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TRANSPORT OF NONMETABOLIZABLE AMINO ACIDS IN RAT LIVER SLICES

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SUMMARY

Transport of α-amino [1-^{14}C]isobutyric acid (AIB) by rat liver slices against a concentration gradient has been demonstrated; uptake was improved by including a preincubation step. Similarities to other systems included linearity of uptake over an extended period of time, as well as indications of saturability of the system with increasing concentrations of substrate. The transport of AIB was inhibited by anoxia and by 2,4-dinitrophenol, while glucose was without effect; inhibition also occurred in the presence of ouabain. No evidence for the active transport of AIB was seen when Na^+ was totally replaced in the medium. The removal of extracellular K^+ or Ca^{2+} markedly decreased the transport of AIB, although some uptake against a concentration gradient still occurred. The replacement of Mg^{2+} had little or no effect on the gradients achieved. Uptake was lower in a Krebs–Ringer phosphate than in a Krebs–Ringer bicarbonate buffer. The Na^+ and K^+ concentrations of slices incubated in the presence of AIB were similar to those of fresh liver. Cycloleucine was also transported against a concentration gradient but to a lesser extent than AIB.

INTRODUCTION

Entry into parenchymal cells is the essential first step in the disposition of many of the numerous compounds transported to the liver *via* the circulatory system. There have been many studies *in vitro* of the transport of amino acids in various tissues[1–12], but it has been stated that no really satisfactory preparation is available for studying the transport of amino acid into liver cells *in vitro*[13]. Although NEAME[3] included liver in a study of the transport in various tissues, his results implied that little or no active transport took place. Recently, CRAWHALL AND SEGAL[14] have also studied the uptake of amino acids by rat liver slices. However, their experiments were somewhat limited in that several characteristics of transport commonly found in other preparations were not examined.

An obvious drawback in the investigation of the transport of amino acids in liver is that the naturally occurring amino acids are actively metabolized by this organ. This in turn leads to difficulties in distinguishing between those factors which

Abbreviation: AIB, α-aminoisobutyric acid.

affect transport alone and those which affect metabolic processes. The use of nonmetabolizable amino acids provides a means of studying active transport while circumventing the problem of metabolic alteration of the substrate.

This report describes experiments which we have carried out in an effort to establish suitable conditions for studying the transport of α-aminoisobutyric acid (AIB) in the rat liver slice.

MATERIALS AND METHODS

Male albino rats (Holtzman), 3–4-weeks old, were employed. They received a standard stock diet (Wayne Lab-Blox) and water *ad libitum*.

The radioactive compounds used were [1-^{14}C]AIB, 1-aminocyclopentane [1-^{14}C]-carboxylic acid (cycloleucine) and [*carboxyl*-^{14}C]inulin (New England Nuclear). The amount of radioactive amino acid added to each flask was 0.05 μC in a volume of 3 ml; the final concentration of amino acid was 1 mM, unless otherwise specified. Where inulin was used, 0.24 μC per 3 ml of medium was added to each flask; specific activity of the inulin was 3.03 μC/mg.

Na^+ and K^+ analyses were made using atomic absorption spectrophotometry (Perkin–Elmer) and were performed by the Wisconsin Alumni Research Foundation (Madison).

Preparation and Incubation of Slices

Slices 0.4-mm thick were cut from chilled livers with a McIlwain tissue chopper; 200 ± 10 mg were placed in 3 ml of Krebs–Ringer bicarbonate buffer in micro-Fernbach flasks (10-ml size) and were preincubated by shaking the flasks for 15 min in a water bath at 37°. The slices were then separated from the medium, were transferred to flasks containing the desired substrate in 3 ml of fresh buffer and were incubated for 1 h at 37°. An atmosphere of O_2–CO_2 (95:5, v/v) was maintained at all times. In some instances this basic procedure was modified as described subsequently for the appropriate experiments. After completion of the incubation period, the flasks were chilled. The slices and the medium were separated by filtration on Hirsch funnels, and the tissue was washed with 3 ml of cold buffer; the washings were discarded. The slices were blotted, frozen, weighed and homogenized in 3% sulfosalicylic acid. The homogenates and media were centrifuged at 28 000 × *g* for 20 min, and the supernatants were used for the determination of radioactivity.

Radioactivity measurements

1-ml portions of the supernatants were counted[15] in a Packard Liquid Scintillation Spectrometer; counting efficiency was between 70 and 75%.

Calculation of results

Disint./min per ml of intracellular fluid were calculated from measurements of radioactivity, total tissue water and extracellular space as formulated by ROSENBERG *et al.*[1]. Total water content was determined as the difference in weight between the blotted slices after completion of the incubation procedure and after drying in a vacuum oven at 75° to constant weight. Extracellular space was measured with [*carboxyl*-^{14}C]inulin essentially as described by ROSENBERG *et al.*,[16] except that the tissue was extracted with either sulfosalicylic acid or hot water.

The transport of AIB or cycloleucine was expressed as the distribution ratio:

$$\text{Distribution ratio} = \frac{\text{disint./min per ml intracellular fluid}}{\text{disint./min per ml medium}}$$

In some instances the uptake of AIB was expressed as μmoles per ml of intracellular fluid:

$$\mu\text{moles AIB per ml intracellular fluid} = \frac{\text{disint./min per ml intracellular fluid}}{\text{disint./min per } \mu\text{mole AIB}}$$

RESULTS

Na^+ and K^+ content of liver slices

Apparently, the distribution or behavior of K^+ in liver preparations often differs from that seen *in vivo*[13,17]. Therefore, we felt it important to determine if the Na^+ and K^+ concentrations of incubated liver slices still resembled those of freshly sliced non-incubated liver. If the incubated slices had lost their ability to maintain the high levels of K^+ and low levels of Na^+ seen in the tissue *in vivo*, deterioration of the preparation would be indicated.

Livers were prepared for the analysis of the Na^+ and K^+ content as outlined in Table I; the slices were frozen after completion of the procedures. Na^+ content after 75 min of preincubation and incubation was barely higher than that of the fresh untreated slices; the K^+ concentration was also very close to that of the untreated slices. However, slightly different concentrations might be seen in slices incubated in the absence of amino acid; such observations have been made for K^+ in intestine[18] and brain[19] preparations.

Washing freshly prepared slices in sucrose appeared to cause a slight decrease in both the Na^+ and K^+ content. It has been noted that manipulation and temperature changes can reversibly affect the concentrations of these ions in various tissues[17,18,20]. (It was necessary to wash the incubated slices with a Na^+-free solution in order to remove adhering incubation medium; Na^+ content of slices immediately washed in buffer, or both incubated and washed in buffer, was about 0.12–0.15%, while the K^+ content closely resembled that of the comparable sucrose-washed samples as shown in Table I.) Values obtained for the Na^+ and K^+ concentration of the fresh untreated slices were almost identical with those found for intact liver by MEYER *et al.*[21].

TABLE I

Na^+ AND K^+ CONTENT OF RAT LIVER SLICES

Incubations were carried out in Krebs–Ringer bicarbonate buffer at 37°; average of three experiments, mean ± S.E.

Slice treatment	*Na^+* (%)	*K^+* (%)
None	0.067 ± 0.001	0.366 ± 0.013
Washed in sucrose	0.055 ± 0.003	0.294 ± 0.011
Preincubated 15 min, incubated 60 min in 1 mM AIB, washed in sucrose	0.077 ± 0.007	0.333 ± 0.018

Water content

The average total water content of the incubated liver slices was 72.9 ± 0.3% for 12 determinations. NEAME[3] found the total water content of rat liver slices to be 77.5%, while CRAWHALL AND SEGAL[14,22] found values of 71.1 and 75 % (after 30 min incubation).

Extracellular space

Slices were incubated with [*carboxyl*-^{14}C]inulin for 15–75 min. The values for the extracellular space were similar regardless of the extraction procedure used (sulfosalicylic acid or hot water) and were found to be 19.7 ± 1.6% at 15 min, 22.4 ± 0.6% at 30 min, 26.5 ± 0.7% at 60 min and 27.2 ± 0.7% at 75 min; these values are for 4, 11, 11 and 7 determinations, respectively. The results may be compared with the 21.3 and 27% reported by CRAWHALL AND SEGAL[14,22] and the 24% found in experiments *in vivo* by SCHANKER AND HOGBEN[23].

Effect of preincubation procedures

Incubation of slices for 15 min, followed by transfer to fresh medium containing [1-^{14}C]AIB and further incubation for 60 min, resulted in distribution ratios higher than those seen for slices for which the preincubation step was omitted. In analyses performed on livers from eight rats, the distribution ratios for preincubated and non-preincubated samples were 3.81 and 2.68, respectively. Furthermore, upon incubation of preincubated slices with [1-^{14}C]AIB in medium from the 15-min preincubation of a second set of slices, the distribution ratios were 65% of those obtained using the standard incubation procedure (six experiments).

A probable factor contributing to the lower ratios in the non-preincubated slices was the release of relatively large amounts of amino acids into the medium. During the usual 15-min preincubation step, roughly 5–7 times as much acid-soluble ninhydrin-positive material appeared in the medium as during each of three subsequent 15-min periods, after each of which the slices were transferred to fresh medium. Since it is well known that transport of amino acids *in vitro* can be inhibited by certain other amino acids, the lower ratios may be attributable to the relatively high concentration of amino acids in the medium. Leakage of nitrogenous constituents from tissue slices has also been observed by several other workers[24–26].

Effect of time

It was found that he uptake of AIB increased linearly for at least 75 min (Fig. 1). In two experiments in which the incubation mixture was kept in ice for 90 min, the distribution ratios were well below 1.0 (Fig. 1.), indicating that simple diffusion processes contributed insignificantly to the ratios observed at 37°. Since determinations of extracellular space were not made for incubation periods of longer than 75 min, the values shown in Fig. 1 for intervals of 90 min or more may be slightly inaccurate.

Effect of initial substrate concentration

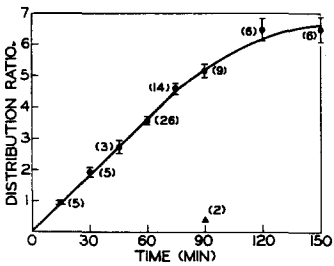
Slices were incubated for 60 min with varying concentrations of [1-^{14}C]AIB (Fig. 2). Uptake of the amino acid appeared to be a saturable process, since a linear increase in the concentration of AIB in the intracellular fluid was not maintained with increasing amounts of substrate; a concentration of 1.0 mM was chosen for

subsequent routine use. Similar indications of saturability have been found in numerous other active transport systems *in vitro*.

A Lineweaver–Burk plot was prepared from the data, and the apparent K_m for the transport of AIB was calculated to be 8.7 mM. It must be realized that this is only an approximate value, since it is based on data from a relatively prolonged incubation period and since the nature of the system is not well defined.

Effects of 2,4-dinitrophenol, anoxia and glucose

The importance of active metabolic processes in cellular transport mechanisms



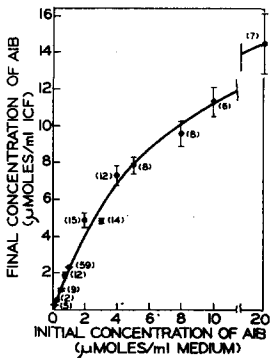


Fig. 1. Changes with time in the uptake of [1-^{14}C]AIB by rat liver slices. Incubations were carried out in Krebs–Ringer bicarbonate buffer at 37°; preincubation period was 15 min; initial AIB concentration was 1 mM; bars represent standard error; number of experiments is indicated in parentheses. ▲, average of ratios for two samples incubated in an ice bath for 90 min.

Fig. 2. Concentration of [1-^{14}C]AIB in intracellular fluid as affected by initial concentration in medium. Incubations were carried out in Krebs–Ringer bicarbonate buffer at 37°; preincubation and incubation periods were 15 and 60 min, respectively; bars represent standard error; number of experiments is indicated in parentheses. Adjustments were made in the NaCl content of the medium to compensate for increased concentrations of AIB at 8 mM and above. ICF, intracellular fluid.

TABLE II

EFFECTS OF 2,4-DINITROPHENOL AND ANOXIA ON TRANSPORT OF [1-^{14}C]AIB BY RAT LIVER SLICES

Incubations were carried out in Krebs–Ringer bicarbonate buffer at 37°; preincubation and incubation periods were 15 and 60 min, respectively; anoxia was produced by incubation in N_2 atmosphere; averages of three experiments ± S.E.

Treatment	*Distribution ratio*
None	3.42 ± 0.15
2,4-Dinitrophenol (0.1 mM)	1.92 ± 0.08
None	3.55 ± 0.20
2,4-Dinitrophenol (0.2 mM)	1.96 ± 0.04
None	2.83 ± 0.42
Anoxia	1.69 ± 0.33
None	4.31 ± 0.54
Anoxia*	2.28 ± 0.25

* Preincubation was also carried out in N_2 atmosphere.

has been shown by the fact that anoxia, or agents such as 2,4-dinitrophenol, CN^- and iodoacetate, can inhibit amino acid transport[1-3,5,6,8,11].

Upon incubation of liver slices in the presence of 2,4-dinitrophenol, an inhibitor of oxidative phosphorylation, the distribution ratios for AIB were depressed to about 55% of the control values (Table II). The inhibitory effects of anoxia on transport were demonstrated by incubating the slices in an atmosphere of N_2 (Table II). The effects were somewhat accentuated in experiments in which the preincubation step was also carried out in the presence of N_2. CRAWHALL AND SEGAL[14] also found anoxia to inhibit the uptake of AIB by rat liver slices.

The need for active metabolic processes in the transport of AIB by liver slices was suggested by these experiments; however, glucose (10 or 20 mM) did not cause a significant change in the distribution ratios. These results may be compared with those reported for other tissues. STERN *et al.*[6] and ABADOM AND SCHOLEFIELD[27] found glucose to stimulate the uptake of amino acids by brain slices. Glucose also has been reported to increase the transport of amino acids by rat intestine[28]. In contrast, it has been found to inhibit the uptake of certain neutral amino acids by kidney cortex slices[29]. Since glucose was ineffective in increasing the uptake of AIB by liver slices, energy for transport of this amino acid apparently can be derived from endogenous substrates. The fact that anoxia inhibited the uptake of AIB by less than 50% suggests the possible importance of glycolysis in providing a source of energy.

Effect of ionic composition of the medium

The importance of cations, especially of Na^+ and K^+, in the transport of certain amino acids by various tissues has been pointed out in numerous reports and reviews[2,7,8,12,18,19,30-34]. We have, therefore, examined the effects of ouabain, an agent which interferes with ion transport, and modifications in the ionic composition of the medium on the uptake of AIB by liver slices.

When slices were incubated for 1 h with 0.2 mM ouabain, the observed distribution ratios were approx. 86% of the control value of 4.08 (three experiments). Upon increasing the ouabain concentration to 0.4 mM, the distribution ratios were 69% of the controls. If ouabain (0.4 mM) were present during both the preincubation and incubation periods, the ratios were decreased further to about 58% of the corresponding control values. Other investigators have noted inhibition of amino acid transport by ouabain[2,8,18,30-32]; such results often have been interpreted to indicate that the uptake of amino acids is linked to the active extrusion of Na^+ from the cell.

Effects of the cations of the Krebs–Ringer bicarbonate buffer were studied by incubating liver slices in buffers in which various ions were completely replaced by suitable iso-osmolar substitutes (Table III). In these experiments both the preincubation and incubation steps were carried out in the modified buffers. A limited number of observations suggested that such a procedure frequently accentuated the effects of the removal of ions on the uptake of AIB; these few experiments also indicated that the uptake of AIB was not greatly affected in control experiments in which the slices were preincubated in the modified buffer followed by incubation in the control buffer.

Replacement of Na^+ caused a marked decrease in the distribution ratios. Since none of the observed ratios were above 1, the results provide evidence that no active transport of AIB occurred in the absence of extracellular Na^+.

If K^+ was replaced, the uptake of AIB was again decreased; the ratios were dis-

TABLE III

EFFECT OF IONIC COMPOSITION OF THE MEDIUM ON UPTAKE OF [1-^{14}C]AIB BY RAT LIVER SLICES

Incubations were carried out in the described buffers at 37°; in those experiments in which Na^+ absence was studied, $NaHCO_3$ of both the experimental and control buffers was replaced with $KHCO_3$; values are for mean ± S.E.; number of experiments is given in parentheses. Composition of control Krebs–Ringer bicarbonate buffer (pH 7.4) was 118 mM NaCl; 4.8 mM KCl; 1.1 mM KH_2PO_4; 2.5 mM $CaCl_2$; 1.2 mM $MgSO_4$; 25.0 mM $NaHCO_3$.

Salt omitted	*Iso-osmolar substitute*	*Distribution ratio*	
		Control	*Experimental*
NaCl, $NaHCO_3$	Choline chloride, $KHCO_3$	4.00 ± 0.38 (6)	0.56 ± 0.05 (6)
NaCl, $NaHCO_3$	LiCl, $KHCO_3$	3.47 ± 0.12 (6)	0.56 ± 0.03 (6)
NaCl, $NaHCO_3$	KCl, $KHCO_3$	2.85 ± 0.22 (5)	0.67 ± 0.02 (9)
NaCl, $NaHCO_3$	Sucrose, $KHCO_3$	3.63 ± 0.42 (7)	0.72 ± 0.06 (7)
KCl, KH_2PO_4	Choline chloride, NaH_2PO_4	3.88 ± 0.27 (3)	1.92 ± 0.09 (3)
KCl, KH_2PO_4	NaCl, NaH_2PO_4	3.11 ± 0.11 (3)	1.69 ± 0.04 (3)
KCl, KH_2PO_4	Sucrose, NaH_2PO_4	4.94 ± 0.18 (3)	2.07 ± 0.10 (3)
$CaCl_2$	Choline chloride	3.80 ± 0.25 (3)	1.38 ± 0.10 (3)
$CaCl_2$	NaCl	3.63 ± 0.18 (3)	1.36 ± 0.07 (6)
$MgSO_4$	Na_2SO_4	3.52 ± 0.13 (6)	3.27 ± 0.17 (6)
KH_2PO_4, $NaHCO_3$	Na_2HPO_4 (as in Krebs–Ringer phosphate*)	4.23 ± 0.16 (3)	2.00 ± 0.16 (3)

* $[Na^+]$ was 6 mM higher than in Krebs–Ringer bicarbonate buffer.

tinctly above 1, indicating that some transport of AIB still occurred in the absence of K^+. It has been found that K^+ is required for much of the normal extrusion of Na^+ from various tissues to take place[20,31,44]. Since it has been proposed that active transport of amino acids and other solutes is coupled to the removal of Na^+ (refs. 32–34) the depression of the net uptake of AIB in the absence of K^+ may be indirectly related to changes in the extrusion of Na^+.

Replacement of $CaCl_2$ also caused a marked decrease in the uptake of AIB, although doubling the amount of $CaCl_2$ had no effect. Ca^{2+} has been reported to increase the transport of amino acids by brain[7,27,35,36] and kidney[37], possibly to inhibit the uptake of amino acids by brain[8], and to have no effect on the transport of amino acids by intestine[38], red blood cells[10] and kidney[30]. MANERY[39] has reviewed studies suggesting that Ca^{2+} is involved in the maintenance of cell permeability in general, as well as of normal intracellular levels of K^+ and certain proteins.

Replacement of Mg^{2+} appeared to have no significant effect on the transport of AIB; similar results also have been found for kidney slices[30].

The uptake of AIB in Krebs–Ringer phosphate buffer was half that obtained in the bicarbonate buffer. Furthermore, the uptake of AIB was about 64% of the control value in experiments in which slices were incubated in the presence of both $NaHCO_3$ and Na_2HPO_4. Part of the calcium of these phosphate buffers is in the form of insoluble $Ca_3(PO_4)_2$; therefore the amounts of Ca^{2+} present may have been inadequate to maintain the uptake of AIB comparable to that seen with the Krebs–Ringer bicarbonate buffer.

Most of the modifications in the ionic composition of the medium had only minor effects on the total and extracellular water contents of the slices. A significant exception occurred when K^+ completely replaced Na^+; obvious swelling of the slices was noted, and total water and extracellular space were found to be 82.1 and 14.7%, respectively. Similar changes in fluid spaces were noted earlier for other tissues incubated in media containing high K^+ and no Na^+ (refs. 7, 18, 30 and 34).

Uptake of cycloleucine

Net transport of cycloleucine by liver slices was investigated in seven rats. Slices were incubated with [1-^{14}C]cycloleucine (1 mM) for 60 min; the distribution ratio was found to be 1.66 $\pm$ 0.03 as compared with 3.37 $\pm$ 0.10 for slices incubated with AIB. Therefore, it is apparent that, under these conditions, cycloleucine is not concentrated to the same degree as is AIB. However, it seems that net transport of this amino acid did occur. These results are in contrast to those of CRAWHALL AND SEGAL[14] who found a distribution ratio of not more than 1.2 and who concluded that no concentration gradient was being maintained. AHMED AND SCHOLEFIELD[40] also found no net accumulation of this amino acid by rat liver slices. However, LAHIRI AND LAJTHA[7] found both AIB and cycloleucine to be concentrated by brain slices.

DISCUSSION

It has been repeatedly demonstrated in experiments *in vivo* that the liver is capable of maintaining an amino acid concentration considerably higher than that of the plasma.The experiments which we have described show that, for AIB and probably for cycloleucine, the liver slice is also capable of establishing a concentration gradient. The distribution ratios for AIB in rat liver slices under control conditions were found to resemble rather closely those reported in several studies *in vivo*. RIGGS AND WALKER[41] found, 39 h after injection of AIB, distribution ratios between liver intracellular water and plasma of about 5.7-7.4. Recalculation of the results presented by NIMNI *et al.*[42] shows that approx. 4 times as much AIB was found in liver cell water as in serum 24 h after injection of the amino acid. CHRISTENSEN AND CULLEN[43] found liver cell water to contain roughly 3–5 times as much AIB as did the plasma 2 h after the injection of 1 or 10 mmoles AIB per kg rat.

Our results together with those of CRAWHALL AND SEGAL[14] indicate that AIB transport by rat liver slices has many features in common with the transport of amino acids *in vitro* by other tissue preparations. We have demonstrated requirements for active metabolic processes in that a partial failure of uptake occurs under anoxic conditions or in the presence of 2,4-dinitrophenol, although glucose itself has no effect on the uptake of AIB. CRAWHALL AND SEGAL also noted that anoxia interferes with transport of this amino acid. Both laboratories have shown that the uptake of AIB is linear over varying periods of time.

Our observations have enlarged and extended those of CRAWHALL AND SEGAL in that we have found: (1) the inclusion of preincubation procedures to increase the amount of AIB subsequently concentrated by the slices; (2) the concentration of Na^+ and K^+ in the incubated tissue to remain close to that seen in fresh slices; (3) saturability of the system with increasing concentrations of AIB; (4) inhibition of transport

by ouabain; and (5) requirements for the presence of Na^+, K^+ and Ca^{2+} in the incubation medium.

Development of a system permitting the study of the uptake of amino acids by rat liver slices will permit detailed examination *in vitro* of the effects of various nutritional and hormonal states in the intact animal. The method should be of particular value in this organ which so actively metabolizes amino acids. In conjunction with separate observations on the metabolism of naturally occurring amino acids, the technique might also permit estimation of the influence of changes in metabolic activity on the actual transport of such compounds into the tissue.

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