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Amino Acid Uptake in Growing and Arrested Human Diploid Cell Populations (38843)

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(Introduced by M. K. Patterson, Jr.)

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Human diploid cells (HDC) but not their virus-transformed counterparts can be maintained in culture as essentially nonmitotic populations by reducing the serum concentration of the incubation medium from 10% to either 0.1% or 0.5% (1, 2). Cells maintained in this manner for extended periods of time remain viable and can reenter a state of rapid proliferation upon subcultivation with medium containing 10% serum (3, 4). This method for arresting and recovering the mitotic activity of cells *in vitro* may offer a more appropriate model system for the study of those tissues *in vivo* which exhibit rapid proliferation only when subjected to the proper stimulus, i.e., fibroblasts of connective tissue during wound healing (5).

Before this *in vitro* system can be used to investigate the regulatory processes of normally nonmitotic human cell populations, it is necessary to characterize it with respect to various biochemical and physiological parameters. One such parameter is the uptake of amino acids. While a considerable amount of information is available on the transport of metabolites by cells in culture, relatively few investigations have employed HDC as the experimental system; and no investigation has employed HDC during a long-term quiescent state (e.g., 6–13). Therefore, the objectives of these studies were to determine the uptake of amino acids in HDC at different stages of growth, log phase preconfluent and plateau phase confluent cell densities, and to compare these to the uptake in HDC subjected to the long term restriction of mitotic activity.

The results show that there are reductions in the rate of amino acid accumulation in arrested cells as compared to cells cultured under growth conditions. These decreases are reflected in alterations in the kinetic parameters of the uptake and appear compatible with the lower metabolic activity previously observed in arrested cultures (14).

Materials and Methods. Human diploid cell strain HFMD was derived from newborn foreskin tissue and was kindly supplied by Dr. Warren Stinebring of the Department of Medical Microbiology, University of Vermont, Burlington. These cells have a characteristic *in vitro* lifespan of 50 ± 10 population doublings. There was no apparent effect of *in vitro* "age" on the results reported in this communication. Procedures for routine cell culture and inducing an essentially nonmitotic state have been described previously (2). Cells were tested for mycoplasma contamination at weekly intervals by routine procedures (15) and by this criterion were free of infection. Experiments were performed 72 hr after the last medium renewal. The results were essentially unaffected by other feeding schedules.

Transport experiments were done at 37° with cells attached to T-25 Falcon tissue culture flasks 3 or 7 days after subculture, preconfluent and confluent, respectively, and 21 days after initiation of the arrested state. Previous studies indicated that mitotic indices at these three time points were 3.5%, 0.2%, and 0%, respectively (3). Cells were washed three times with warm modified Earle's balanced salt solution (MEBSS) [142 m*M* NaCl, 5.4 m*M* KCl, 1 m*M* NaH_2PO_4, 1.8 m*M* $CaCl_2$, 0.8 m*M* $MgSO_4$, 2 m*M* *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES), pH 7.4]. To study amino acid uptake, amino acid pools were first depleted for 1 hr by incubation with MEBSS containing 0.1% (w/v) glucose. Uptake was determined using 3 ml MEBSS containing 0.1% glucose and 2 m*M* ^{14}C-leucine or 2 m*M* ^{14}C-α-aminoisobutyrate (AIB) at a specific activity of 0.1 μCi/μmole. The isotope mixture was removed at various

time intervals and the cells were washed three times with ice-cold MEBSS. Cells were ruptured in 3 ml of 0.1 *M* citric acid and aliquots were taken for protein determination and isotope counting. Protein was determined by the method of Lowry *et al.* (16). Radioactivity was determined by liquid scintillation with quenching corrected by the channels ratio method (17). Values reported are the results from at least two experiments using replicate cultures at each time point and have been corrected for unremoved incubation medium by determining inulin spaces (18). Uptakes were proportional to cellular protein in the cell ranges utilized as reported by Foster and Pardee (6).

In studies to determine kinetic parameters, cells were prepared as described above and incubated for 3 min with 3 ml of the appropriate isotope mixture. Uptakes were measured from 0.1–4.0 m*M* ^{14}C-AIB of a constant specific activity (0.1 μCi/μmole). α-Aminoisobutyrate uptake was linear during the assay time over these concentration ranges. Data were fitted to a weighted least-square line by linear regression to calculate values for K_m and V_{max}.

Both inulin binding data and dye exclusion tests with trypan blue (19) indicated the conditions utilized in these studies were not deleterious to the cells.

^{14}C-α-Aminoisobutyric acid (12.3 mCi/mmole), ^{14}C-L-leucine (280 mCi/mmole), and ^{14}C-inulin (2.56 mCi/g) were purchased from New England Nuclear Corporation, and HEPES was purchased from Calbiochem. All other reagents were analytical grade.

Results. Figure 1 illustrates the uptake pattern of the amino acids leucine (1a) and AIB (1b) in preconfluent, confluent, and arrested human diploid fibroblasts. A significant reduction in both leucine and AIB uptake was evident only in arrested cultures. Leucine uptake is most rapid in the first 3 min with equilibrium being reached between 5 and 10 min; however, AIB did not reach equilibrium by 30 min. The reduction in leucine and AIB uptake in arrested cultures occurred within 7 days after placing the cells on 0.5% serum and essentially remained constant thereafter. Metabolic conversion of leucine was not a significant factor, as less than 5% of the total leucine accumulated by the cells in 30 min was present in trichloroacetic acid-precipitable material.

The data in Fig. 2 show the concentration dependence of the initial rate of AIB uptake in log phase preconfluent, plateau phase confluent, and arrested populations over the range 0.1–4.0 m*M* AIB. Preconfluent and confluent cultures showed similar rates, while arrested cultures accumulated AIB considerably slower (two to four times).

The apparent K_m and V_{max} for AIB uptake under the three culture conditions are presented in Table I. The K_m value for preconfluent cultures was 1.52 $\pm$ 0.07. A similar value was obtained for confluent cultures, while a somewhat smaller value, 0.90 $\pm$ 0.10, was obtained for arrested cultures. The V_{max} values were 4.41 $\pm$ 0.21, 3.03 $\pm$ 0.13, and 1.04 $\pm$ 0.11 nmoles of AIB uptake per min per mg of protein for preconfluent, confluent, and arrested cultures, respectively.

Discussion. Nonmetabolizable analogs are extensively utilized in elucidating the pathways of amino acid transport in cultured cells. α-Aminoisobutyrate is such a model for the uptake of several neutral amino acids, but it is not an analog for the leucine transport system (20). Foster and Pardee (6) reported confluent 3T3 cells accumulated AIB about 30% less rapidly than did preconfluent 3T3 cells. Figure 1b shows no significant difference in AIB uptake between preconfluent and confluent HDC at an external AIB concentration of 2 m*M*. Also, no significant differences were found in initial rates of AIB uptake between preconfluent and confluent HDC above AIB concentrations of 0.5 m*M* (Fig. 2). Griffiths (10) has reported a lower intracellular AIB and leucine concentration in confluent diploid human lung cells as compared to preconfluent cultures. While this does not appear to agree with the results reported here, it should be emphasized that initial rates of accumulation were measured in the current studies, whereas Griffiths determined equilibrium concentrations.

Classical interpretation of the AIB kinetic parameters shown in Table I suggests that the reduced AIB uptake in arrested cultures is principally the result of fewer functional

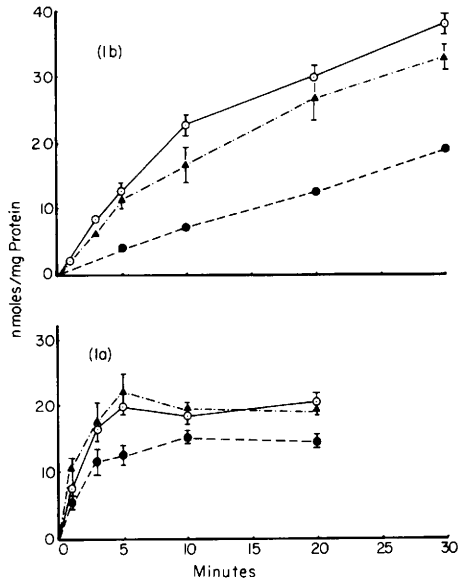

FIG. 1. Uptake of leucine (1a) and α-aminoisobutyrate (1b) in preconfluent (○——○), confluent (▲—·—▲) cultures under growth conditions and cultures arrested (●- - -●) for 21 days with medium containing 0.5% serum. Leucine and α-aminoisobutyrate were at an external concentration of 2 m*M* and specific activity of 0.1 μCi/μmole. Each time point is the mean for replicate samples from at least two separate experiments. Bars denote SE.

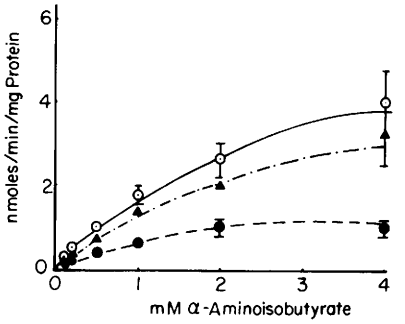

FIG. 2. Concentration dependence of α-aminoisobutyrate uptake in preconfluent (○——○), confluent (▲—·—▲) cultures under growth conditions and cultures arrested (●- - -●) for 21 days with medium containing 0.5% serum. Incubation was for 3 min and uptake was linear over this time period. Concentration range: 0.1–4.0 m*M*, 0.1 μCi/μmole. Each point is the mean for replicate samples from at least three separate experiments. Bars denote SE.

TABLE I. KINETIC PARAMETERS OF α-AMINOISOBUTYRATE UPTAKE.[a]

Culture condition (% serum)	K_m (m*M*)	V_{max} (nmoles/min/mg)
Preconfluent (10%)	1.52 ± .07	4.41 ± .21
Confluent (10%)	1.50 ± .06	3.03 ± .13[b]
Arrested (0.5%)	0.90 ± .10[b,c]	1.04 ± .11[b,c]

[a] K_m and V_{max} are expressed as value ± SE and are calculated from data in Fig. 2 by weighted least-squares method.

[b] Significantly different from preconfluent value at $P \leq 0.05$.

[c] Significantly different from confluent value at $P \leq 0.05$.

transport sites in these cultures. An apparent small increase in the affinity of AIB for its transport sites occurred in arrested cultures but is heavily outweighed by the decrease in the number of functional transport sites in this state. These results indicate that cells arrested in an essentially nonmitotic state present a different cell surface to their environment than those in preconfluent and confluent cultures. It is not clear at this time whether this effect on membrane function is dictated primarily by culture conditions or by internal signals generated by the cell.

Other attempts to attain stable nonproliferating populations have proved to be unsuitable. When HDC have been depleted of nutrients to inhibit division, degenerative changes were observed (21); and when division was restricted by altering the feeding regimen of confluent cultures to weekly intervals, the stability of the population was disrupted by continuing mitotic activity (22, 23). By reducing the serum concentration of the incubation medium, these problems have not been encountered; on the contrary, stable arrested populations have been maintained in a viable state for as long as 6 mo (3). Additionally, cultures arrested in this manner have been shown to differ from both preconfluent log phase and confluent plateau phase cultures by a variety of criteria (2–4, 14, 24). This report shows that certain membrane-associated phenomena also follow this pattern. Although most of the parameters studied indicated that arrested cells were in a reduced state of activity, others indicated that the cells were in a quasi-differentiated state in which specialized cell functions were more

fully expressed (24, 25). It can be postulated, therefore, that arrested populations may offer a better *in vitro* model system for those *in vivo* populations that normally do not exhibit rapid proliferation and that exist in a stable, relatively well-defined differentiated state.

Summary. Human diploid fibroblasts cultured *in vitro* were monitored for amino acid uptake in preconfluent and confluent cultures under conditions amenable to growth and in confluent cultures arrested in an essentially nonmitotic state. Preconfluent and confluent cultures in growth medium showed similar uptake patterns for leucine and α-aminoisobutyrate; arrested cultures exhibited a reduced uptake of both amino acids. Kinetic measurements revealed a 4-fold reduction in apparent V_{max} for α-aminoisobutyrate influx in arrested cultures. These results suggest that the culture conditions used in this study to produce restrictions in mitotic activity likewise influence amino acid accumulation.

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