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Unidirectional flux of phenylalanine into Vero cells. Measurement using paired tracers in perfused cultures

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The uptake of phenylalanine by Vero cells in perfused culture was measured using a double-label technique. Cells were anchored in microcarrier beads and maintained in a column perfused at a constant rate. The extracellular tracer [^{14}C]mannitol and the test tracer [^{3}H]phenylalanine were injected as a bolus, and the column effluent was sampled at 10-s intervals. The proportion of the test tracer retained by the cells was calculated by analysis of time-dilution curves of test and reference tracers. Uptake measurements were specific and highly reproducible. Uptake of [^{3}H]phenylalanine was inhibited by unlabelled phenylalanine and by other amino acids that utilize transport system L. This new approach proved useful for rapid measurement of unidirectional uptake, and for determination of kinetics parameters of uptake under steady state conditions. This rapid technique obviates some of the limitations associated with uptake measurements in whole organs and with measurements in conventional cell cultures.

Introduction

Membrane transport of substrates is a process which involves more than one potentially rate-limiting step. Even the most simplified model of transport has to account for at least a binding or recognition event at one surface of the membrane, a translocation event across the membrane, and a dissociation step at the opposite surface. The binding and dissociation steps are fast relative to the time resolution of most experimental methods that measure the rate of the transport process. For this reason, the rate of the translocation event is what, in most cases, determines the measured rate of substrate uptake [1,2]. However, if the resolution of the experimental measurement is too low with respect to time, secondary factors such as metabolic transformation and efflux of substrate can influence the rate measured. This consideration is especially pertinent to in vitro transport measurements in cell and tissue cultures, where metabolites accumulate in the extracellular space. Therefore, measurement of the rate of accumulation of a labeled substrate inside cells may not be a good estimate of the rate of substrate influx unless the time resolution of the measurement is such that only the initial unidirectional uptake or translocation event is measured.

The problem of differentiating between unidirectional flux of substrates and secondary rate altering steps, has been approached by the use of paired-tracer techniques in studies with perfused organs [3–5]. In these studies, two radioactive tracers, test and reference tracer, are injected into the arterial inflow of an isolated organ, and the venous effluent is sampled at short time intervals. The concentrations of the test and reference tracers

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are determined in the samples and normalized with respect to the total amount of tracer injected. The reference tracer is a molecule which remains extracellular but which has physicochemical characteristics similar to those of the test tracer.

In this report we describe a new technique in which the paired-tracer principle is applied to the in vitro study of unidirectional flux of substrates in homogeneous cell populations. Data from a study on the uptake of phenylalanine by vero cells is presented to illustrate the methodology. Parts of this work were presented at the Third World Congress for Microcirculation, London 1984, and at the November meeting of the Physiological Society, London 1984.

Rapid paired-tracer techniques, as applied to uptake measurements are based on analysis of dilution curves obtained in continuous flow systems [3–5]. Application of these techniques to homogeneous cell populations requires culture conditions which allow for controlled perfusion of the cells. Such conditions were established for Vero cells using microcarrier beads as an anchorage surface and a column perfused at a constant flow rate. Similar perfused-cultures for other cell types and for other applications, have been described [6–8].

Methods

Vero cells, a fibroblast-like cell line, originally isolated from the kidney of an African green monkey [9] were obtained from the American Type Culture Collection, and were maintained by serial passage in RPMI, 10% FCS. $(8–10) \cdot 10^6$ cells were mixed with 10 ml of microcarrier beads at 10 mg/ml (Flow laboratories, McLean, VA) and incubated in polystyrene petri dishes (100×15 mm Lab-Tek Division, Miles Laboratories, Inc., Naperville, IL 60540). The microcarrier covered with cells were packed into a thermo-regulated (37°C) column (Pharmacia, Piscataway, NJ) fitted with flow adaptors. The section of the column occupied by the packed beads measured 0.5×1 cm and was perfused at a continuous flow rate maintained at 10 μl/s with a peristaltic pump (VarioPerpex LKB, Rockville, MD). Before each series of uptake measurements the column was equilibrated for at least 90 min, with Hanks' salt solution (Life Technologies, Grand Island, NY) containing 25 mM Hepes buffer. The uptake of ^{3}H-labelled phenylalanine with respect to the extracellular marker [^{14}C]mannitol (Amersham, Arlington Heights, IL) was determined as follows: A mixture of [^{3}H]phenylalanine (7 μCi, 47.5 Ci/mmol) and [^{14}C]mannitol (2.8 μCi, 60 mCi/mmol) in 100 μl of Hanks' salt solution, was rapidly injected into the inflow tubing of the column. After the tracers reached the outflow opening of the column (30 s), twenty-four, 50-μl samples were collected. The amounts of test ([^{3}H]phenylalanine) and reference ([^{14}C]mannitol) tracer in each sample were estimated by scintillation counting in a Packard 2000 counter, and normalized as percentage of the total amount of each tracer injected. Percent uptake (U) in each sample was calculated from the normalized concentration of the tracers according to the formula $\%U = (1 - c_t/c_r)$, were c_t is the normalized concentration of [^{3}H]phenylalanine, and c_r is the normalized concentration of [^{14}C]mannitol [3,4].

Results and Discussion

The [^{3}H]phenylalanine present in the injection mixture was selectively retained during passage through the column. This is indicated in Fig. 1A which depicts the profiles of the two tracers expressed as percentage of the total amount injected. The retention of [^{3}H]phenylalanine by the column was almost completely inhibited in the presence of 100 mM unlabelled phenylalanine. Under these conditions the elution times of both tracers were essentially identical, indicating lack of non-specific binding or artifactual delay of [^{3}H]phenylalanine (Fig. 1B). The percent uptake calculated from the elution profiles reached a maximum 30 s after initial detection of label in the effluent and then decreased slightly at later sampling times. Fig. 2A. This slight decrease in percent uptake may reflect some efflux of [^{3}H]phenylalanine occurring at these times. Uptake measurements were very reproducible and in 12 measurements the mean maximal uptake $\pm$ S.E. was $82 \pm 1.2\%$. The percent recovery of the reference tracer was not significantly different from 100 ($95.77\% \pm 9.9\%$ for ten experiments).

These measurements were performed at very

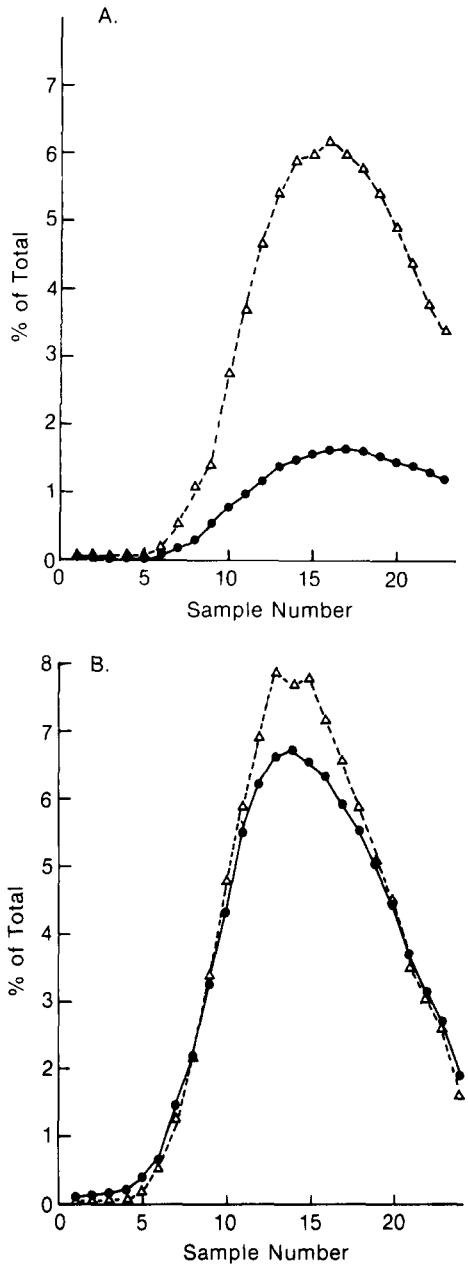

Fig. 1. Elution profiles of [^{14}C]mannitol (△) and [^{3}H]phenylalanine (●). Data represent the normalized concentrations of each tracer in the effluent of a column packed with Vero cells ($\times 10^7$) and perfused at a flow rate of 10 μl/s with buffer devoid of unlabelled amino acids. The volume of each sample was 50 μl and samples were collected continuously. (A) Profiles of tracers injected without unlabelled amino acids. (B) Profiles of tracers injected mixed with 100 mM unlabelled L-phenylalanine. Normalized concentrations were calculated as explained in the text (Methods).

low concentrations of the [^{3}H]phenylalanine, at short time intervals, and in the absence of unlabelled phenylalanine. Under these conditions, the uptakes obtained were directly proportional to the substrate concentration. This can be deduced from Fig. 2B which shows a linear relationship between the amounts of [^{3}H]phenylalanine retained by the cells and the concentrations of phenylalanine in the perfusate. The concentration of [^{3}H]phenylalanine in the perfusate was calculated from the normalized concentration of mannitol in perfusate sampled during the interval elapsed be-

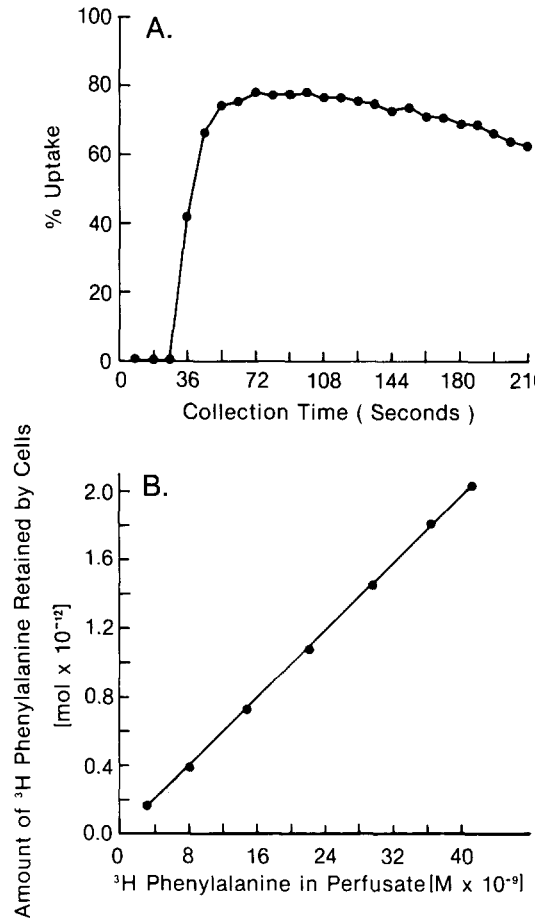

Fig. 2. Uptake of phenylalanine by Vero cells. (A) Percent uptake during the complete sampling interval. (B) Uptake of phenylalanine as a function of the concentration of phenylalanine in perfusate, measured during the interval elapsed between the 54th and 108th second. Percent uptake and phenylalanine concentrations were calculated as explained in text (Results).

tween the 54th and 108th second. (As shown in Fig. 1B, this interval corresponds to the time during which the concentration profiles of [^{3}H]phenylalanine and [^{14}C]mannitol were superimposable, and therefore, at these times the normalized concentration of mannitol reflects accurately the normalized concentration of [^{3}H]phenylalanine in the perfusing fluid entering the column). In the experiment represented in Fig. 2 the concentration of [^{3}H]phenylalanine in perfusate =

$$(\%[^{14}\text{C}]\text{mannitol} \times (\text{total mol } [^3\text{H}]\text{phenylalanine injected}) \times 1.58 \times 10^{-4})/100.$$

The volume of each sample was 63 μl, thus, the factor 1.58×10^{-4} transform the concentration of [^{3}H]phenylalanine from mol/63 μl, to M. The amount of [^{3}H]phenylalanine retained by the column at each of the sampling times was determined from the percent uptake ($\%U$):

$$\text{mol } [^3\text{H}]\text{phenylalanine retained} = (\text{mol } [^3\text{H}]\text{phenylalanine in perfusate}) \times \%U/100$$

Since the intervals between samples were equal and the flow rate remained constant, these data (Fig. 2B) indicate that the rate of phenylalanine uptake by Vero cell was directly proportional to the concentration of phenylalanine in perfusate.

The ability of the cells for phenylalanine uptake remained constant for at least 4 h of culture in the perfused column. This was demonstrated in seven consecutive measurements performed in the same cell preparation. After each measurement, the culture was equilibrated for approximately 30 min with buffer devoid of phenylalanine. As indicated in Fig. 3, the maximal uptakes measured after each equilibration period did not change significantly, indicating that this type of perfused culture can be used for studies requiring multiple measurements.

To further determine the specificity of the uptake, inhibition experiments were performed by mixing 50 mM concentrations of various unlabelled amino acids with the tracers in the injection (Table I). The pattern of inhibition observed was as expected for an amino acid such as phenylalanine which is transported primarily by transport system L [2].

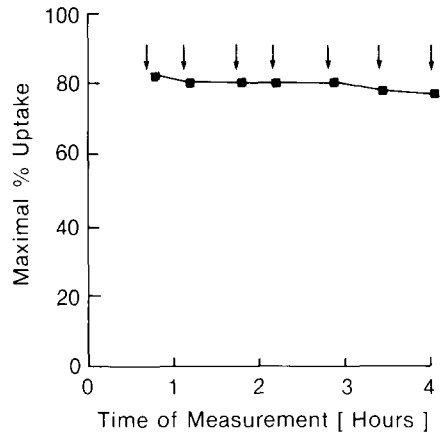

Fig. 3. Percent maximal uptakes obtained after repeated measurements on cells maintained for 4 h in perfused culture. Arrows indicate the times at which tracers were injected and uptake measurements initiated. Each measurement was performed as indicated in the text (Methods) and Figs. 1 and 2.

In addition to rapid measurements of uptake at low substrate concentrations, the double-tracer dilution technique was also applied to the study of kinetics parameters of uptake under steady-state conditions. Steady-state conditions were assumed after perfusing the cultures with a constant concentration of unlabelled phenylalanine for at least 10 min. Measurements were performed as explained above, except that the tracers were injected mixed with unlabelled phenylalanine at

TABLE I

SELECTIVE INHIBITION OF [^{3}H]PHENYLALANINE UPTAKE BY VARIOUS UNLABELLED AMINO ACIDS

The uptake of [^{3}H]phenylalanine by Vero cells was measured in the presence of various unlabelled L-amino acids (50 mM). Percent uptakes were calculated as indicated in text and compared with uptakes measured in the absence of unlabelled L-amino acids. The data represent the means ± S.E. of values of the maximal uptakes ($\%U$) obtained in three experiments. Percent inhibition = 100($\%U$ − $\%U$ with inhibitor)/$\%U$.

Amino acid	[^{3}H]phenylalanine	
	% Uptake	% Inhibition
None	81.3 ± 3.3	0
Phenylalanine	14.3 ± 0.7	82.3 ± 0.7
Proline	14.3 ± 0.7	6.4 ± 2.3
Glycine	68.5 ± 1.9	15.3 ± 4.6
Threonine	37.9 ± 7.8	52.4 ± 11.0
Methionine	19.5 ± 0.4	76.5 ± 1.6

the same concentration used in the perfusing fluid. Using this approach the changes in the concentration of substrate entering the column during measurement are negligible and the labelled phenylalanine retained per unit of time (s) by the cells can be used to estimate the total amount of phenylalanine (labelled and unlabelled) retained per second. Measurements in the presence of 0.5, 1, 2, 5 and 10 mM concentrations of unlabelled phenylalanine were performed at random, and repeated in three different cell preparations. The velocity of the uptake (influx) reaction at each concentration of phenylalanine was calculated for each one of the concentrations tested according to the formula

$$V = F(\%U \cdot C/100)$$

where F is the flow rate in ml/min, $\%U$ is maximal percent uptake, and C is the concentration of phenylalanine in the perfusion fluid. Since in these experiments maximal $\%U$ was below 50%, correction for the decrease of tracer along the length of the column was not necessary [11]. At millimolar concentrations of phenylalanine in perfusate, the influx displayed saturation kinetics as indicated in Fig. 4 which represents the best fit line obtained by polynomial regression analysis of the data. The multiple correlation-coefficient for the quadratic equation was 0.938. A double-reciprocal plot of the points predicted by the best fit line is included

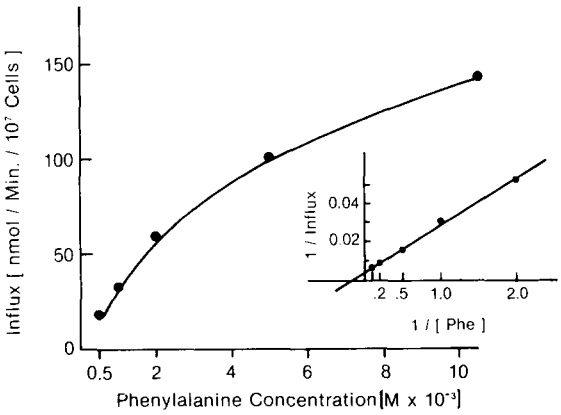

Fig. 4. Rate of phenylalanine influx into Vero cells maintained in perfused cultures. Influx was measured at five different concentrations of phenylalanine in three different cell preparations. Data were fitted by polynomial regression analysis. Inset represents a double-reciprocal plot of the calculated points.

TABLE II

RATE OF PHENYLALANINE UPTAKE BY VERO CELLS UNDER STEADY STATE CONDITIONS

The rate of uptake (influx) of phenylalanine by Vero cells, was measured at the concentrations indicated, as explained in the text. Data are the means ± S.E. from the number of experiments indicated in parenthesis.

Phenylalanine (mM)	Influx (nmol/min per 10^7 cells)
0.5	18.8 ± 3.8 (4)
1.0	31.9 ± 14.5 (3)
2.0	59.8 ± 10.5 (4)
5.0	106.0 ± 17.0 (3)
10.0	143.3 ± 5.7 (3)

as an insert. The actual values obtained are listed in Table II. The estimated apparent K_m was 3.8 mmol/l which is close to values reported for the uptake of phenylalanine measured in whole organ systems [11].

Measurement of transport in homogeneous cell suspensions is a necessary complement to uptake studies in whole organs since the cellular heterogeneity of organs makes it difficult to determine the relative contribution of each cell type. In addition, transport studies in cell cultures using tracer techniques are simple to analyze because only one extracellular compartment need to be considered. However, the time resolution of conventional culture techniques is limited by the time required for complete mixing of cells and tracers and for the subsequent separation of cells from media containing the tracers. Furthermore, accumulation of cell metabolites in the extracellular compartment can interfere with measurements and replicate cultures are necessary for experiments requiring repeated sampling.

In continuously perfused cultures 'mixing' and 'separation' of tracers and cells do not slow the measurements, and cell products, including the substrate of interest (or its labelled metabolites), do not accumulate in the extracellular space. Uptake is monitored continuously and therefore, the time resolution of the measurements is limited only by the flow rate and the size of the sample collected. With the column utilized for the experiments described here, with a flow rate of approx. 10 μl/s and a void volume of 300 μl, a 50 μl

sample corresponds to a period of 5 s of contact between perfusate and each cell in the column. Shorter times can be achieved by collecting smaller samples or by increasing the flow rate.

The application of this rapid technique to the measurement of uptake of metabolizable molecules by homogeneous cell populations permit the study of the process implicated in unidirectional transport (specific recognition by receptors and internalization) without interference from secondary metabolic events.

An additional advantage of the system described here is its potential applicability to primary cultures established with biopsy material. Since a single preparation of cells can be used repeatedly for uptake measurements complete inhibitory and kinetics studies may be performed in a single culture. This approach, therefore, fills a gap in the methodology for study of uptake by homogeneous cell populations and is presented as an alternative or complementary technique to conventional approaches.

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