, liver slices were incubated after the desired preincubation period for 10 min with [^{14}C]AIB. Earlier studies had shown that AIB uptake by the liver slices is linear within this time period [9].

Alanine transport was measured in the presence of 0.2 mM aminooxyacetate which inhibits alanine transaminase without affecting alanine transport [10]. On the basis of preliminary experiments, a 10 min-period was chosen to measure initial rates of alanine transport.

In all experiments with cysteine, dithioerythritol was present at 5 mM in order to keep the amino acid in the reduced state.

The concentration of free alanine in the liver slices was estimated by the enzymic procedure described by Williamson [11], which was modified for smaller sample sizes.

In order to avoid influences of the diurnal rythm of AIB transport, all experiments were started at 10.00 h.

Results

The measurement of initial rates of AIB uptake in media containing concentrations of [^{14}C]AIB between 0.1 and 1.0 mM revealed that a 3 h-preincubation of rat liver slices in Krebs-Ringer buffer resulted in a significant increase of transport compared to slices which were preincubated for the routinely used 15 min-period. Lineweaver-Burk transformations of the uptake velocities are depicted in Fig. 1. The data show that the increase of AIB transport due to the extended preincubation can not be ascribed to an augmentation of the maximal uptake velocity but to a lowering of the apparent K_t value (2.4 mM AIB versus 4.2 mM AIB).

The increase of transport seems to be restricted to a Na^+-dependent mediation. This is indicated by the findings that omission of Na^+ from the medium in which AIB transport was measured caused a significant reduction of AIB uptake, and, furthermore, that under this condition the AIB uptake rates did not differ anymore after 180 and 15 min of preincubation (Table I).

As the saturable Na^+-dependent transport of AIB into the liver cells seems to occur through two distinct systems (A and ASC), AIB uptake was measured in the presence of excess MeAIB, *i.e.*, conditions under which the transport of AIB by system A is largely inhibited [12, 13]. The data show that AIB transport is markedly diminished in the presence of 5 mM MeAIB. The resulting AIB uptake

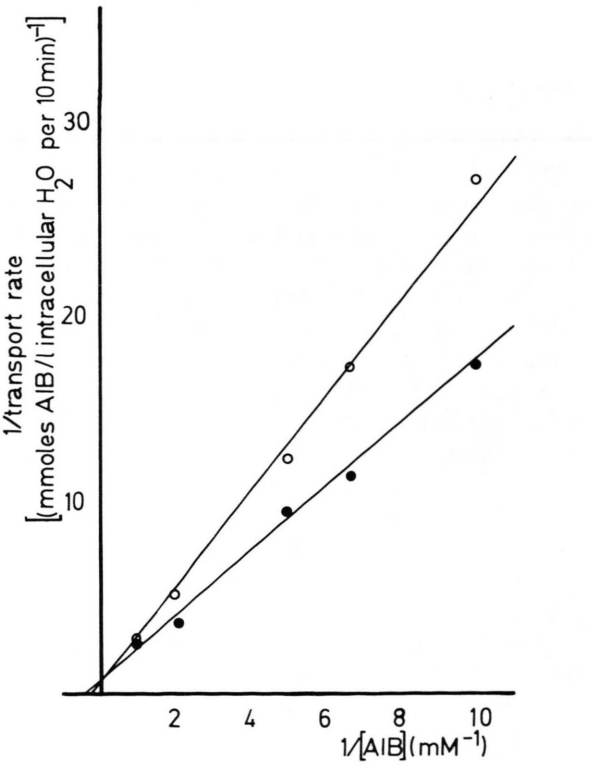

Fig. 1. Lineweaver-Burk plots of AIB transport rates after 15 min and 180 min of preincubation. Liver slices were preincubated 15 min (○) or 180 min (●) in Krebs-Ringer bicarbonate buffer (pH 7.4). AIB transport was measured during 10 min in fresh buffer containing [^{14}C]AIB (0.1–1.0 mM, 0.05 μCi/ml). The straight lines were obtained by linear regression analysis. The points represent the means of 3–8 individual measurements.

Table I. Effect of a prolonged preincubation of Na^+-dependent, MeAIB-inhibitable AIB transport in rat liver slices. Rat liver slices were preincubated in Krebs-Ringer bicarbonate buffer (pH 7.4) either for 15 min of for 180 min. After the preincubation period the slices were transferred into fresh medium which was either Krebs-Ringer bicarbonate buffer ($Na^+ = 143$ mM) or modified buffer (NaCl replaced by choline chloride, $NaHCO_3$ by $KHCO_3$, $Na^+ = 0$ mM). In this medium, which contained [^{14}C]AIB (0.1 mM, 0.05 μCi/ml) and, if desired, MeAIB (5 mM), the liver slices were further incubated for 10 min. Results are expressed as the mean ± S. D. The numbers of experiments are given in parentheses.

Duration of preincubation [min]	Concentration of Na^+ during AIB uptake [mM]	5 mM MeAIB present during AIB uptake	AIB uptake (μmol/l of intracellular water)
15	143	−	36.9 ± 1.3 (5)
15	0	−	25.2 ± 2.9 (5)
15	143	+	25.1 ± 4.1 (6)
180	143	−	57.8 ± 11.7 (8)
180	0	−	25.8 ± 4.8 (5)
180	143	+	26.6 ± 7.2 (6)

rates were hardly to distinguish from those observed in Na^+-free media (Table I). This observation indicates that the bulk of Na^+-dependent AIB transport in the liver slices is mediated by the A system and, furthermore, that the stimulatory effect of the extended preincubation is restricted to this transport system.

In control experiments, the uptake of cysteine, which seems to be a specific substrate for the ASC system in liver cells [13], was measured after 15 min of preincubation as well as after a 3 h-preincubation period. The data show that the uptake of cysteine into the liver slices was not affected by the prolonged preincubation (Table II). This result strengthen the view that the effect of the extended preincubation period is restricted to the A system.

In order to find out whether the increase of AIB transport due to the extended preincubation period can be attributed to a derepression of the synthesis of transport proteins, liver slices were preincubated for 3 h in buffer containing cycloheximide or 5-azacytidine in concentrations that suppress completely the induction of AIB transport in the liver slices by cAMP and glucagon (Ehrhardt, unpublished results). Both antibiotics, however, had no effect on the increase of AIB uptake due to the extended preincubation (Table III).

The possible role of transinhibition was studied by supplementing the medium during the 3 h-preincubation with 5 mM concentrations of several individual amino acids. A comparison of the AIB uptake rates after incubation in amino acid-free and amino acid-supplemented media revealed the the supplementation of the preincubation medium with AIB or alanine, which have been reported to be transported into the liver cell predominantly by the A system [10], produced a significant reduction of the increase of transport (Table III).

The inhibition of AIB transport observed in the experiments with csyteine can obviously not be ascribed to the amino acid itself but to dithioerythritol which was added together with cysteine to the preincubation medium in order to keep this amino acid in the reduced state (Table III).

Table II. Effect of a prolonged preincubation on cysteine transport in rat liver slices. Rat liver slices were preincubated in Krebs-Ringer bicarbonate buffer (pH 7.4) either for 15 min or for 180 min. After the preincubation the slices were transferred into fresh buffer containing [^{14}C]cysteine (0.1 mM, 0.05 μCi/ml) and dithioerythritol (5 mM). In this medium, cysteine transport was measured during 10 min. Results are expressed as the mean ± S. D. The numbers of experiments are given in parentheses.

Duration of preincubation [min]	Cysteine uptake (μmol/l of intracellular water)
15	187 ± 26 (6)
180	196 ± 30 (6)

Table III. Effect of adding several individual amino acids or inhibitors of macromolecular synthesis to the preincubation medium (180 min-preincubation) on subsequent uptake of AIB in rat liver slices. Rat liver slices were preincubated in Krebs-Ringer bicarbonate buffer (pH 7.4) for 180 min. Where indicated, cysteine (5 mM), alanine (5 mM), AIB (5 mM), lysine (5 mM), leucine (5 mM), glutamic acid (5 mM), 5-azacytidine (0.5 mM), cycloheximide (2 μg/ml) or dithioerythritol (5 mM) were added to the preincubation medium. After the preincubation the liver slices were transferred into fresh Krebs-Ringer bicarbonate buffer containing [^{14}C]AIB (0.1 mM, 0.05 μCi/ml) and further incubated for 10 min. Results are expressed as the mean ± S. D. The numbers of experiments are given in parentheses.

Addition to preincubation medium	AIB uptake (μmol/l of intracellular water)
none	57.8 ± 11.7 (8)
cysteine + dithioerythritol	28.0 ± 5.1 (5)
dithioerythritol alone	31.4 ± 1.5 (6)
AIB	29.0 ± 6.6 (8)
lysine	47.8 ± 5.8 (6)
leucine	51.2 ± 5.7 (7)
glutamic acid	51.7 ± 9.7 (6)
5-azacytidine	57.2 ± 9.5 (6)
cycloheximide	57.4 ± 11.2 (6)

The other amino acids tested, lysine, leucine, and glutamic acid, had only weak, insignificant effects on the stimulation of AIB transport (Table III).

The possible role of transinhibition was further investigated by studiing the interrelations between changes of the uptake of a natural substrate for system A and changes in the pool size of this amino acid. Alanine was chosen for these experiments as the addition of this amino acid to the preincubation medium inhibited the increase of AIB transport markedly. The analyses of the alanine content of the liver slices showed that the alanine concentrations did not decrease during the extended preincubation. Furthermore, cycloheximid and 5-azacytidine had no influence on the pool size of alanine. All alanine concentrations measured in the incubated liver slices were nearly the same as in freshly excised liver (Table IV).

In contrast to these observations on the alanine concentrations, however, the uptake of alanine into the liver slices was found to be increased after 3 h of preincubation compared to 15 min preincubated slices. As in the case of AIB, the enhancement of alanine transport due to the extended preincubation can be ascribed to a lowering of the apparent K_t (0.4 mM alanine versus 1.3 mM alanine) without alterations of V_t (Fig. 2).

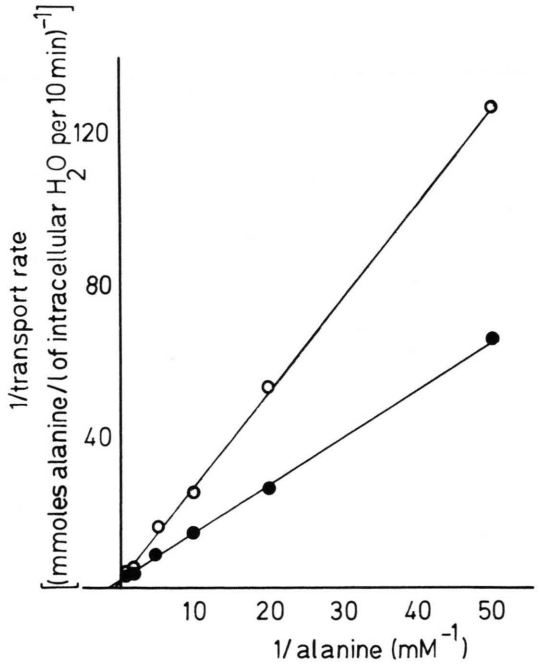

Fig. 2. Lineweaver-Burk plots of alanine transport rates after 15 min and 180 min of preincubation. Liver slices were preincubated 15 min (○) or 180 min (●) in Krebs-Ringer bicarbonate buffer (pH 7.4). Alanine transport was measured during 10 min in fresh buffer containing [^{14}C]-alanine (0.02–1.0 mM, 0.05 μCi/ml) and aminooxyacetate (0.2 mM). The straight lines were obtained by linear regression analysis. The points represent the mean of at least 2 measurments.

Table IV. Effect of duration of preincubation on alanine concentrations in rat liver slices. Alanine concentrations were estimated in freshly excised liver and in liver slices which were preincubated for 15 min or for 180 min in Krebs-Ringer bicarbonate buffer (pH 7.4). Where indicated, cycloheximide (2 μg/ml) or 5-azacytidine (0.5 mM) were added to the buffer. Results are expressed as the mean ± S. D. The numbers of experiments are given in parentheses.

Duration of preincubation [min]	Addition to preincubation medium	Alanine concentration (μmol/g fresh weight)
0	–	1.03 ± 0.22 (5)
15	–	0.89 ± 0.45 (6)
180	–	0.99 ± 0.47 (6)
180	cycloheximide	1.02 ± 0.42 (6)
180	5-azacytidine	1.17 ± 0.19 (6)

Discussion

The data presented here indicate the existence of a control mechanism regulating the uptake of amino acids into the liver cells which is expressed upon extended preincubation of liver slices. Three lines of evidence suggest that the enhancement of transport which is based on a reduction of the amino acid concentration for half-maximal transport velocity, can be attributed to a stimulation of the A system: this is demonstrated by the inhibiting effect of the omission of Na^+ during AIB uptake, by the inhibiting effect of adding excess MeAIB to the medium in which AIB uptake was measured, and, furthermore, by the finding that the uptake of cysteine, a specific substrate for system ASC in hepatocytes [13], is not affected by the prolonged preincubation period.

On the basis that cycloheximide and 5-azacytidine failed to inhibit the observed stimulation of AIB transport, the assumption seems to be justified that the increase of transport due to the prolonged preincubation is not caused by a derepression of *de novo* synthesis of proteins involved with the transport process. The failure to detect with liver slices any indication for the involvement of a derepression mechanism that participates in the increase of amino acid transport, may probably be due to the fact that the liver slices were preincubated

only up to 3 h. In view of the limited cell viability in the liver slice system, however, more prolonged preincubation periods appeared not to be useful.

As the transport of AIB has been reported to be subject to transinhibition by intracellular system A-reactive amino acids [14, 15], the possibility that the enhancement of AIB transport after the prolonged preincubation of the liver slices reflects the release from transinhibition, AIB uptake was measured in liver slices which were preincubated with 5 mM concentrations of several individual amino acids. If transinhibition accounts for the increase of AIB uptake, the presence of high concentrations of single amino acids in the preincubation medium should increase the intracellular concentrations of these amino acids sufficiently to inhibit AIB uptake. The results of these experiments revealed that of the amino acids tested, only AIB and alanine, which are presumably taken up into the liver cell mainly through the A system [10], suppressed the expected increase of AIB transport. This finding alone, however, cannot be taken as a cogent proof for the existence of a regulatory mechanism involving transinhibition. If the enhancement of amino acid transport through the A system after extended preincubation periods reflects really a release from transinhibition, a decrease of the intracellular concentrations of naturally occurring A site-reactive amino acids should be paralleled by an increase of uptake of these amino acids. A comparison of the alanine concentrations, in the incubated liver slices with the alanine uptake rates, however, argue against the assumption that the preincubation-induced increase of transport is due to a release from transinhibition: alanine transport was stimulated while the alanine concentrations in the incubated liver slices did not change significantly.

By using a combination of inhibitors of protein synthesis along with addition or omission of AIB from an amino acid-deficient medium, Kelley and Potter demonstrated that in hepatocyte monolayer cultures the major part of the increase of AIB transport mediated by amino acid starvation is due to the derepression of the synthesis of transport proteins. The other part of the enhancement of transport was ascribed by the authors to a relief from transinhibition [8]. This supposition, however, have to be considered with reservation, as interrelationships between changes of the intracellular pool size of naturally occuring system A-reactive amino acids and changes of the amino acid uptake rates have not been established.

At present, it remains an open question which mechanism is responsible for the observed enhancement of amino acid transport in liver slices. Possibly, an augmentation of the electrical potential difference across the plasma membrane of the liver cells occurs during the extended preincubation of the liver slices which may contribute to the observed enhancement of amino acid uptake. Heinz *et al.* have presented evidence that the transport of AIB is accelerated in Ehrlich Ascites tumor cells by raising the electrical potential difference [16]. Investigations in this direction are currently in progress with isolated hepatocytes, which seem to be a more suitable system for such studies.

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[1] G. G. Guidotti, A. F. Borghetti, and G. C. Gazzola, Biochim. Biophys. Acta **515,** 329 (1978).
[2] J. K. Tews, N. A. Woodcock, and A. E. Harper, J. Biol. Chem. **245,** 3026 (1970).
[3] V. Ehrhardt, Chronobiologia **4,** 205 (1977).
[4] G. G. Guidotti, G. C. Gazzola, A. F. Borghetti, and R. Franchi-Gazzola, Biochim. Biophys. Acta **406,** 264 (1975).
[5] A. Le Cam and P. Freychet, Biochim. Biophys. Res. Commun. **72,** 893 (1976).
[6] D. B. Donner, K. Nakayama, U. Lutz, and M. Sonenberg, Biochim. Biophys. Acta **507,** 322 (1978).
[7] R. F. Kletzien, M. W. Pariza, J. E. Becker, V. R. Potter, and F. R. Butcher, J. Biol. Chem. **251,** 3014 (1976).
[8] D. S. Kelley and V. R. Potter, J. Biol. Chem. **253,** 9009 (1978).
[9] V. Ehrhardt, J. Interdiscipl. Cycle Res. **10,** 125 (1979).
[10] J. W. Edmundson, L. Lumeng, and T.-K. Ling, J. Biol. Chem. **254,** 1653 (1979).
[11] D. H. Williamson, Methoden der enzymatischen Analyse (H. U. Bergemeyer, ed.), 3. edition, pp. 1724–1727, 1774–1777, Verlag Chemie, Weinheim 1974.
[12] A. Le Cam and P. Freychet, J. Biol. Chem. **252,** 148 (1977).
[13] M. S. Kilberg, H. N. Christensen, and M. E. Handlogten, Biochem. Biophys. Res. Commun. **88,** 744 (1979).
[14] D. L. Oxender, M. Lee, and G. Cecchini, Membranes and Neoplasia, Progress in Clinical and Biological Research (C. F. Fox, ed.), pp. 41–47, A. Liss Inc., New York 1976.
[15] H. N. Christensen, Biological Transport, 2nd edition, W. A. Benjamin Inc., Reading 1975.
[16] E. H. Heinz, P. Geck, C. Pietrzyk, G. Burckhardt, and B. Pfeiffer, J. Supramol. Structure **6,** 125 (1977).