α -Aminoisobutyrate Transport into Cells from R3230AC Mammary Adenocarcinoma

EVIDENCE FOR SODIUM ION-DEPENDENT AND -INDEPENDENT CARRIER-MEDIATED ENTRY AND EFFECTS OF DIABETES

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In the presence of Na^+ , α -aminoisobutyrate was transported by saturable and non-saturable processes into R3230AC mammary tumour cells isolated by enzymic treatment. Eadie–Hofstee analysis for the saturable process gave a curvilinear plot, suggesting that transport occurred by more than one carrier. In the absence of Na^+ , α -aminoisobutyrate was also transported by both saturable and non-saturable processes. This Na^+ -independent saturable process gave a linear plot according to Eadie–Hofstee analysis: V , 708 ± 105 pmol/min per 5×10^6 cells; K_m , 0.36 ± 0.33 mM (mean $\pm$ S.E.M.). Subtracting α -aminoisobutyrate entry in the absence of Na^+ from total α -aminoisobutyrate uptake (in the presence of Na^+) showed the presence of another saturable process (Na^+ -dependent), accounting for 75% of total α -aminoisobutyrate uptake. This component gave a linear Eadie–Hofstee plot: V , 2086 ± 213 ; K_m , 1.75 ± 0.16 . α -(Methylamino)isobutyrate, a substrate specifically taken up by the A system, inhibited 80% of α -aminoisobutyrate entry. The presence of both α -(methylamino)isobutyrate and phenylalanine inhibited α -aminoisobutyrate entry completely. 2-Aminobicyclo[2.2.1]heptane-2-carboxylate, an analogue specifically taken up by the Na^+ -independent system, inhibited completely the Na^+ -independent entry of α -aminoisobutyrate. In the presence of Na^+ , the distribution ratio, which is defined as the amino acid concentration in the intracellular space divided by that in the incubation medium for α -aminoisobutyrate, at 90 min was 19, and in the absence of Na^+ at 60 min was 5. These concentrative processes were sensitive to the metabolic inhibitor pentachlorophenol. The Na^+ -dependent, but not the Na^+ -independent, α -aminoisobutyrate uptake was increased in cells from diabetic rats. This was primarily due to an increase in the V for the Na^+ -dependent component (164%) with no effect on the K_m . We conclude, therefore, that α -aminoisobutyrate entry into cells from this mammary tumour is mediated by two transport systems, one Na^+ -dependent and another Na^+ -independent. Furthermore, the Na^+ -dependent component of α -aminoisobutyrate is sensitive to alterations of insulin *in vivo*.

The mode of entry into cells of neutral amino acids shows extensive overlap between three transport carriers: the A (alanine-preferring), ASC (alanine, serine, cysteine) and the L (leucine-preferring) systems (Christensen, 1975a). The entry of those amino acids transported by the A and ASC systems is Na^+ -dependent, whereas entry by the L system is Na^+ -independent. The effect of insulin on amino acid transport has been studied in a variety of tissues (Elsas *et al.*, 1968, 1971; Goldfine *et al.*, 1972; Guidotti *et al.*, 1974a, 1976). Results from these studies indicate that insulin stimulated the entry of these amino acids transported primarily by the A system (Guidotti *et al.*, 1974b; Riggs &

McKirahan, 1973). α -Aminoisobutyrate, a non-metabolizable amino acid analogue (Noall *et al.*, 1957), was found to be transported only by the A system in Ehrlich ascites-tumour cells (Christensen, 1969), and isolated rat diaphragm (Riggs & McKirahan, 1973). Since α -aminoisobutyrate is not metabolized, and thus offers the advantage for study of transport without potential interference by subsequent metabolic events, it has been extensively used as a probe for the A system. However, α -aminoisobutyrate entry by more than one transport system has been reported for certain tissues. For example, it appears to be transported by both the A and ASC systems in isolated hepatocytes (Le Cam & Freychet, 1977), by Na^+ -dependent and Na^+ -independent systems in foetal rat calvaria (Finerman & Rosenberg,

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1966), and by both an A and L system in chick-embryo heart cells (Gazzola *et al.*, 1972). Thus it appears that the mode of α -aminoisobutyrate transport reflects the tissue under study.

We have observed that insulin *in vivo* inhibited uptake of proline and α -aminoisobutyrate, but not of phenylalanine, into R3230AC adenocarcinoma cells (P. J. Hissin & R. Hilf, unpublished work). From preliminary observations, it appeared that α -aminoisobutyrate transport into these tumour cells was mediated by more than one system. These observations have been extended, and the present report describes for the first time, to our knowledge, the characteristics of α -aminoisobutyrate entry into enzymically dissociated cells obtained from a mammary tumour. Our findings, based on kinetic and inhibitor studies, indicate that α -aminoisobutyrate is transported across the cell membrane of these tumour cells by two transport carriers, whose properties resemble those described for the A and L systems (Christensen, 1975a). In addition, only one of these systems is responsive to the presence of insulin *in vivo*.

Materials and Methods

Female Fischer rats (80–90g) were obtained from Charles River Breeding Laboratory (Wilmington, MA, U.S.A.). The rats were offered food and water *ad libitum*. The R3230AC tumour was implanted subcutaneously in the axillary region on both sides by a sterile trocar technique as described by Hilf *et al.* (1965).

Since the goal of these studies is to examine the influence of insulin on α -aminoisobutyrate transport, diabetes was induced by administration of streptozotocin into the jugular vein 2 days before tumour implantation (Cohen & Hilf, 1975). Streptozotocin was dissolved in 0.9% NaCl and rapidly adjusted to pH 4.5 with 0.025M-citric acid. Owing to the instability of the drug, all injections were made within 30min of dissolving the drug. The dose administered was 60mg/kg. Blood glucose was determined by the glucose oxidase method (Gluco-stat; Worthington Biochemical, Freehold, NJ, U.S.A.) and the urinary glucose was measured by Clinistix (Ames Co., Elkhart, IN, U.S.A.). Animals were classified as diabetics if their blood glucose was greater than 250mg/100ml and their urine glucose exceeded 0.5g/100ml.

Animals were killed by cervical dislocation 2–3 weeks after tumour implantation. Tumours were excised as quickly as possible and placed in ice-cold 0.9% NaCl. After removal of connective and necrotic tissues, 3g of tumour tissue was minced into 1mm × 1mm pieces on a McIlwain tissue slicer (Brinkmann Instruments, Westbury, NY, U.S.A.). The minced tissue was incubated in 250ml Erlenmeyer flasks

with 10ml of Hanks' balanced-salt solution (Ca^{2+} - and Mg^{2+} -free) (Grand Island Biological Co., Grand Island, NY, U.S.A.), containing 0.1% (w/v) hyaluronidase (Sigma Chemical Co., St. Louis, MO, U.S.A.) and 0.05% (w/v) collagenase (type II, Worthington), for 25min at 37°C in a shaking water bath (approx. 50 cycles/min). At the end of the incubation period, the mixture was poured through a 140 μ m stainless-steel strainer (Cistron Corp., Lebanon, PA, U.S.A.). The non-dissociated tissue remaining on the strainer was returned to the flask with a fresh 10ml portion of enzyme solution and incubated for an additional 55min; the filtrates from the first incubation mixture were discarded, owing to considerable contamination by erythrocytes and cellular debris. At the end of the second hour, the incubation mixture was again strained, and the non-dissociated pieces were rinsed with Hepes [N-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer. The filtrates and the wash mixtures were combined and centrifuged for 5min at 80g. The cell pellet was washed and centrifuged three more times with 10vol. of buffer. Cell viability was estimated by Trypan Blue exclusion, and cell number was determined by the use of a haemocytometer. Cell preparations used had over 90% viability (Harmon & Hilf, 1976) and most preparations yielded approx. 2.5×10^8 cells per 3g of tissue.

Transport studies

Transport was studied with 5×10^6 cells in plastic (Falcon, Oxnard, CA, U.S.A.) tubes in a final volume of 1.0ml. The medium consisted of 25mM-Hepes buffer, pH 7.5, 10mM- NaHCO_3 , 130mM-NaCl, 3mM- K_2HPO_4 , 1mM- MgSO_4 , 1mM- CaCl_2 , 11mM-glucose and 1mg of bovine serum albumin/ml with a final osmolality of 310mosm. In experiments where Na^+ was omitted or varied, NaCl and NaHCO_3 were replaced by choline chloride and choline bicarbonate at equimolar concentrations, thereby maintaining the same osmolality throughout. Cells were incubated for 30–45min in solutions containing various $[\text{Na}^+]$, and transport rates were determined by adding the amino acid (labelled and unlabelled) to cells in a shaking water bath (80 cycles/min) at 37°C. α -Aminoisobutyrate uptake was linear for up to 10min at 0.1mM concentration; however, the range of linearity when α -aminoisobutyrate was 10 and 40mM decreased to 3 and 2min respectively. Therefore initial-velocity measurements for all α -aminoisobutyrate concentrations used were based on samples taken at 15, 30, 45 and 60s. Transport was terminated by rapidly adding 8ml of cold 0.9% NaCl (0–2°C) to each tube, followed immediately by centrifugation for 1min at 3°C (900g). After decanting the supernatant, the cells were washed with another 8ml of cold 0.9% NaCl, centrifuged for 2.5min (900g),

and the supernatant was decanted. Since the washing required approx. 4–5 min, the efflux of α -aminoisobutyrate during this procedure was examined. Cells were allowed to accumulate α -aminoisobutyrate at 37°C for 5 min. The cells were then placed on ice for 0, 1, 5, 10, 15 or 20 min and then washed as described above. No significant efflux of α -aminoisobutyrate occurred (<4%). The tubes were inverted for 15 min to drain; any remaining droplets on the sides of the tubes were removed. The cell pellet was dissolved in two successive 5 ml portions of Aqueous Counting Scintillant (Amersham, Arlington Heights, IL, U.S.A.), transferred to vials and the samples were counted for radioactivity in a liquid-scintillation counter (Nuclear–Chicago, Isocap 300). The efficiency for ^{14}C was 60% and for ^3H , 40%.

Intracellular and extracellular spaces were determined by use of 3-*O*-[^3H]methylglucose and [^{14}C]inulin in the same preparation. The cells were incubated under the same conditions as described above, for at least 45 min, when 3-*O*-methylglucose has reached equilibrium; 3-*O*-methylglucose was shown by Harmon & Hilf (1976) to be transported into these tumour cells by facilitated diffusion, reaching equilibrium at approx. 30 min at 20°C. In experiments where prolonged incubation was required, the tubes were incubated with 3-*O*-methylglucose and inulin for the same period under study; the intracellular space so calculated should reflect this parameter at the time when transport was measured. The tubes were centrifuged at 900g for 5 min; 100 μl samples of the supernatant were taken and the remaining supernatant was decanted. The tubes were wiped and radioactivity (^3H and ^{14}C) in the pellet was determined.

The intracellular space was calculated in the following way:

$$\frac{{}^3\text{H c.p.m. (pellet)} \times 100 \mu\text{l}}{{}^3\text{H c.p.m. (supernatant)}} = \text{intracellular + extracellular space} \quad (1)$$

$$(\mu\text{l per } 5 \times 10^6 \text{ cells})$$

$$\frac{{}^{14}\text{C c.p.m. (pellet)} \times 100 \mu\text{l}}{{}^{14}\text{C c.p.m. (supernatant)}} = \text{extracellular space} \quad (2)$$

$$(\mu\text{l per } 5 \times 10^6 \text{ cells})$$

$$(1) - (2) = \text{intracellular space } (\mu\text{l per } 5 \times 10^6 \text{ cells})$$

After the intracellular volume was thus obtained, the concentration of labelled amino acid taken up by the cells was calculated and a distribution ratio was calculated by the following formula:

Distribution ratio

$$= \frac{\text{concentration of the amino acid in the intracellular space}}{\text{concentration of the amino acid in the medium at the end of the incubation}}$$

Uptake is reported either as the distribution ratio or as pmol/min per 5×10^6 cells. Cells prepared as described above were found to be suitable for transport studies for prolonged incubation periods (Hissin & Hilf, 1978). Further, α -aminoisobutyrate accumulation against a concentration gradient was maintained. The cells displayed minimal changes in viability (3–4% change) and in intracellular space ($4.3 \pm 0.14 \mu\text{l}/5 \times 10^6$ cells), in the absence of amino acids over a period of 4 h at 37°C.

Chemicals

Hepes, NaCl, choline chloride, choline bicarbonate, serine, phenylalanine, glycine, α -aminoisobutyrate and hyaluronidase were obtained from Sigma; D-glucose was from Mallinckrodt Chemical Works, St. Louis, MO, U.S.A.; α -(methylamino)isobutyrate was from Aldrich, Metuchen, NJ, U.S.A.; 2-aminobicyclo[2.2.1]heptane-2-carboxylate was generously given by Dr. Robert Zand, University of Michigan (Ann Arbor, MI, U.S.A.); bovine serum albumin (lot 55) was from Kupits, Forked River, NJ, U.S.A.; collagenase (type II) was from Worthington; α -amino[1- ^{14}C]isobutyric acid (2–10 mCi/ μmol), 3-*O*-[^3H]methyl-D-glucose (4–5 Ci/mmol) and [*carboxy*- ^{14}C]inulin (1–3 mCi/g) were from New England Nuclear, Boston, MA, U.S.A.

Results

α -Aminoisobutyrate entry into tumour cells

The mode of α -aminoisobutyrate entry into mammary tumour cells was investigated by measuring the initial velocity (v) of uptake at various concentrations, ranging from 0.05 to 40 mM. At low concentrations (0.05–5.0 mM), v increased rapidly with increasing concentration, demonstrating curvilinear characteristics. For 10–40 mM α -aminoisobutyrate, v increased linearly with increasing concentration (Fig. 1a). This behaviour suggested that entry was mediated by two transport processes, one saturable, and the other non-saturable. The linear portion of the graph at high concentrations (>5 mM) was used to calculate the diffusion coefficient, K_D . The K_D was then used to determine the non-saturable entry at each concentration, which, when subtracted from total (saturable and non-saturable) entry, yielded a plot (v^c versus [α -aminoisobutyrate]) depicting saturation kinetics (Fig. 1a). When the data for this saturable process were plotted according to Eadie-Hofstee analysis, v^c versus v^c/α -aminoisobutyrate, a curvilinear relationship was obtained (Fig. 1b). Thus it appeared that α -aminoisobutyrate entry was mediated by more than one system.

Fig. 1. Initial velocity of α -aminoisobutyrate transport as a function of its concentration

(a) v was determined at various concentrations (0.05–40 mM). The rate of entry at high concentrations (10–40 mM) was used to calculate the rate for the non-saturable entry (K_D). The use of K_D to correct for non-saturable entry at each concentration yielded a saturable process. v , Initial velocity; $\bullet$, total entry (saturable and non-saturable); $\circ$, saturable entry (v^C values). (b) Eadie-Hofstee plot of data for the saturable entry (a). The curvilinear relationship suggested α -aminoisobutyrate entry by more than one carrier.

Fig. 2. Effect of Na^+ on α -aminoisobutyrate transport

The v for α -aminoisobutyrate was determined in the absence or presence of increasing $[Na^+]$; NaCl and $NaHCO_3$ were replaced by equimolar concentration of choline chloride and choline bicarbonate respectively. v , initial velocity; $\bullet$, total α -aminoisobutyrate entry; $\circ$, corrected for non-saturable entry.

Effect of Na^+ on α -aminoisobutyrate transport

As one approach to characterize the heterogeneity of α -aminoisobutyrate uptake, the effect of $[Na^+]$ on its transport was investigated. The v for α -aminoisobutyrate (0.1 mM) was determined at concentrations of Na^+ ranging from 0 to 125 mM. The data for the total (saturable and non-saturable) and saturable (after correcting for the non-saturable component) entry are shown in Fig. 2. A considerable amount

was transported in the absence of Na^+ , indicating that a portion of the total saturable entry might be mediated by a Na^+ -independent carrier. Fig. 2 also shows that entry increased with increasing Na^+ concentrations. α -Aminoisobutyrate entry in the presence of Na^+ was measured in all subsequent experiments in medium containing 130 mM NaCl, the maximum that could be included and still satisfy optimal osmotic conditions (310 mosM).

Kinetic parameters for the Na^+ -dependent and -independent uptake of α -aminobutyrate

The entry in the absence of Na^+ also demonstrated both saturable and non-saturable processes, similar to those seen (Fig. 1a) in the presence of Na^+ . Fig. 3(a) includes a plot of the saturable uptake in the absence of Na^+ . A plot of v^C (in the absence of Na^+) versus v^C/α -aminoisobutyrate (Fig. 3b) gave a linear representation, suggesting that, in the absence of Na^+ , α -aminoisobutyrate was transported by a single carrier-mediated process. After subtraction of this saturable Na^+ -independent entry from the total saturable entry (in the presence of Na^+), it appeared that the remaining Na^+ -dependent entry was also a saturable process (Fig. 3a). This latter process displayed a single component when analysed by an Eadie-Hofstee representation (Fig. 3b). Thus the kinetic analysis resolved the curvilinear characteristics of the total carrier-mediated entry into two components; one, Na^+ -dependent with a K_m of 1.75 mM, and another, Na^+ -independent with a K_m of 0.22 mM. The K_m (mean $\pm$ S.E.M.) obtained from nine separate experiments was 1.75 ± 0.16 and 0.36 ± 0.03 mM for the Na^+ -dependent and -independent components respectively. The capacities of the Na^+ -dependent and -independent entry were 75 and 25%

Fig. 3. α -Aminoisobutyrate transport in the presence and absence of Na^+ as a function of α -aminoisobutyrate concentration

The initial velocity for uptake in the presence and absence of Na^+ was determined at various α -aminoisobutyrate concentrations and v^C was calculated as described in Fig. 1. Data for the total (saturable and non-saturable) uptake are not shown. (a) v^C for transport at various α -aminoisobutyrate concentrations (0.05–40 mM) in the absence of Na^+ demonstrated a saturable process. Subtracting the saturable entry in the absence of Na^+ (Na^+ -independent) from the total saturable entry in the presence of Na^+ yielded another saturable component (Na^+ -dependent). $\circ$, Total saturable α -aminoisobutyrate uptake in the presence of Na^+ ; $\blacktriangle$, Na^+ -dependent uptake; $\triangle$, Na^+ -independent uptake. (b) Eadie-Hofstee plot of data for the saturable uptake (a). Best fitting line was determined by linear-regression analysis (correlation coefficient 99%).

respectively of the total α -aminoisobutyrate entry. Although variation in V was observed in cells from different animals, the relative capacity for the Na^+ -dependent and -independent entry was similar. The V for the Na^+ -dependent and -independent entry from nine separate experiments was 2086 ± 213 and 708 ± 3.6 pmol/min per 5×10^6 cells respectively.

Inhibition studies

To elucidate the specificity of the mechanism responsible for the entry of α -aminoisobutyrate, inhibition analysis was performed, an approach that has been successful in identifying the class of carriers that mediate the entry of neutral amino acids (Christensen, 1966).

Inhibition of α -aminoisobutyrate (0.1 mM) entry by amino acids that are known to utilize the A, ASC and/or L systems is shown in Fig. 4(a). The results suggested that α -aminoisobutyrate may be transported by more than one system. First, α -(methylamino)isobutyrate was tested for inhibitory effects. This non-metabolizable amino acid analogue was found to be transported by the A system in these tumour cells, on the basis of results of saturation experiments that yielded a single component by Eadie-Hofstee plots, and completely inhibited proline uptake, which we reported to occur exclusively by the A system (Hissin & Hilf, 1978). As shown here (Figs. 4a and 4b), α -(methylamino)isobutyrate was effective in inhibiting α -aminoisobutyrate entry at low concentrations of the inhibitor. However, α -(methylamino)isobutyrate did not completely inhibit entry even when its concentration was raised to

50 mM (500-fold that of α -aminoisobutyrate). Similar results were obtained when proline was used to inhibit the entry of α -aminoisobutyrate (results not shown). Second, unlike α -(methylamino)isobutyrate, low concentrations of phenylalanine, which enter primarily by the L system, were not as effective in inhibiting α -aminoisobutyrate entry as high concentrations of phenylalanine, when phenylalanine is more likely to be transported by both the L and A systems (Christensen, 1975a). Third, when both α -(methylamino)isobutyrate and phenylalanine (Fig. 4b) were added together at concentrations equal to or at one-half the concentrations used for unlabelled α -aminoisobutyrate, the uptake of α -aminoisobutyrate was completely inhibited. Fourth, the entry of glycine into foetal rat calvaria was found to be mediated by both Na^+ -dependent and Na^+ -independent systems, and glycine and α -aminoisobutyrate showed similar uptake patterns (Finerman & Rosenberg, 1966). Our data show (Fig. 4a) that glycine was more effective than α -(methylamino)isobutyrate in inhibiting α -aminoisobutyrate entry. However, glycine entry in these tumour cells has not been characterized, and therefore, definite conclusions regarding the mode of entry of α -aminoisobutyrate cannot be drawn from these particular findings. Fifth, serine completely inhibited α -aminoisobutyrate entry. As with glycine, serine uptake in these tumour cells has not been characterized; however, since serine was reported to enter other cells by A, ASC and L systems (Christensen, 1975a), it is possible that the inhibition by serine may be a result of competition for more than one carrier.

Therefore, on the basis of inhibition analysis,

Fig. 4. Inhibition of α -aminoisobutyrate transport by amino acid inhibitors

(a) Transport at 0.1 mM α -aminoisobutyrate was determined in the presence of increasing concentrations of amino acid inhibitors (0.1–50 mM): ●, unlabelled α -aminoisobutyrate (control); ▲, α -(methylamino)isobutyrate; △, phenylalanine; ◆, glycine; ○, serine. α -Amino[14 C]isobutyrate and unlabelled amino acid inhibitor were added simultaneously. (b) Inhibition (%) of transport by various concentrations of α -(methylamino)isobutyrate and phenylalanine. ●, Unlabelled α -aminoisobutyrate (control); ▲, α -(methylamino)isobutyrate; △, phenylalanine; □, α -(methylamino)isobutyrate + phenylalanine, each at concentrations equal to that of the control unlabelled α -aminoisobutyrate; ■, α -(methylamino)isobutyrate + phenylalanine, each at concentrations one-half that of unlabelled α -aminoisobutyrate.

we conclude that α -aminoisobutyrate entry was mediated by more than one transport system. In view of the fact that α -(methylamino)isobutyrate and phenylalanine are not transported by the ASC system, the complete inhibition of α -aminoisobutyrate uptake by the combination of α -(methylamino)-

isobutyrate plus phenylalanine indicated that α -aminoisobutyrate entry by the ASC system (one possible interpretation from the studies with serine) was not likely to occur in these tumour cells, as was reported for α -aminoisobutyrate entry into hepatocytes (Le Cam & Freychet, 1977).

Additional evidence to support the suggestion that α -aminoisobutyrate entered by more than one carrier was obtained when α -(methylamino)isobutyrate was used to block α -aminoisobutyrate entry by the A system. Saturation experiments, performed in the presence of excess α -(methylamino)isobutyrate (20 mM) (results not shown), demonstrated both saturable and non-saturable processes for α -aminoisobutyrate entry; the saturable process depicted a linear relationship in the Eadie-Hofstee plot. When the saturable uptake in the presence of α -(methylamino)isobutyrate was subtracted from total α -aminoisobutyrate uptake [in the presence of α -(methylamino)isobutyrate], another saturable process was obtained, which also showed a linear relationship in the Eadie-Hofstee plot. The kinetic parameters and the relative capacities for the α -(methylamino)isobutyrate-sensitive and -insensitive components of α -aminoisobutyrate were similar to those for the Na^+ -dependent and -independent components, confirming the results reported above. Taken together, these results suggest that α -aminoisobutyrate is mediated by two transport systems, one with properties similar to those of the A system, and the other with properties similar to the L system.

One approach to quantifying the extent of α -aminoisobutyrate entry by the A and L systems used the techniques suggested by Inui & Christensen (1966), in which data were plotted as $1/(v_0 - v_i)$ versus $1/[I]$, where v_0 and v_i represent the values for α -aminoisobutyrate uptake in the absence and presence of various inhibitor concentrations respectively. Data plotted in this manner permit an estimate of the magnitude of the inhibitable portion of α -aminoisobutyrate uptake at infinite concentration of the amino acid inhibitor (the reciprocal of the intercept on the y axis). Fig. 5(a) illustrates the inhibition obtained when the entry of two different concentrations of α -aminoisobutyrate (0.25 and 1.0 mM) were measured in the presence of increasing concentrations of α -(methylamino)isobutyrate. At both concentrations of α -aminoisobutyrate, uptake was inhibited by about 80%. When $1/(v_0 - v_i)$ was plotted against $1/[I]$ (Fig. 5b), a linear relationship was obtained for each α -aminoisobutyrate concentration used and the reciprocal of the intercept was approx. 80% of that of the control (α -aminoisobutyrate transport in the absence of inhibitor).

It is of interest that the Inui-Christensen (1966) expression could also be represented in a form that allowed for analysis in a way analogous to an

Fig. 5. Inhibition of α -aminoisobutyrate transport by α -(methylamino)isobutyrate

(a) Inhibition of entry of α -amino[14 C]isobutyrate ($\circ$, 0.25 mM; $\bullet$, 1.0 mM) by increasing concentrations of α -(methylamino)isobutyrate. v^c represents the initial velocity for uptake after correction for the non-saturable component. (b) Data from (a) plotted as $1/(v_0 - v_i)$ versus $1/[\alpha$ -(methylamino)isobutyrate] (Inui & Christensen, 1966). v_0 and v_i represent v^c for α -aminoisobutyrate uptake in the absence and presence of various concentrations of α -(methylamino)-isobutyrate respectively.

Fig. 6. Inhibition of the Na^+ -independent uptake of α -aminoisobutyrate by 2-aminobicyclo[2.2.1]heptane-2-carboxylate

(a) Inhibition (%) of uptake of 0.1 M- α -amino[14 C]isobutyrate in the absence of Na^+ by increasing concentrations of ($\bullet$) unlabelled α -aminoisobutyrate (control) or ($\circ$) 2-aminobicyclo[2.2.1]heptane-2-carboxylate. (b) Data presented in (a) plotted as $1/(v_0 - v_i)$ versus $1/[I]$ (Inui & Christensen, 1966). v_0 and v_i represent the v^c for uptake in the absence and presence of various concentrations of the inhibitors respectively.

Eadie-Hofstee representation. When the following formula:

$$(v_0 - v_i) = \left[\frac{-K_i(K_m + [A])}{K_m} \times \frac{(v_0 - v_i)}{[I]} \right] + \frac{v[A]}{K_m + [A]}$$

was derived and the data were plotted as $(v_0 - v_i)$ versus $(v_0 - v_i)/[I]$, a straight line resulted, in which the intercept at the y axis represented the inhibitable portion of α -aminoisobutyrate uptake at infinite concentration of the inhibitor. This calculation provided a value of approx. 80%, which agreed well with the analysis shown above.

Inhibition of the Na^+ -independent uptake of α -aminoisobutyrate

The data presented above suggest the presence of two components for α -aminoisobutyrate uptake.

Moreover, the partial inhibition of α -aminoisobutyrate uptake by α -(methylamino)isobutyrate and the demonstration of the existence of a Na^+ -independent carrier-mediated entry suggested that α -aminoisobutyrate utilized an L-like system. To examine the specificity of this Na^+ -independent entry further, we chose to use 2-aminobicyclo[2.2.1]heptane-2-carboxylate, an analogue used successfully by Christensen *et al.* (1969) as a probe for the Na^+ -independent system. Fig. 6(a) shows that the entry in the absence of Na^+ was progressively inhibited by increasing concentrations of 2-aminobicyclo[2.2.1]heptane-2-carboxylate, with complete inhibition at 40 mM, a result similar to that obtained with unlabelled 40 mM- α -aminoisobutyrate. This was confirmed by a plot of $1/(v_0 - v_i)$ versus $1/[I]$ (Fig. 6b); the intercepts on the y axis were identical for 2-aminobicyclo[2.2.1]heptane-2-carboxylate and unlabelled α -aminoisobutyrate, indicat-

Fig. 7. Effect of Na⁺ and pentachlorophenol (0.75 mM) on α-aminoisobutyrate accumulation

Tumour cells were allowed to accumulate α-aminoisobutyrate from medium containing 0.1 mM, in the absence and presence of Na⁺ for periods up to 4 h. In the presence of Na⁺, pentachlorophenol was added at zero time to one group of cells (◆). To another group, pentachlorophenol was added after the cells accumulated α-aminoisobutyrate for 90 min (▲), and to the third group of cells, no pentachlorophenol was added (●). In the absence of Na⁺, pentachlorophenol was added to one group of cells after the cells had accumulated α-aminoisobutyrate for 60 min (△), and to the other group no pentachlorophenol was added (○). α-Aminoisobutyrate accumulation was measured at the indicated time points and distribution ratios were calculated (see the Materials and Methods section).

ing complete inhibition of the Na⁺-independent entry by 2-aminobicyclo[2.2.1]heptane-2-carboxylate.

Effect of Na⁺ on α-aminoisobutyrate accumulation and effects of pentachlorophenol

The time course of accumulation in the presence or absence of Na⁺ was examined for periods up to 4 h. As shown in Fig. 7, in the presence of Na⁺, α-aminoisobutyrate accumulated rapidly, reaching a distribution ratio (see the Materials and Methods section) of 19 by 60 min, which was maintained for up to 4 h. When 0.75 mM-pentachlorophenol, a potent inhibitor of mitochondrial ATP synthesis (Parker, 1958), was added after the cells had been allowed to accumulate α-aminoisobutyrate for 90 min, the amount retained in these cells rapidly decreased, reaching a distribution ratio of less than 1 by 60 min after addition of pentachlorophenol. These results show that the tumour cells were capable of accumulating α-aminoisobutyrate against its concentration gradient and that this process was energy-dependent.

Also in the absence of Na⁺, α-aminoisobutyrate

Fig. 8. Effect of pH on α-aminoisobutyrate (0.1 mM) transport

The uptake was measured in the presence (●) or absence (○) of Na⁺ (see the Results section).

was found to accumulate against its concentration gradient. At 60 min, the distribution ratio was 5, and decreased gradually to a value of 2 by the fourth hour. Addition of pentachlorophenol to cells that had accumulated α-aminoisobutyrate for 60 min decreased the amount retained by the cells, such that the distribution ratio was less than 1 by 30 min after inhibitor addition. The effects of pentachlorophenol, when added after α-aminoisobutyrate accumulation, were similar to those obtained when it was added at zero time and α-aminoisobutyrate accumulation (in the presence of Na⁺) was determined at various times up to 4 h.

Effect of pH

Fig. 8 shows the pH profile for α-aminoisobutyrate uptake in the presence and absence of Na⁺. Entry in the presence of Na⁺ was pH-sensitive, with optimum transport at pH 7.5; however, in the absence of Na⁺, entry was not pH-sensitive. These results are similar to those reported for the A and L systems (pH-sensitive and -insensitive respectively) in Ehrlich ascites-tumour cells (Christensen, 1969).

Effect of diabetes on the Na⁺-dependent and -independent α-aminoisobutyrate uptake

Since insulin has been shown to affect the entry of amino acids transported only by the A system (Guidotti *et al.*, 1974b; Riggs & McKirahan, 1973), the effects of the presence or absence of insulin, i.e. diabetes, *in vivo* on transport of α-aminoisobutyrate were examined. Data in Table 1 show that total saturable transport in the presence of Na⁺ (i.e. Na⁺-dependent + Na⁺-independent components) was

Table 1. *Effect of diabetes on transport of α -aminoisobutyrate, proline and phenylalanine into R3230AC tumour cells*

The v^c for α -aminoisobutyrate (in the presence of Na^+), proline and phenylalanine was determined, as described in the Materials and Methods section, in cells from intact and diabetic rats. Amino acids were present at 0.1 mM. Phenylalanine transport was determined in the presence of unlabelled 50 mM-proline. v^c is expressed as pmol/min per 5×10^6 cells. Data presented are means $\pm$ S.E.M. (each transport-determination performed in triplicate); the number of separate experiments is indicated in parentheses.

	Intact	Diabetic	Percentage of intact
α -Aminoisobutyrate			
Total	147.2 $\pm$ 30.6 (6)	224.3 $\pm$ 29.0 (12)	152
Na^+ -dependent	56.1 $\pm$ 6.0 (4)	111.9 $\pm$ 11.2 (9)	199
Na^+ -independent	118.0 $\pm$ 14.1 (4)	134.6 $\pm$ 14.9 (11)	114
Proline	150.1 $\pm$ 12.0 (10)	258.7 $\pm$ 25.0 (17)	172
Phenylalanine	2298 $\pm$ 64 (5)	2485 $\pm$ 240 (5)	108

Table 2. *Effect of diabetes on the kinetic parameters of α -aminoisobutyrate transport in R3230AC tumour cells*

Transport was measured at various α -aminoisobutyrate concentrations in cells from intact or diabetic rats, in the presence and absence of Na^+ . V and K_m for the Na^+ -dependent and -independent components were calculated. V is expressed as pmol/min per 5×10^6 cells; K_m as mM; data presented are means $\pm$ S.E.M. for four experiments.

	Intact	Diabetic	Percentage of intact
Na^+ -dependent			
V	1372 $\pm$ 165	2249 $\pm$ 210	164
K_m	2.32 $\pm$ 0.07	2.08 $\pm$ 0.05	
Na^+ -independent			
V	596 $\pm$ 63	750 $\pm$ 14	125
K_m	0.348 $\pm$ 0.03	0.392 $\pm$ 0.028	

greater in cells from diabetic rats than in cells from intact rats. Whereas transport in the absence of Na^+ was similar in cells from diabetic or intact rats, the Na^+ -dependent components for α -aminoisobutyrate uptake in cells from diabetic rats was twice that in cells from intact rats.

Table 1 also shows that proline transport, which has been shown to be mediated primarily by the A system in these tumour cells (Hissin & Hilf, 1978), was also greater in cells from diabetic rats (172%) than in intact rats, results supporting those for the Na^+ -dependent component for α -aminoisobutyrate entry. The results obtained with phenylalanine, which enters primarily by the L system, are similar to those obtained with the Na^+ -independent α -aminoisobutyrate entry. In these experiments, phenylalanine (0.1 mM) transport was determined in the presence of 50 mM-proline to inhibit phenylalanine entry by the A system. Thus the presence or absence of insulin *in vivo* affected the transport of amino acids that are mediated by the A system and not those transported by the L system.

Effect of diabetes on the kinetic parameters of α -aminoisobutyrate entry

Since insulin has been reported to modify transport processes by affecting either the V (Elsas *et al.*, 1975; Guidotti *et al.*, 1971, 1974b; Hahn *et al.*, 1971; Neville & Lobitz, 1977), or the K_m (Akedo & Christensen, 1962) or both V and K_m (Elsas *et al.*, 1971; Goldfine *et al.*, 1972), the effect of insulin on the maximum capacity and affinity for the Na^+ -dependent and Na^+ -independent entry of α -aminoisobutyrate was examined. The rate of uptake was determined at various α -aminoisobutyrate concentrations (0.05–40 mM) in the presence or absence of Na^+ in cells from either diabetic or intact rats; V and K_m were determined as described above. Table 2 shows that the K_m for both the Na^+ -dependent and -independent components was similar in cells from diabetic and intact rats. However, the V for the Na^+ -dependent component in cells from diabetic rats was significantly greater than that in cells from intact rats (164%). The V for the Na^+ -independent component was somewhat higher in cells from diabetic rats (125%).

Discussion

As part of our overall goal to elucidate the effects of insulin and other hormones on tumour growth and metabolism, we chose α -aminoisobutyrate as one probe for measurement of amino acid transport. Since this analogue was shown by Christensen (1969) to enter Ehrlich ascites-tumour cells by a single carrier system (A system), we had not expected to find that, even after correcting for the non-saturable entry, the saturable process revealed a curvilinear relationship according to the Eadie-Hofstee plot of the data. This result suggested that the entry of α -aminoisobutyrate was mediated by more than one transport carrier, and experiments were conducted to characterize this process before examining the effects of hormonal perturbation.

The approach we employed was to measure α -aminoisobutyrate uptake as it was influenced by Na^+ , by pH, by the presence of selected inhibitors of transport and by its energy-dependence. Data were obtained indicating that: (1) the Na^+ -dependent saturable component for α -aminoisobutyrate transport displayed a linear Eadie-Hofstee plot; (2) in the presence of Na^+ , uptake was sensitive to the pH of the medium; (3) addition of either α -(methylamino)-isobutyrate or proline inhibited the Na^+ -dependent entry of α -aminoisobutyrate, and since both of these compounds were shown to enter these tumour cells exclusively by the A system (Hissin & Hilf, 1978), we interpret this as evidence that α -aminoisobutyrate uptake in the presence of Na^+ occurs by the A system; (4) the metabolic inhibitor, pentachlorophenol, inhibited this concentrative process. Thus the entry of a major portion of α -aminoisobutyrate occurred by a process with characteristics described for the A carrier system.

However, our data indicated that in these tumour cells, transport of α -aminoisobutyrate also occurred by another process, which showed the following properties: (1) a Na^+ -independent saturable process that displayed a linear Eadie-Hofstee plot and was not sensitive to pH; (2) entry was not inhibited by either α -(methylamino)isobutyrate or proline alone but was inhibited when phenylalanine was added with α -(methylamino)isobutyrate; (3) the Na^+ -independent entry of α -aminoisobutyrate was completely inhibited by 2-aminobicyclo[2.2.1]heptane-2-carboxylate, an analogue specific for the Na^+ -independent system (Christensen, 1969). These data imply that entry of α -aminoisobutyrate into these tumour cells also occurred by a carrier with properties similar to those described for the L system. Thus we conclude that uptake of α -aminoisobutyrate by R3230AC mammary adenocarcinoma cells occurs by two classes of carriers, results that closely resemble those reported by Gazzola *et al.* (1972) for chick-embryo heart cells and by Finerman & Rosenberg (1966) for foetal rat calvaria. It appears that there may be similarities in α -aminoisobutyrate transport between certain neoplastic and embryonic tissues; biochemical similarities between tumour and embryonic tissues have been noted by others (Knox, 1972).

An interesting finding reported here related to the effects of pentachlorophenol on the concentrative uptake process in the presence, as well as in the absence, of Na^+ . These data implicate an alkali-metal gradient as a major supplier of energy required by the concentrative process, although other sources of energy, such as mitochondrial ATP or the membrane potential, could also play a role. It should be noted that the Na^+ -independent (L system) carrier does not usually exhibit uphill transport, although concentrative transport by this system has been

implied under certain conditions (Christensen, 1975b). One could postulate therefore that either the L system in these tumour cells has the capability for concentrative uptake or that these cells possess an additional transport system with properties that are similar to those described for the L system.

The effects of diabetes on α -aminoisobutyrate transport are intriguing. The increase in V of the Na^+ -dependent α -aminoisobutyrate component as well as proline uptake in cells from diabetic rats suggested that the transport activity of the A system was responsive to the presence or absence of insulin *in vivo*. The specificity of the effect of insulin is indicated by the finding that the Na^+ -independent α -aminoisobutyrate transport as well as phenylalanine uptake were similar in cells from diabetic or intact rats. These observations agree with those reported in the chick-embryo heart cells (Guidotti *et al.*, 1974b) and the isolated rat diaphragm (Riggs & McKirahan, 1973). Furthermore, the effect of insulin appears to be directed towards the maximum capacity rather than the affinity of these carriers. It is pertinent that the increased transport of proline and α -aminoisobutyrate transport in tumour cells from diabetic rats may correlate with the effect of diabetes on tumour growth; tumour growth was more rapid in diabetic rats than in intact rats. Furthermore, daily administration of insulin to tumour-bearing rats produced an inhibition of tumour growth and returned proline transport to that observed in intact animals (P. J. Hissin & R. Hilf, unpublished results).

Taken together, the results presented above indicate that α -aminoisobutyrate is transported by a Na^+ -dependent α -(methylamino)isobutyrate-inhibitable pH-sensitive system, whose properties are consistent with those known for the A system (Christensen, 1969), as well as by a Na^+ -independent 2-aminobicyclo[2.2.1]heptane-2-carboxylate-inhibitable α -(methylamino)isobutyrate- and pH-insensitive system, whose properties are consistent with those described for an L-like system. It is not possible to say with certainty whether the two carriers identified for α -aminoisobutyrate are (a) unique to mammary tumours as compared with Ehrlich ascites tumours, (b) are artifacts of enzymically dissociated cells rather than a mechanism of naturally occurring free-cell systems or (c) are peculiar to the R3230AC mammary adenocarcinoma as compared with other rodent breast cancers. Regardless of the reasons for the findings, it is clear that careful and adequate characterization of the mode of entry of amino acids must precede any experiments utilizing agents, e.g. insulin, to perturb the system.

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