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Membrane Transport During Erythroid Differentiation

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Summary. Transport, unidirectional flux, of a monosaccharide, a nucleoside and three amino acids, all of which enter cells by independent, discrete carriers, was compared at three stages of erythroid maturation, the normal (anucleate) mouse erythrocyte, and in differentiated and undifferentiated Friend erythroleukemia cells. We found specific transport alterations during this developmental program. Transport of 3-O-methylglucose increased with each successive developmental stage. Aminoisobutyrate transport was maintained during Friend cell differentiation, but fell slightly in erythrocytes. Leucine, lysine and uridine transport began to fall two days after dimethylsulfoxide exposure, and diminished further in red cells. These studies of transport are not directly comparable to uptake studies reported by others.

Median cell volume and thus surface area decreased more during differentiation than amino acid transport declined, so flux, transport past a unit area of membrane, actually increased. Monosaccharide flux also increased. Only uridine transport fell in parallel to surface area. Perhaps sites for nutrient transport required for energy production are preferentially maintained.

Key words Friend cells · membrane transport · erythroid differentiation

Introduction

Friend erythroleukemia cells proliferate in culture as a homogeneous population of large, nucleated primitive cells committed to erythroid differentiation but arrested at a stage biochemically and morphologically like proerythroblasts [16, 24, 34]. Treatment with dimethylsulfoxide (DMSO)[1] and other agents [16, 34, 47] stimulates their further *in vitro* biochemical and morphological maturation into cells which finally look like orthochromatophilic normoblasts.[2] Within 5 to 6 days, 95% of the DMSO-challenged population undergoes five divisions [16, 34] and is converted from rapidly dividing, undifferentiated Friend cells into nucleated but nondividing cells that contain large amounts of hemoglobin, differentiated Friend cells.

Erythropoiesis, including the segment of differentiation for which Friend cells are used as a model, is accomplished with generalized decreases in protein and nucleic acid synthesis in spite of increased production of erythrocyte-specific proteins [11, 16, 29]. Several studies have led to the hypothesis that membrane transport falls during erythrogenesis [30, 31], both specifically with the decision to differentiate further and generally as a function of differentiation [13, 16, 20, 32, 33] in Friend cells and other erythroid cells [26, 52]. A different formulation of that hypothesis is: do transport systems characteristic of relatively undifferentiated cells disappear during maturation? When do pathways associated with or unique to mature erythroid cells appear?

This paper examines both transport and metabolic trapping, where appropriate [25], of a saccharide, a nucleoside and several amino acids during differentiation of nucleated erythroid cells (Friend cell maturation) and in normal erythrocytes of the DBA/2J mouse, during the very short times at which transport of nutrients across cell membranes is unidirectional, linear and unaffected by backflux or subsequent intracellular phenomena [3]. The dependence of amino acid transport upon a sodium-gradient, and the degree to which cells concentrated amino acids was also determined.

Materials and Methods

Materials

New England Nuclear supplied L-α-amino [3-^{14}C]-isobutyric acid (AIB) (40 mCi/mmol) and L-[^{14}C-(U)]-lysine (270 mCi/mmol). ICN

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[1] The abbreviations used are: DMSO, dimethyl sulfoxide; AIB, α-aminoisobutyric acid; 3-OMG, 3-O-methylglucose; DMEM, Dulbecco's modified Eagle's Medium with 10% fetal calf serum, 1X nonessential amino acids, 2 mM glutamine and 100 U/ml each penicillin and streptomycin; DPBS, Dulbecco's phosphate-buffered saline, pH 7.4; BSA, bovine serum albumin; v^0, initial rate of transport; [*S*], substrate concentration; K_m, half-saturating concentration of substrate or affinity of carrier for substrate; V_{max}, maximal rate of transport; tRNA, transfer

[2] An operational definition of differentiation.

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supplied L-[4,5-^{3}H]-leucine (46 Ci/mmol) and uridine-[2-^{14}C] (50.8 mCi/mmol). Amersham-Searle supplied inulin-[^{14}C]-carboxyl (5 mCi/mmol), L-[^{14}C-(U)]-leucine (324 mCi/mmol), and 3-O-methyl-D-[U-^{14}C]-glucose. Unlabeled amino acids, 3-O-methylglucose, and bovine serum albumin (BSA) were obtained from Sigma. Unlabeled uridine was obtained from Pabst Labs. Tissue culture supplies, including fetal calf serum, Dulbecco's Modified Eagle's Medium powder, penicillin, streptomycin, and nonessential amino acid concentrate, were purchased from Grand Island Biological. All other chemicals were reagent grade. GF/C glass fiber filters were obtained from Whatman, and HAWP filters from Millipore.

Cell Culture

Friend erythroleukemia cells (clone 745a) were grown at 37 °C in suspension culture in Dulbecco's Modified Eagle's Medium with 10% fetal calf serum, 1x nonessential amino acids, 2 mM glutamine, and 100 U/ml each penicillin and streptomycin (DMEM) with a humidified atmosphere of 92% O_2, 8% CO_2. Cells were maintained in log phase ($< 2 \times 10^6$ cells/ml) by dilution every other day [43]. Saturation density under these conditions is $\sim 3.5 \times 10^6$ cells/ml. Cells (2×10^5/ml) were stimulated to differentiate by adding DMSO to the medium (final concentration 2%) and incubating without other additions for 5 days. The percentage of cells containing hemoglobin was determined with tetramethylbenzidine using a modification of Orkin's benzidine stain [36]. Benzidine-positive cells began to appear on day 2. They represented 75% of the cells on day 3, 80–85% on day 4 and at least 95% of the cells after 5 days of exposure to DMSO. Cell viability was monitored by trypan blue exclusion at each step of the experiments.

Normal Erythrocytes

Friend erythroleukemia cells were originally obtained from DBA/2J mice. Erythrocytes were obtained from normal male DBA/2J mice by retro-orbital bleeding into tubes containing sodium citrate. The cells were washed in 0.15 M NaCl, 5 mM sodium phosphate buffer, pH 7.9.

Partial depletion of monosaccharide, nucleoside or amino acid pools was achieved by washing cells three times in Dulbecco's phosphate-buffered saline, pH 7.4 (DPBS) at 37 °C, and incubating cells 10 min in DPBS. Cells were then resuspended in DPBS containing 4% fetal calf serum, or in DMEM lacking both the specific nutrient, whose transport was being studied, and serum. Cells were equilibrated to 37 °C in a shaking water bath for 10–15 min, centrifuged and resuspended. All centrifugations were done in a clinical centrifuge.

Influx Studies

Conditions were established to measure the rate of substrate movement from the extracellular side of the plasma membrane, where the substrate concentration was varied, to the cytoplasmic side of the membrane, where substrate was at a limiting low level [23, 39], zero-trans conditions. The five transported substrates enter the cell by independent pathways. Leucine, lysine and uridine are metabolized, and AIB and 3-OMG are not. Transport was initiated by rapidly mixing $2–10 \times 10^5$ cells/0.5 ml medium with 0.5 ml medium containing a mixture of labeled and unlabeled transportable substrate. At the appropriate time, cells were filtered under vacuum onto GF/C glass fiber filters which had been wet with DPBS containing 0.1% BSA. Filters were then washed several times with ice-cold DPBS containing 0.1% BSA or with DMEM; each wash required only 0.2–0.3 sec, and the filtrates contained essentially no radioactivity. Protein determinations on filtered cells and pelleted cells were the same; in addition, no hemoglobin was found in the filtrate of differentiated Friend cells (*data not shown*). Sampling times as early as 2 sec were obtained by this method. Air-dried filters and all other samples were counted in 5 ml of Triton-toluene-Omnifluor scintillation fluid [38]. Inulin-[^{14}C]-carboxyl was added to the substrate solution as an extracellular marker to allow calculation of the volume of medium trapped on the filters. The trapped volume was negligible, less than 5% of the total intracellular counts. Nonspecific binding of radioactivity to filters was determined by adding 5 ml of cold DPBS or DMEM (4 °C) containing labeled substrate and 100-fold excess of unlabeled substrate to 1×10^6 cells and filtering immediately, and accounted for less than 3% of the counts. All transport data were corrected for trapped and nonspecifically bound radioactivity.

Sodium Dependence of Amino Acid Transport

Cells were washed and equilibrated to 37° C in DPBS or sodium-free buffer in which sodium chloride and sodium phosphate were replaced by equimolar choline chloride and potassium phosphate (choline medium). Choline, a poorly permeating cation, replaced sodium in the extracellular medium in experiments designed to determine whether the chemical gradient of sodium provided the motive force for concentrative uptake of amino acids [6]. Influx studies were performed as described above. Transport in the presence of DPBS comprised both sodium-dependent and sodium-independent processes. Sodium-dependent transport was determined as the difference between total transport and sodium-independent transport, transport occurring in the absence of extracellular sodium.

Expression of Results

K_m (the concentration of substrate required to achieve half-maximal transport) and V_{max} (the maximum rate of transport) were determined graphically from Lineweaver-Burk plots, $1/v^0$ *vs.* $1/[S]$, and from Hofstee plots, v^0 *vs.* $v^0/[S]$ [45]. K_m and V_{max} values obtained by these two methods were in good agreement. On Hofstee plots, points are more evenly distributed allowing easier fit to a straight line, and potential deviations from Michaelis-Menten kinetics are amplified [45]. Data were analyzed for statistical significance using Student's *t*-test for unpaired populations [14].

Amino Acid Metabolism

Amino acids entering the acid-soluble pool are incorporated into aminoacyl transfer ribonucleic acid (tRNA) and then into protein. Both are precipitated by trichloroacetic acid leading to erroneously high values for protein synthesis. Transport was terminated as described and cells were resuspended in 10% trichloroacetic acid at 0 °C for 10 min. The suspension was centrifuged and the supernatant, the acid-soluble pool, removed and sampled. To distinguish between amino acid incorporation into protein and charging of tRNA, the acid-precipitated material was resuspended in trichloroacetic acid, boiled for several minutes to hydrolyze aminoacyl-tRNA and filtered onto HAWP filters which were then washed with ice-cold 10% trichloroacetic acid and water [21]. The difference between the total acid-precipitable radioactivity and the radioactivity retained on the filter represents the charged tRNAs.

Results

3-O-Methylglucose Influx

Entry into cells of the non-utilizable monosaccharide 3-OMG was linear for 10–15 sec, when presented at low concentrations at the external surface of the plas-

ma membrane. Influx rapidly became nonlinear as the external concentration increased (Fig. 1). Initial rates of 3-OMG transport under zero-trans conditions obtained from the slopes of lines in Fig. 1 were used to obtain K_m and V_{max} from a Hofstee plot (Table 1, Fig. 2). Equilibration with the medium occurred within 6–8 min (Fig. 1). Saccharide transport increased during erythroid development (Table 1): V_{max} increased to 0.75 nmol/min/10^6 cells after Friend cells were exposed to DMSO for 72 hr, although K_m was unchanged. Differentiated Friend cells, exposed to DMSO 5 days, had much higher V_{max} and K_m. In the normal erythrocyte V_{max} was still higher, and K_m much lower (Table 1, Fig. 2). Transport was maximal in DPBS containing 4% fetal calf serum or in DMEM lacking both serum and glucose (Table 1).

Uridine Influx

Initial rates of uridine transport occurred over 10–15 sec and the highest intracellular levels of uridine were reached in 8–10 min in Friend cells and in erythrocytes. V_{max} for uridine transport fell continuously after 48 hr of exposure to DMSO from 1.02 nmol/min/10^6 undifferentiated cells to 0.85 at 48 hr, 0.63 at 72 hr and 0.48 at 5 days (Table 1), but without a change in affinity of the carrier for its substrate (K_m) (Table 1). V_{max} was even lower and K_m more than doubled in erythrocytes (Table 1, Fig. 2).

Aminoisobutyric Acid Transport

AIB transport was linear for < 1 min, and achieved steady state in ~ 7–10 min. Differentiated Friend cells exhibited the same maximal influx rate for AIB as undifferentiated cells but transport was half-saturated at a lower concentration (Table 2, Fig. 3), an increase in transport during differentiation of the nucleated erythroid cells. However, AIB transport was somewhat lower in the anucleate mature erythrocyte (K_m increased, V_{max} fell: Table 2, Fig. 3).

Friend cells concentrated AIB fivefold in the presence of an external sodium gradient and two- to threefold in its absence, but transport into the erythrocyte was sodium-independent although still twofold concentrative (Table 3).

Leucine Transport

In undifferentiated Friend cells, leucine influx increased linearly with time during the first 10 sec of exposure to 0.5–0.95 μM leucine. Higher leucine concentrations did not increase transport further (upper half, Fig. 4). Leucine entry into differentiated Friend cells was also linear during the first 10 sec over a much greater range of concentrations, 0.5 to 50 μM

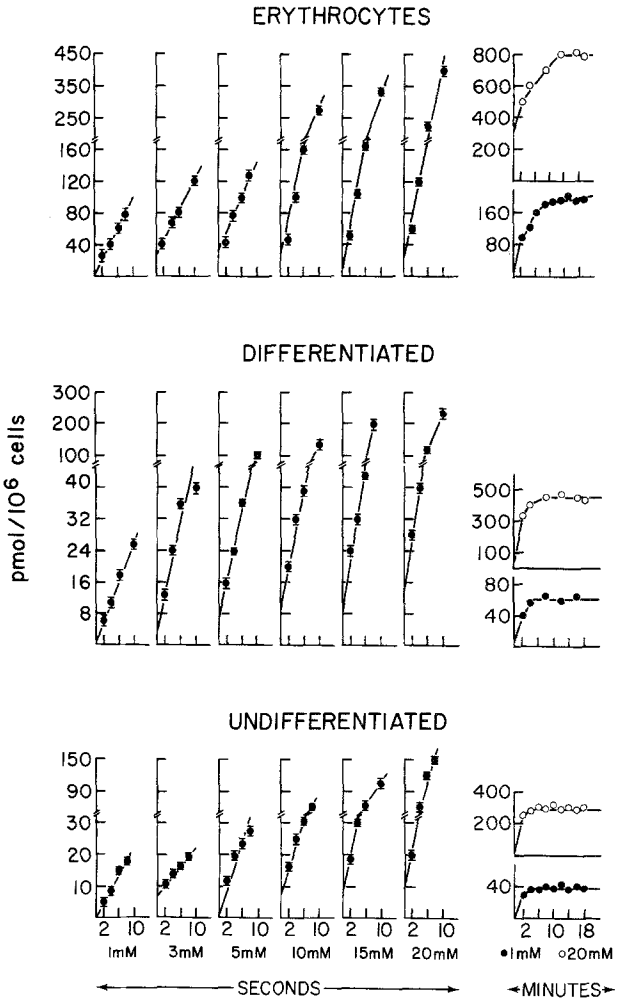

Fig. 1. 3-OMG entry into erythroid cells. Washed cells resuspended in DPBS supplemented with 4% fetal calf serum were equilibrated to 37 °C. Initial rates of transport into undifferentiated and differentiated Friend cells and erythrocytes were measured during 2–10 sec in the presence of increasing concentrations of 3-OMG. Transport was also measured over a longer interval to determine steady-state levels. The last frame represents 3-OMG uptake for several minutes in the presence of 1.0 (●) and 20 (○) mM 3-OMG in the medium. The size of the bar describes the standard error of the measurements for at least 15 determinations. Note the differences in scale in the first 3 frames and in the last frame of each panel

(Fig. 4, lower half). Transport into normal erythrocytes was also linear during the first 10 sec throughout that concentration range (*data not shown*). Unlike AIB transport, leucine transport was not maintained during differentiation of nucleated erythroid cells. In differentiated Friend cells leucine transport had a higher K_m and lower V_{max} than in undifferentiated Friend cells. K_m was still higher and V_{max} lower in DBA/2J erythrocytes (Table 2, Fig. 3).

Leucine reached its highest intracellular levels within 10 min, and was concentrated fivefold in acid-

Table 1. 3-O-methylglucose and uridine influx into erythroid cells

	Friend cells				Erythrocytes	
	Undifferentiated		Differentiated			
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
3-O-Methylglucose:						
DPBS	4.1±0.3	0.35±0.01	4.8±0.3	0.54±0.00	nd	nd
DPBS+4% serum	4.0±0.2	0.60±0.01	4.6±0.1*	0.96±0.01*	0.75±0.05*	1.28±0.05*
DMEM, no glucose, no serum	4.2±0.2	0.62±0.00	4.6±0.3*	0.98±0.01	nd	nd
Uridine:						
DPBS+4% serum	1.5±0.1	1.02±0.01	1.5±0.2	0.48±0.01*	3.5±0.1*	0.29±0.00*

3-O-Methylglucose and uridine transport were assessed as described in Materials and Methods. Fetal calf serum was used. K_m and V_{max} (nmol/min/10^6 cells) were obtained from Hofstee plots, Fig. 2. K_m for 3-O-methylglucose, mM; K_m for uridine, μM.
Mean±SE for at least 3 determinations.
nd=not determined.
* $p<0.05$ as compared to values for undifferentiated cells.

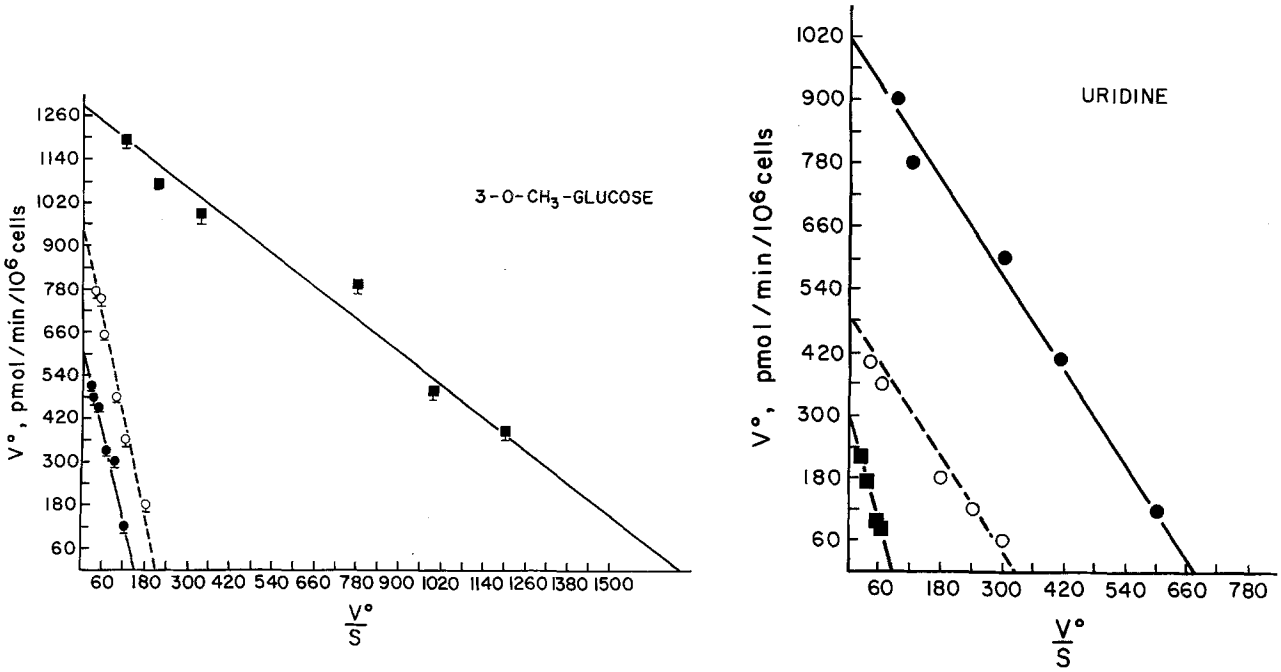

Fig. 2. K_m and V_{max} determinations for 3-OMG and uridine transport into undifferentiated (●) and differentiated (○) Friend cells and normal erythrocytes (■). Hofstee plot of data obtained as in Fig. 1. Bar represents standard error for at least five experiments

soluble pools as compared to the medium in 15 min (Table 4). Even after 15 min, >90% of the total leucine remained in the medium. As will be discussed later, "trapping" by incorporation into protein lagged 1.5 min behind uptake.

Using the growth, substrate depletion and transport conditions described herein, changes in transport kinetics became evident only 48 hr after DMSO addition. Cells exposed to DMSO for two or three days had lower V_{max} (1.0 and 0.96 nmol/min/10^6 cells, respectively) but the same K_m for leucine as undifferentiated Friend cells (*data not shown*).

The same K_m and V_{max} were obtained for leucine uptake whether the medium used was DPBS containing 4% fetal calf serum or DMEM lacking leucine and serum (*data not shown*). Transport was not increased in DPBS containing more than 4% fetal calf serum. However, transport was markedly decreased in DPBS with less than 4% serum, or after incubation of washed cells in DPBS for as much as an hour. In fact, after 30 min incubation in DPBS, more than half the cells were permeable to the dye trypan blue.

Because DMSO or its metabolites conceivably could alter amino acid permeability directly, leucine

Table 2. Amino acid influx into erythroid cells

	Friend cells				Erythrocytes	
	Undifferentiated		Differentiated			
	K_m	V_{max}	K_m	V_{max}	K_m	V_{max}
Amino acid:						
leucine	1.5±0.3	1.2 ±0.01	2.5±0.5*	0.74±0.01*	3.5±0.2*	0.48±0.01*
lysine	4.5±0.3	0.67±0.00	6.8±0.4*	0.47±0.00*	8.0±0.1*	0.30±0.00*
AIB	3.5±0.1	0.69±0.00	2.9±0.3*	0.67±0.00	5.0±0.5*	0.60±0.00*
After daily feeding:						
leucine	1.4±0.2	1.1±0.02	2.4±0.2*	0.76±0.00*		
lysine	4.3±0.2	0.68±0.01	6.6±0.1*	0.49±0.01*		
AIB	3.3±0.1	0.67±0.00	2.7±0.3*	0.65±0.00		

K_m (μM) and V_{max} (nmol/min/10^6 cells) were obtained from data represented in Figs. 3 and 4.
* $p<0.05$ as compared to undifferentiated cells.

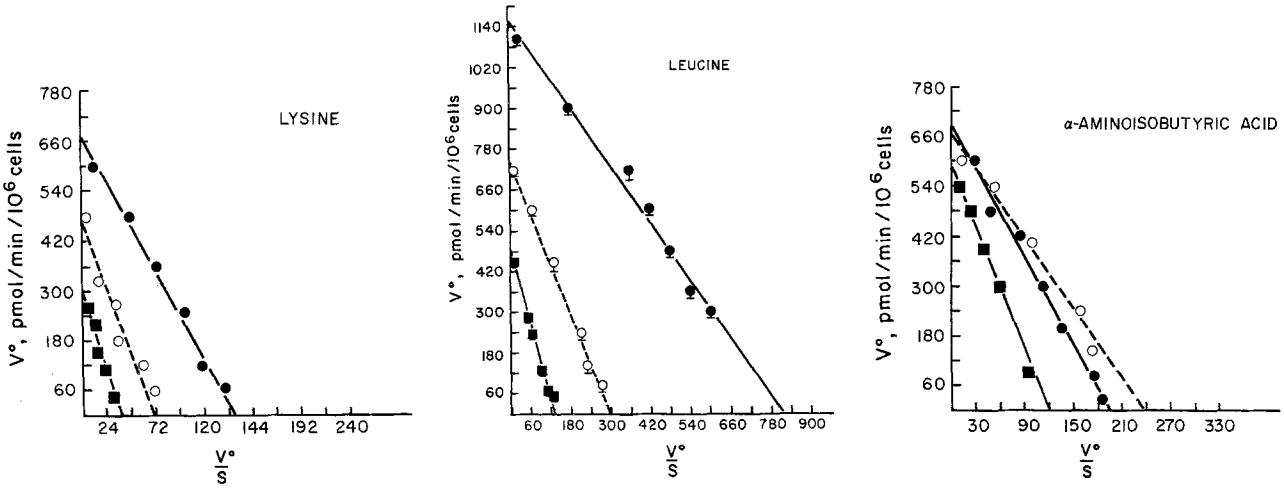

Fig. 3. K_m and V_{max} determinations for AIB, leucine and lysine transport into erythroid cells. Symbols as in Fig. 2

transport in DBA/2J mouse erythrocytes was studied after incubation in DMEM containing 2% DMSO for one hour to five days. V_{max} and K_m for leucine entry into erythrocytes were unaffected by DMSO incubation (*data not shown*).

In undifferentiated Friend cells, leucine was concentrated three- to fourfold in the absence of extracellular sodium, and five- to 10-fold in its presence. In differentiated Friend cells, leucine was concentrated three- to fourfold and transport was less sodium-dependent (Table 3). In mature erythrocytes, leucine was concentrated threefold and independent of the sodium gradient (Table 3).

Membrane functions often show discontinuities in temperature dependence attributed to lipid phase changes and altered lipid-protein interactions in the membrane [39, 50, 54, 57]. We ascertained initial rates of leucine entry at temperatures between 0 ° and 37 °C in the same manner as shown in Figs. 3 and 4 for

Table 3. Sodium dependence of leucine and AIB influx

	Sodium dependent		Sodium independent	
	K_m	V_{max}	K_m	V_{max}
Leucine:				
Friend cells				
Undifferentiated	1.3	600	1.9	640
Differentiated	2.7	345	2.1	392
Erythrocytes	none		3.6	470
AIB:				
Friend cells				
Undifferentiated	3.4	399	3.6	330
Differentiated	2.8	330	3.0	340
Erythrocytes	none		5.0	603

Amino acid influx into Friend cells and erythrocytes was performed in DPBS or choline buffer as described in Materials and Methods. K_m expressed as μM, V_{max} as pmol/min/10^6 cells, at least 2 determinations.

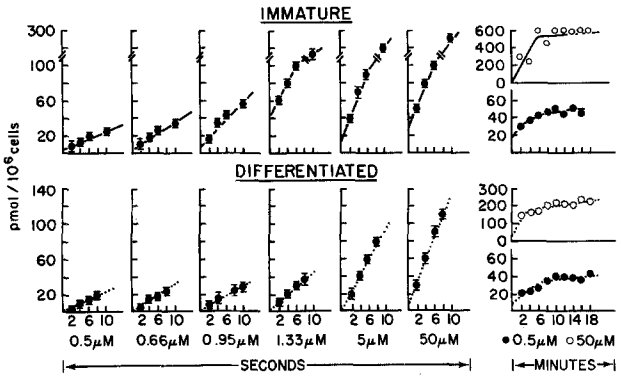

Fig. 4. Leucine entry into erythroid cells. Symbols and methods as for Fig. 1, except that in the last frame, the steady-state equilibrium is shown for 0.5 (●) and 50 (○) μM leucine

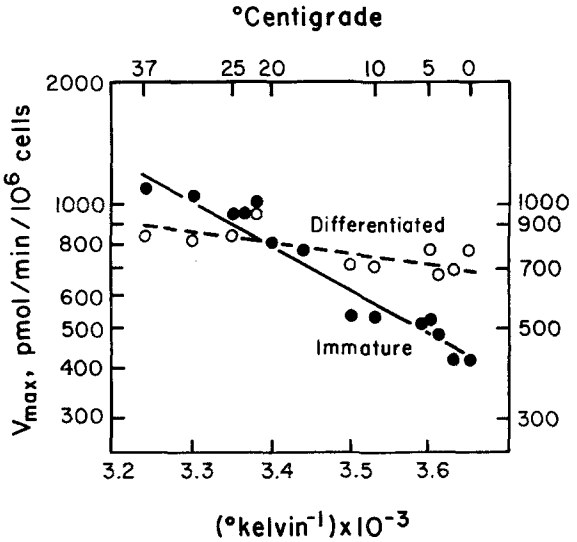

Fig. 5. Dependence of initial rate of leucine transport on temperature. Arrhenius plot of V_{max} (from Hofstee plots as in Fig. 3) for undifferentiated (●) and differentiated (○) Friend cells equilibrated at temperatures between 0 ° and 37 °C

Table 4. Metabolic trapping of leucine and lysine

	Acid soluble	Acid insoluble	
		Aminoacyl-tRNA	Protein
Leucine			
Undifferentiated	4683±12	1279±17	1590±15
Differentiated	1325±10	215± 7	226± 6
Lysine			
Undifferentiated	1537±18	225± 8	914± 9
Differentiated	2941±11	102± 7	257± 5

Friend cells (10^6) were loaded with 10 μM labeled amino acid for 15 min at 37 °C. Labeled amino acid (cpm) in acid-soluble and insoluble pools were measured as described in Materials and Methods.

Mean ±SE for 3 experiments.

V_{max} for leucine incorporation into protein was 0.60±0.002 and 0.54±0.003 nmol/min/10^6 cells in undifferentiated and differentiated populations; K_m was 1.5±0.3 and 2.7±0.3 μM ($p<0.05$ for both; at least 10 determinations).

37 °C. The half-maximal concentration for leucine transport rose slightly with decreasing temperature, from 1.5±0.3 μM at 37 °C to 2.0±0.3 μM at 0 °C ($p<0.05$, $n=5$) in undifferentiated Friend cells, and was unaffected by temperature in differentiated Friend cells. No discontinuities were apparent in Arrhenius plots (log V_{max} *vs.* °K^{-1}, Fig. 5), but from the slopes of the lines very different "energies of activation" for leucine transport were calculated: ~5–7 kcal/mol for undifferentiated and ~0.6 kcal/mol for differentiated Friend cells.

Lysine Transport

As with leucine, the transport system for lysine in undifferentiated Friend cells had a lower K_m and a higher V_{max} than in cells treated with DMSO for 5 days. Normal erythrocytes exhibited still higher K_m and lower V_{max} (Table 2, Fig. 3).

Lysine transport was sodium-independent and strongly concentrative during Friend cell differentiation, but was less concentrative in mouse erythrocytes.

In some systems, incubation in the same medium for several days, which might lead to growth arrest by starvation, induces transport of charged amino acids like AIB [37] and depresses transport of neutral amino acids that travel by the same path as leucine [22, 48]. To determine whether induction or depression of transport occurs in Friend cells, we assessed amino acid transport into cells that received fresh medium (and DMSO where appropriate) daily. Refeeding did not affect transport into Friend cells (Table 2). The growth arrest resulting from Friend cell differentiation remained associated with a fall in leucine and lysine transport, and a slight increase in AIB entry, and could not be attributed to depletion of growth factors. That these changes are maturation- rather than feeding-dependent is also indicated by still lower rates of leucine and lysine entry into erythrocytes, and a slight decrease in AIB entry.

Metabolic Trapping of Amino Acids

Appreciable leucine incorporation into protein occurred after a 1.5–2 min lag, increased at a constant rate during the following 10–11 min, and then fell off sharply. However, leucine transport had reached steady state in about 6 min (Fig. 4). Immature cells incorporated sevenfold more leucine into protein than differentiated Friend cells over a 15-min period.

Threefold more radioactive leucine was present in acid-soluble pools in the immature than in the differentiated cells (Table 4). Leucyl-tRNA accounted for 17 and 12% of the labeled intracellular leucine in undifferentiated and differentiated Friend cells (Table 4).

During Friend cell differentiation, the leucine concentration which half-saturated incorporation into protein equalled that for influx, and varied identically with temperature (data not shown). However, the maximal rate of leucine incorporation into protein was much less than for transport: at 37 °C, 0.60 ± 0.002 and 0.54 ± 0.003 nmol/min/10^6 cells for undifferentiated and DMSO-stimulated cells (compare Table 2). V_{max} for protein synthesis fell much more sharply in undifferentiated cells than DMSO-differentiated cells with decreasing temperature (*data not shown*), as did V_{max} for transport.

Incorporation of lysine into protein also occurred with the same half-saturating concentration but decreased V_{max} as compared to its uptake: 0.51 nmol/min/10^6 undifferentiated cells and 0.41 nmol/min/10^6 differentiated cells (*compare* Tables 2 and 4). Lysine remaining in the acid-soluble pool was 57% and 89% of total cellular lysine in undifferentiated and differentiated Friend cells, respectively. Undifferentiated cells incorporated fourfold more lysine into protein than differentiated cells, and contained about twice as much lysyl-tRNA (Table 4).

Discussion

We initiated our studies to characterize nutrient transport systems for amino acids, nucleoside and hexose during the course of DMSO-stimulated differentiation of Friend cells, and compared those systems to paths present in murine erythrocytes. Since changes in nutrient transport may directly participate in controlling cell division and differentiation by regulating nutrient availability, it is essential to assess the kinetic properties of transport at each stage of maturation. Studies aimed at understanding the role of transport during commitment [13, 15–18, 20, 32, 33, 45, 46] are not easily interpreted without prior definition of kinetic and differentiation-dependent properties of the transport systems. Notions about net uptake may largely reflect differentiation-dependent alteration in metabolic paths in Friend (erythroid) cells, rather than intrinsic changes in regulation and kinetic properties of transport systems.

Three definitions will help clarify comparison of our findings with those of others. First, *transport* or influx refers to initial rates of transport, unidirectional flow across the plasma membrane. *Uptake* includes both transport and "trapping", that is, metabolism. *Flux* is used as flow past a given area of surface membrane, V_{max}/μ^2.

The salient findings were that although influx of leucine, lysine and uridine decreased as Friend cells differentiated, AIB transport was maintained, and 3-OMG transport increased. Alterations in uridine and 3-OMG transport were detectable as early as 2 days after exposure to DMSO (Table 1, Figs. 1 and 2). These findings are not consistent with the "generalized transport decrease" hypothesis of Friend and colleagues [15, 16] nor with specific changes shown by others [18, 20, 22, 32, 46]. Erythrocytes transported less leucine, lysine, AIB and uridine, and more 3-OMG than even differentiated Friend cells.

Our studies differ from these others in two significant ways: *growth conditions* and *experimental design.*

Growth Conditions

First, our growth conditions did not produce a growth lag upon cell dilution; the growth rate in DMSO approximated that of controls for at least three days; and saturation density was $\sim 3.5 \times 10^6$ cells/ml, corroborating the findings of Rubin [43], but quite different from those of others [15, 16; for review *see* 34]. DMEM is much richer in amino acids and cofactors than the alpha medium used in many Friend cell studies. Growth conditions may have substantial effects on membrane components as well as intracellular functions. Serum starvation and depletion of growth factors [22, 37, 48] was not a significant regulatory factor during Friend cell differentiation. Cultures fed daily showed the same decreases in leucine and lysine transport and maintenance of AIB transport as nonfed cultures (Table 2). DMSO or its metabolites did not significantly alter transport into erythrocytes exposed to it for 60 min to 5 days (*data not shown*).

The median cell volume of undifferentiated Friend cells, 1,000 μ^3, decreased after 10 hr exposure to DMSO to 750 μ^3 and fell slowly thereafter to 640 μ^3 in fully differentiated Friend cells (5 days in DMSO) (*data not shown*), partially corroborating other studies [1, 19, 31] although we used unsynchronized cells and found smaller decreases. The volume of normal DBA/2J erythrocytes was 90 μ^3.

After partial substrate depletion as described in Materials and Methods, at least 95% of the cells excluded trypan blue. Conditions described by other authors left as many as half the cells permeable to dye.

Experimental Design

Second, our experiments were designed to measure unidirectional flux, in the absence of backflux (as

intracellular substrate content increased or metabolic trapping [23, 25]. Accurate measurement of initial transport rates requires short sampling times. The plot of entry *vs.* time should be linear and extrapolate back to zero. The period of unidirectional flux, usually occupying $< {}^1/_2$ min, with cells taking up no more than 0.2% label from the medium, may be followed by a second period which appears to be linear but depends on metabolism [3, 12, 25, 30, 51]. For example, as is clear in Figs. 1 and 4, 3-OMG and leucine influx into Friend cells was linear for much less than ${}^1/_2$ min. Influx rates are independent of subsequent metabolism.

Amino Acid Transport

Transport systems with affinities for discrete groups of neutral and charged amino acids have been characterized in another mouse line, Ehrlich ascites cells, and in many other mammalian cells [6–9, 22]. Transport of leucine and AIB, but not lysine, into Friend cells and normal erythrocytes differed in some respects from the usual well-defined paths.

Lysine transport remained sodium-independent and strongly concentrative during Friend cell differentiation but was less concentrative in mouse erythrocytes. Its properties were generally characteristic of the Ly^+ system described in other cells [6, 22]. The level to which the cells concentrated a solute was assessed from the specific activity of solute in medium and cells at equilibrium.

The neutral amino acid leucine enters some cells by the L system, which is poorly concentrative and independent of the chemical gradient of sodium [7]. In other cells, leucine transport can be highly concentrative [8, 10, 22]. The sodium-independent pathway which concentrated leucine three- to fourfold was maintained during Friend cell differentiation and was also present in the erythrocyte. The sodium-dependent pathway, which allowed (total) five- to 10-fold concentrative uptake of leucine in undifferentiated cells, was sharply curtailed in differentiated Friend cells and lost in erythrocytes. As in other cells, however, leucine transport rapidly reached a steady state, and V_{max} was, as in other cells, ~ 1 nmol/min/10^6 cells (Table 2 and ref. [7]).

Transport of AIB into both Friend and Ehrlich ascites cells is sodium-dependent and concentrative. However, Friend cell AIB transport equilibrated in < 10 min and V_{max} was 0.69 nmol/min/10^6 cells (Table 2) whereas ascites cells achieve steady-state levels of AIB after 30 min and have a low V_{max}, ~ 0.09 pmol/min/10^6 cells. About half of AIB transport into Friend cells depended on the presence of extracellular sodium (Table 3). In choline medium, AIB was concentrated two- to threefold, and in 140 mM sodium-containing medium, fivefold. However, AIB transport into erythrocytes, though still twofold concentrative, was not sodium-dependent (Table 3).

The decreased dependence of AIB and leucine transport on the chemical gradient of sodium shown here corroborates and extends studies of rabbit and rat anucleate erythroid cells [41, 42, 52, 53] in that primitive cells have more, and more complex, paths for transport.

If some AIB entered Friend cells by the leucine carrier, the high V_{max}, rapid attainment of equilibrium and significant independence of external sodium might be explained. But excess extracellular leucine did not stimulate AIB efflux (*data not shown*), so AIB did not permeate the membrane by the leucine carrier. Transstimulation of labeled AIB egress by unlabeled AIB (*data not shown*) was comparable to that shown in other systems [28, 44]. The independence of entry and exit of all three amino acids was clear from transstimulation studies with and without an imposed sodium gradient (*data not shown*).

Substrate Flux during Erythropoiesis

If the surfaces of erythroid cells were entirely unspecialized, their surface areas could be estimated from the corresponding median cell volumes (p. 17) as 480, 360 and 100 μ^2 for undifferentiated and fully DMSO-differentiated Friend cells, and erythrocytes. (During differentiation, cell volume varies greatly.) In fact, microvilli on undifferentiated cells [4, 27, 29] increase their surface area much more than do the "blebs" on differentiated cells [4], so the estimates represent lower limits for the true surface areas of Friend cells, and the flux changes are really greater than our figures suggest.

Friend cells exposed to DMSO 10 hr had a median cell surface area 83% that in undifferentiated cells. As a result, although transport was unchanged, flux of all substrates increased. Fully differentiated Friend cells had transport decreases corresponding to flux decreases of 18, 5 and 40% for leucine, lysine and uridine. AIB transport was maintained, so flux increased 31%, and 3-OMG transport increased, so 3-OMG flux rose 100%. More 3-OMG and less uridine, leucine, lysine and AIB entered erythrocytes than entered differentiated Friend cells, but the 80% fall in surface area compared to undifferentiated Friend cells led to corresponding flux increases of 10- to 20-fold for 3-OMG and two- to fourfold for amino acids. Only for uridine was the transport decrease of the same magnitude as the fall in surface area, so uridine flux was unchanged.

The apparent specificity as to degree, timing, and preferential maintenance of some carriers, perhaps to provide metabolic energy for other cell functions even though metabolism has diminished and macromolecular synthesis almost ceased, implicates these alterations in the developmental program, rather than as triggering- or commitment-dependent phenomena.

Substrate Uptake

We designed uptake studies to measure leucine and lysine incorporation into tRNA and protein during 15-min incubations in buffer containing low concentrations of the appropriate amino acid and 4% fetal calf serum. Transport experiments (*see* Fig. 4 for leucine) indicated amino acid equilibrated between cells and medium in 7–10 min, and serum was included to alleviate possible inhibition of protein synthesis by limitingly low levels of other amino acids.

Most examinations of differentiation-dependent alterations in plasma membrane permeability and subsequent metabolism comparing maturing Friend cells and red cells [13, 15–18, 20, 32, 33, 35, 45, 46] have been concerned with necessary and sufficient requirements for Friend cell triggering; many have confused transport and uptake. The differences from our results, which showed maintenance of transport, by and large, until late in differentiation *vs.* rather early decreases in uptake, may be at least partly ascribed to our richer growth medium, and to changes in pool size and metabolism which would affect transport.

In Friend cells, as in other cells [5, 40], K_m for amino acid influx equalled that for incorporation into protein (Tables 2 and 4), and V_{max} was greater, consonant with substrate entry being the rate-limiting step. Most leucine or lysine entering Friend cells remained in acid-soluble pools (Table 4). However, incorporation of labeled amino acid into acid precipitable material which is not dissolved by trichloroacetic acid declined only after 4 days in DMSO, to 0.56 nmol/min/10^6 cells ($p < 0.05$, compare Table 4). Decreased protein synthesis during Friend cell differentiation [13, 15–17, 35, 45] probably reflects not only developmental changes but also different pool sizes of endogenous amino acids.

Temperature Dependence of Leucine Transport

Studies on the temperature dependence of transport may provide evidence for major phase separations of lipid or other alterations of lipid-lipid or lipid-protein interactions in the vicinity of the transport sites. Some carrier-mediated transport systems show discontinuities (transition temperatures), but others do not [2, 39, 50, 54, 57]. Leucine transport into Friend cells had no discontinuities on an Arrhenius plot (Fig. 5). For each population, K_m's were the same for leucine transport and incorporation into protein at all temperatures (*data not shown*).

The "energy of activation" calculated for leucine transport into undifferentiated Friend cells (5–7 Kcal/mol) is similar to other carrier-mediated systems [39]. Leucine transport into differentiated Friend cells appeared to be nearly independent of temperature (Fig. 5). This unusual plot suggests that the lipid environment of the leucine carrier is very different in the differentiated and undifferentiated cells. Although such plots have been interpreted to indicate diffusion-controlled processes, leucine transport is clearly saturable and carrier-mediated.

Although Arrhenius calculations have been principally used in studies of energetics of enzyme catalysis in solution, studies other than ours [2, 57] have calculated "energies of activation" for membrane transport systems. These data should be interpreted cautiously, though, because the activity of transport proteins is affected by their interaction with other membrane lipids and proteins which are themselves altered by temperature; the system is one of multiple interrelationships.

In summary, we did not reproduce previous studies of early depression in Friend cell uptake of substrates from the medium after DMSO stimulation, but because of the volume decreases entrained by DMSO, flux across the membrane of all substrates was higher at early times because transport was unchanged. Furthermore, 48 hr after DMSO treatment, specific increases, decreases or maintenance of transport of the various substrates was demonstrated. As Friend cell maturation progressed, these differences from the undifferentiated erythroleukemia cell became greater, and still greater in erythrocytes. In addition, the sodium dependence of leucine and AIB transport decreased during erythrogenesis, but while the cells were still nucleated! Thus, maturation from "blast" to mature erythrocyte was characterized by systematic loss in specificity and number of transport systems of amino acids.

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