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Uptake of α-aminoisobutyric acid by cultured human fibroblasts

Recent advances in understanding transmembrane transport in microbial systems have depended on the availability of large quantities of purified cell membranes from single cell lines[1]. This requirement can be met in man by using human cells grown in tissue culture. A preliminary step to the use of purified membrane systems is the definition of transport phenomena in intact cells. The present study was undertaken to determine if human diploid fibroblasts, grown in monolayer culture, are applicable to the study of amino acid transport. Our results with α-aminoisobutyric acid, a model amino acid which is not metabolized intracellularly, indicate that cultured fibroblasts can be readily used to investigate transport processes in man.

Human fibroblasts were obtained from skin biopsies of healthy children and adults. They were grown in a medium containing 10 % fetal calf serum and 0.1 mg/ml neomycin and were used for transport studies between the 4th and 21st passage in culture. Cells were removed from the glass surfaces either by gentle scraping or with 0.25 % trypsin. The cells were then rinsed twice, suspended, and incubated in a phosphate buffered saline (pH 7.34) containing the following concentrations of ions in mequiv/l: Na^+, 156; K^+, 4.2; Ca^{2+}, 1.8; Mg^{2+}, 1.6; Cl^-, 145; PO_4^{3-}, 29; SO_4^{2-}, 1.6. The final cell suspension contained 0.8–1.2 million cells/ml (8–12 mg wet cell weight/ml).

Incubation was carried out with 0.5 ml cell suspension in 10 ml glass erlenmeyer flasks at 37° in a Dubnoff metabolic shaker. Radioactivity of the substrate, [1-^{14}C]-α-aminoisobutyric acid, in the incubation medium was 0.1–0.2 μC/ml. When inhibitors were used, the inhibitor was added to the cell suspension for a 2 min preincubation period before adding the labelled substrate. One series of experiments used low sodium concentrations in the incubation medium. This was done by equimolar substitution of Tris–HCl for sodium salts in the phosphate buffered saline. This Tris buffer was used for harvesting as well as incubating the cells.

To terminate the incubation we followed the method used previously with renal tubules[2]. The cell suspension was poured from incubation flask to 3 ml plastic tared tubes precooled to 0° and immediately spun at 48000 × *g* in a refrigerated centrifuge. Medium was removed, the cell pellet rinsed and weighed, and the cells lysed by boiling in distilled water. Aliquots of boiled incubation medium and of tissue supernatant were counted in a liquid scintillation counter.

A distribution ratio of intracellular to extracellular radioactivity was calculated as previously described[3]. Trapped extracellular fluid volume (determined with [^{14}C]-inulin) was 14 ± 2 % (mean ± S.D.) of the wet cell weight and total fluid volume was 78 ± 2 % of the wet weight.

Trypan blue was used to assess apparent cell viability and we assumed those cells which excluded the dye were viable. When cells were harvested by scraping them from the growth surface, less than 20 % were viable and no net uptake of α-aminoisobutyric acid could be shown in subsequent experiments. Trypsinization yielded cells that were 90–95 % viable and all of the studies reported below used trypsinized cells.

After 15 min incubation, the percentage of viable cells had dropped to 78–88 % but no further decrease occurred for incubation intervals up to 45 min. Starting in the first minutes of incubation, however, and increasing with time there was a loss

of total cells from the incubated suspension. Some of these cells adhered to the flasks while others fragmented during shaking. Those remaining in suspension, however, continued to have a high viability count and it was these cells that were recovered for determination of intracellular radioactivity. The magnitude of cell loss ranged as high as 33 % after 60 min incubation. We have since learned that this loss can be kept lower by using plastic incubation vessels.

The relationship of net uptake to duration of incubation was studied at an initial α-aminoisobutyric acid substrate concentration of 0.1 mM. Fig. 1 shows the uptake curve in three different cell lines, each of which accumulated α-aminoisobutyric acid to intracellular concentrations much greater than that of the incubation medium. Triplicate determinations, made at each point, had a variation of 10 % or less for incubation intervals less than 30 min but as great as 25 % for longer intervals. Net uptake was linear for the first 20–30 min and equilibrium was approached between 30 and 60 min for cell lines A and C but not until 90 min for cell line B.

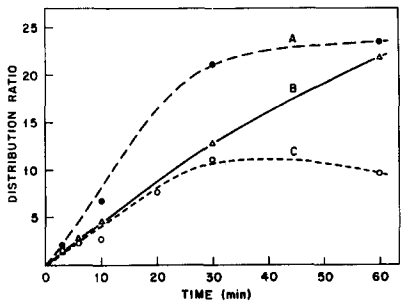

Fig. 1. Time-course of α-aminoisobutyric acid uptake (0.1 mM) by three human fibroblast lines, denoted A, B, and C. Each point is the mean of triplicate observations.

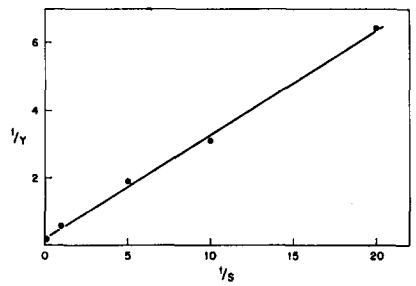

Fig. 2. Double reciprocal plot of transport velocity, $1/Y$ (mmoles/l per 6 min), against medium substrate concentration, $1/S$ (mM), for α-aminoisobutyric acid uptake by human fibroblast line B. Each point is the mean of duplicate observations. Incubation time was 6 min.

To demonstrate saturation kinetics for α-aminoisobutyric acid uptake, seven initial substrate concentrations from 0.05 to 40 mM were chosen and an incubation period of 6 min was used. The apparent diffusion constant, K_D, was 0.26 per 6 min. A Lineweaver–Burk plot from cell line B is shown in Fig. 2 and gives an apparent K_m of 2.2 mM and v_{max} of 6.7 mmoles/l per 6 min. These values for cell line C were 1.8 mM and 7.1 mmoles/l per 6 min, respectively.

α-Aminoisobutyric acid uptake was inhibited significantly by ouabain, cyanide, *p*-chloromercuribenzoate and by glycine (Table I). Uptake was also impaired by reducing the Na^+ concentration of the incubation medium. After a 20-min incubation period, the Na-free medium had a measured Na^+ concentration of 0.7 mequiv/l, but even at this low Na^+ concentration, α-aminoisobutyric acid was still accumulated against a concentration gradient (distribution ratio = 2.6).

Amino acid transport by cultured cells in monolayer has been studied by a few investigators. PLATTER AND MARTIN[4] demonstrated that human fibroblasts attached to coverslips accumulated tryptophan. HARE[5] studied phenylalanine transport in hamster embryo cells in monolayer. FOSTER AND PARDEE[6] also used cell monolayers to investigate α-aminoisobutyric acid uptake in an aneuploid mouse embryo cell line

TABLE I

EFFECT OF METABOLIC AND COMPETITIVE INHIBITORS AND OF MEDIUM SODIUM ION CONCENTRATION ON α-AMINOISOBUTYRIC ACID UPTAKE BY HUMAN FIBROBLASTS

*Experimental conditions**	*Inhibition* (%) *at 20 min*
Ouabain ($1 \cdot 10^{-4}$ M)	46
Sodium cyanide ($5 \cdot 10^{-3}$ M)	52
p-Chloromercuribenzoate ($1 \cdot 10^{-4}$ M)	82
Glycine ($2 \cdot 10^{-3}$ M)	54
Medium Na^+, 110 mequiv/l	29
Medium Na^+, 50 mequiv/l	57
Medium Na^+, 0 mequiv/l	79

* Human fibroblasts were preincubated with the inhibitors for 2 min prior to the addition of α-aminoisobutyric acid (0.1 mM). Uptake was measured after 6 and 20 min periods, and % inhibition was similar at both intervals. The % inhibition compares the distribution ratio achieved by cells in the presence of inhibitor with the ratio in control cells studied in the same experiment. All values were calculated from the mean of duplicate observations.

and estimated a transport K_m for α-aminoisobutyric acid of 1.3–1.8 mM compared to our estimate of 1.8–2.2 mM in the human fibroblasts.

We chose to study transport in suspension to more readily quantitate parameters of cell number or mass and to provide preliminary data for experiments requiring large quantities of membranes for chemical analysis. Our results indicate that human fibroblasts accumulate α-aminoisobutyric acid by processes which are energy-dependent, Na-sensitive, and saturable. They further indicate that cells prepared by trypsin can be used to study transport by intact cells and presumably for more detailed membrane analysis.

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