Amino acid transport and rubidium-ion uptake in monolayer cultures of hepatocytes from neonatal rats

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Amino acid and K^+ transport during development has been investigated in hepatocyte monolayer cultures with either α -amino[1- ^{14}C]isobutyrate or $^{86}Rb^+$ used as a tracer for K^+ . Parenchymal cells from neo- and post-natal rat livers have been isolated by an improved non-perfusion technique [Bellemann, Gebhardt & Mecke (1977) *Anal. Biochem.* **81**, 408–415], and the resulting hepatocyte suspensions purified from non-hepatocytes before inoculation. In the presence of Na^+ (Na^+ -dependent component), the rates of amino acid uptake in neonatal hepatocytes were markedly enhanced compared with cells from 30-day-old rats. When Na^+ was replaced by choline (Na^+ -independent component) the accumulation of α -aminoisobutyrate was decreased and it was not affected by the age of the animals. Kinetic analysis of Na^+ -dependent α -aminoisobutyrate transport revealed the existence of a high-affinity low- K_m component (K_m 0.91 mM) with a V_{max} of 2.44 nmol/mg of protein per 4 min, which later declined gradually with progressive development. Rates of Rb^+ transport were concomitantly enhanced in neonatal hepatocytes and thereafter declined with postnatal age. The increased Rb^+ influx was effectively inhibited by ouabain and reflected elevated activity of the electrogenic Na^+/K^+ -pump during early stages of development. Kinetic evaluation of the enhanced rates of Rb^+ uptake indicates multiple and co-operative binding sites of the enzyme involved in the Rb^+ uptake, and the transport system is positively co-operative (the Hill coefficient h is >1.0). In short, amino acid transport in neonatal rat hepatocytes is increased as a result of an existing low- K_m component for the Na^+ -dependent α -aminoisobutyrate uptake, which endows the hepatocytes with a high capability for concentrating amino acids at low ambient values. The concomitant enhancement of K^+ transport reflects changes in the electrochemical gradient for Na^+ across the hepatocellular membrane and, along with this, presumably alterations in the membrane potential; the latter might be the driving force for the enhanced α -aminoisobutyrate transport in the alanine-preferring system during postnatal age.

The hepatocellular membrane is an important locus for the transport of substrates involved in the regulation of metabolic events related to cell differentiation, proliferation and malignancy (Holley, 1972; Bhargava, 1977). Cellular growth and proliferation are strongly associated with increased capacities to concentrate amino acids that occur particularly during the transition of the G_1 to the S phase in the cell cycle. Growth-initiated enhanced amino acid uptake has been previously demonstrated in a wide variety of cell types *in vitro* (Mendelson *et al.*, 1971; Sander & Pardee, 1973;

Tramacere *et al.*, 1973; Villereal & Cook, 1977), and most recently during early events of compensatory growth in the regenerating liver *in vivo* (Wondergem & Potter, 1978) and *in vitro* (Short *et al.*, 1974; LeCam *et al.*, 1979; Wondergem *et al.*, 1979). Furthermore, alterations in cation fluxes across the membrane have also been studied with regard to cellular growth (Rozengurt & Heppel, 1975; Tupper *et al.*, 1977), and results underline the importance of the cell membrane for many regulatory processes during cell growth, e.g. gluconeogenesis, nucleotide synthesis and, especially, protein synthesis. However, investigations concerning kinetics of nutrient and cation transport into hepatocytes have been very limited during early stages of development.

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Amino acid transport in the liver has been investigated in detail, and generally three distinct transport systems have been identified for neutral amino acids: the A- (alanine-preferring), ASC- (specific for alanine, serine and cysteine) and L- (leucine-preferring) systems (Christensen, 1969, 1975; LeCam & Freychet, 1977). Among these the one preferentially studied is the A-system, which is strictly Na^+ -dependent, Na^+ -dependent amino acid transport therefore being directly affected by changes in the extracellular $[\text{Na}^+]$. However, the Na^+ gradient across the hepatocellular membrane is also maintained by the electrogenic pumping of Na^+ out of the cell and K^+ into the cell by the $(\text{Na}^+ + \text{K}^+)$ -stimulated ATPase. Furthermore, the intracellular $[\text{K}^+]$ plays an important role not only in maintaining the membrane potential, but also in protein synthesis and cell growth (Lubin, 1964, 1967; Ledbetter & Lubin, 1977).

The present study reports the application of a novel technique for isolating hepatocytes from small and non-perfusible pieces of tissue (Fry *et al.*, 1976; Bellemann *et al.*, 1977), e.g. human liver samples (Bellemann *et al.*, 1978) or rat kidney cortices (Curthoys & Bellemann, 1979), to neo- and post-natal rat livers and demonstrates the maintenance of newborn rat parenchymal cells in primary monolayer cultures. This new technique provides for the first time kinetic evaluation of the enhanced amino acid uptake during postnatal age, and correlates it with a concomitantly increased K^+ transport. Furthermore, greater activity of the electrogenic Na^+/K^+ -pump is reflected by a ouabain sensitivity of the enhanced Rb^+ influx.

Parts of this work have been presented at the Conference on Differentiation and Carcinogenesis in Liver Cell Culture, at the New York Academy of Sciences on October 9–12, 1979 (Bellemann & Potter, 1980).

Materials and methods

Materials

Pregnant albino rats (250–300 g) were received 10 days after mating (Sprague–Dawley, Madison, WI, U.S.A.) and were housed in individual solid-bottom drawer-type cages with wood chips for nesting material. They were allowed free access to water and to a standardized carbohydrate-rich (30% protein) laboratory diet (TEKLAD), and were kept on controlled light/dark (12h/12h) cycles. The pregnant rats delivered naturally in the early morning on day 22 after mating. Groups of 15–19 newborn rats (day 0) were randomly selected for the experiments, which were started immediately after at least three mothers had completed their delivery, which amounted to about 27–35 young.

Waymouth's MB 752/1 medium was obtained from KC Biologicals Inc. (Lenexa, KS, U.S.A.) and

was supplemented with 0.2% albumin, 0.02 mm-oleate, 0.5 mm-serine, 0.4 mm-alanine and 6.8 mm-glutamine; for convenience, this medium is termed WO/BA-M2 medium below; Swim's medium was purchased from GIBCO (Grand Island, NY, U.S.A.). Collagenase (type IV), unlabelled α -aminoisobutyrate and fatty acid-free serum albumin (fraction V) were supplied by Sigma (St. Louis, MO, U.S.A.); α -amino[1- ^{14}C]isobutyrate (sp. radioactivity 40–60 Ci/mol) and $^{86}\text{Rb}^+$ (sp. radioactivity 10 Ci/mol) were from New England Nuclear (Boston, MA, U.S.A.). All other reagents were of the highest commercially available grade. Culture dishes were from Falcon Plastics, Oxnard, CA, U.S.A. Nylon mesh (150 μm pore size) was obtained from Tetko Inc., Elmsford, NY, U.S.A. All solutions used for the preparation of hepatocytes were routinely bubbled with 100% O_2 before use and contained 5.8 mm-glucose. All procedures were carried out under aseptic conditions.

Surgical procedure

The isolation of neo- and post-natal rat hepatocytes was preceded by a complete flushing of the liver *in situ* with Ca^{2+} - and Mg^{2+} -free Swim's S-77 medium containing 1 mg of serum albumin/ml to wash out erythrocytes and plasma. Weanling rats were briefly anaesthetized with diethyl ether and killed by cervical dislocation. The abdomen and the thorax were quickly opened, and a catheter connected to an infusion pump was inserted into the inferior vena cava between the right auricle and the right ventricle of the heart. The ligature surrounding the inserted catheter was tied, the vena cava and the vena portae distal to the liver were cut open, and the organ was flushed by retrograde perfusion with prewarmed Ca^{2+} - and Mg^{2+} -free Swim's S-77 medium containing 1 μg of heparin/ml (Heparin sodium; Upjohn, Kalamazoo, MI, U.S.A.) for 2 min at a rate of 2.5 ml/min. The livers were gently removed from 4–18 random rats (from five litters), pooled in cold perfusion solution, dried between ashless paper, weighed and subsequently submitted to an improved slicing technique (Bellemann *et al.*, 1977).

Isolation and cultivation of hepatocytes

Preparation of hepatocyte suspension from newborn and 5-, 10-, 20- and 30-day-old rats was performed in an analogous way to a technique recently reported for isolation of adult hepatocytes from liver samples (Fry *et al.*, 1976; Bellemann *et al.*, 1977). The method, modified for neonatal liver, results primarily in the isolation of hepatocytes. Each liver was cut by hand with a heavy-duty blade (Microtome Knife Blades, American Optical) so that slices were obtained of less than 0.50 mm in thickness. Liver slices totalling approx. 4–5 g were

then aseptically (Bellemann, 1980) disaggregated with collagenase (0.05%, w/v) dissolved in 20 ml of Ca^{2+} - and Mg^{2+} -free Swim's S-77 medium, as described previously for isolating kidney tubules (Curthoys & Bellemann, 1979). The resulting hepatocyte suspension was washed three times with Swim's S-77 medium and concomitantly separated from most erythropoietic cells by low-speed centrifugation (400 *g* for 5 min; 200 *g* for 5 min; 100 *g* for 7 min). The pellet was finally resuspended in Ca^{2+} - and Mg^{2+} -free Swim's S-77 medium and stored on ice. Cell yield and viability were estimated cytochemically (Cummings, 1970). Sufficient cells were obtained to form monolayers in 120–150 culture dishes.

Cells were resuspended to a final concentration of $1.5 \times 10^6/\text{ml}$ in WO/BA-M2 medium containing insulin (13 $\mu\text{g}/\text{ml}$) and gentamycin (50 $\mu\text{g}/\text{ml}$); 2–3 ml of the cell suspension was then inoculated on to 60-mm petri dishes (Falcon) that had previously been coated with native rat-tail collagen. The culture dishes were maintained in an incubator kept at 37°C and an atmosphere of CO_2/air (1:19). After the 4-h period of attachment, the unattached cells were removed with the medium, and transport studies were initiated immediately thereafter.

Assay for α -aminoisobutyrate and Rb^+ transport

The culture dishes were placed in a water bath at 37°C, the medium was aspirated and the cells were washed twice with 3 ml of prewarmed Hanks balanced salt solution without glucose, modified to contain 20 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethane sulphonic acid] buffer, pH 7.4. Transport linearity was observed at various α -aminoisobutyrate concentrations and beyond (5–6 min) the regularly used 4-min incubation time in hepatocyte cultures from 5-day-old animals; this is consistent with linear uptake rates for up to 30 min reported in hepatocytes of 15-day-old rat hepatocyte cultures (Koch & Leffert, 1979). For the cumulative uptake of α -aminoisobutyrate, the cells were incubated for the indicated time periods (4–300 min) with 2 ml of Hanks balanced salt solution containing 1 mM- α -aminoisobutyrate and α -amino[1- ^{14}C]isobutyrate (sp. radioactivity 0.1 Ci/mol). To measure the concentration-dependent uptake of α -aminoisobutyrate, the cells were incubated for 4 min in Hanks balanced salt solution plus α -aminoisobutyrate concentrations ranging from 0.1 to 250 mM. For α -aminoisobutyrate transport assays in the absence of Na^+ (Na^+ -independent component) NaCl was replaced with iso-osmolar concentrations of choline chloride, NaHCO_3 with KHCO_3 , and NaH_2PO_4 was omitted. All incubations were terminated by aspirating the radioactive solution, placing the tissue culture dishes on ice and immediately washing the cells three times with 3 ml of Hanks balanced salt solution at

4°C. The cells were then collected by being scraped with a rubber policeman with two 1-ml portions of 0.2 M-NaOH. The two scrapings were combined, thoroughly mixed and portions were taken for radioactive assay and protein determination. For radioactivity 1 ml of the cell solution, neutralized with 0.5 ml of 0.4 M-acetic acid, was mixed with 15 ml of RIA Solve II and counted for radioactivity in a Nuclear-Chicago Isocap 300 liquid-scintillation counter.

Rb^+ has been frequently used in many cell systems as a valid tracer for K^+ (for further references see Ledbetter & Lubin, 1977; Putney, 1978). There was no difference in the $^{86}\text{Rb}^+$ uptake, when $^{86}\text{Rb}^+$ was added to the incubation medium supplemented with additional 1–5.8 mM-RbCl or -KCl. Furthermore, the specific radioactivity of $^{86}\text{Rb}^+$ in the cells and in the incubation solution did not differ significantly. Thus in the present paper cell content of $^{86}\text{Rb}^+$ or K^+ are referred to interchangeably (for further references see Ledbetter & Lubin, 1979). For Rb^+ transport, labelled Rb^+ ($^{86}\text{RbCl}$, 0.1 $\mu\text{Ci}/\text{ml}$) was measured in a way similar to that of α -aminoisobutyrate uptake, except that before the scraping procedure, $^{86}\text{Rb}^+$ was extracted from the monolayer culture by incubating the cells twice for 15 min with 1 ml of 5% trichloroacetic acid on ice. The radioactivity in 1 ml of the combined extract was then measured in water (Čerenkov radiation).

Protein was assayed in the suspension collected by scraping by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. The moderate differences in protein content of the different hepatocyte preparations were normalized by a correction factor that also took cell volume into consideration.

Data analyses

The experiments were repeated at least two to three times with separate cell preparations. Each determination within an experiment was carried out at least in triplicate, and the results are means $\pm$ S.E.M. The Na^+ -dependent part of amino acid transport was calculated by subtracting the experimental values obtained in an Na^+ -free medium (Na^+ -independent component) at each α -aminoisobutyrate concentration from the total velocity data measured in the presence of Na^+ . Results were analysed by using the Woolf–Augustinsson–Hofstee plot (Segel, 1976) for initial rates in which the Na^+ -dependent velocity (v) is related to the substrate (α -aminoisobutyrate) concentration ($[\text{S}]$). When curvilinear plots were obtained, the assumption was made that two independent Michaelis–Menten components contributed to the transport. The final kinetic parameters were calculated with a computer (Honeywell DDP-124)-fitting program (MACC,

1972) using non-linear least-squares analyses for all velocity values including each individual standard error. This advanced routine (MACC, 1972) allowed non-linear regression analyses of the data and yielded the same results as a program applied previously (Cleland, 1967) in which previous linearization of the mathematical function was required.

Light and electron microscopy

Primary cultures were continuously checked by visual examination with an inverted light microscope (Wild, Herbruck, Switzerland). Several staining procedures, e.g. eosin-haematoxylin and benzidine (Benzidine-Wight-Giesma stain), were routinely performed to control the separation technique. For electron microscopy the monolayer cultures were post-fixed with 1% (w/v) OsO_4 , dehydrated by increasing ethanol concentrations, and then embedded in Epon-Araldite by the method of Sattler *et al.* (1978). Sections were stained with uranyl acetate and lead citrate, and finally examined in a Hitachi HU IIC (Perkin-Elmer, Mountain View, CA, U.S.A.) electron microscope at 75 kV.

Results

Isolation and cell culture of hepatocytes from developing livers

Retrograde perfusion of livers from developing rats was necessary, since the organs had to be pooled to isolate sufficient numbers of hepatocytes for kinetics in 120–180 monolayer cultures (Table 1). After enzymic dissociation of the liver slices the resulting suspension was carefully purified from debris and non-hepatocytes by low-speed-centrifugation techniques. The hepatocyte suspension obtained was generally free of erythrocytes and contained less than 5% erythropoietic cells (in hepatocyte suspensions from newborn and 5-day-old rats), which was verified frequently by a special staining technique with benzidine (Orkin *et al.*, 1975); in addition several other sensitive assays attested to the successful separation of the parenchymal cells from non-hepatocytes. After 4 h in culture, neo- and post-natal hepatocytes had spread, aggregated and formed an almost confluent layer.

Studies of the ultrastructure (Plate 1) confirmed that hepatocytes from developing rats retained well their structure in monolayer cultures.

Na^+ -dependent amino acid transport

The time-dependent accumulation of α -aminoisobutyrate (1 mM) in the presence of 140 mM- Na^+ was always greater in hepatocytes from newborn and 5-, 10- and 20-day-old rats than in young adult rats (30 days and older). Steady-state values achieved differ significantly within each experimental group depending on the time after birth; they were reached after 4–5 h of incubation in monolayer cultures, whereas in hepatocyte suspension alanine is transported much faster (Joseph *et al.*, 1978). The concentration-dependent uptake of α -aminoisobutyrate shows a similar pattern. With age, the enhanced rates of amino acid transport showed a gradual decline in the α -aminoisobutyrate influx to uptake rates close to that of young adult rats (30-day old). When Na^+ was replaced by isosmolar concentrations of choline the uptake of α -aminoisobutyrate was significantly decreased and it was not affected by the postnatal time periods reported here.

The initial rates of the Na^+ -dependent amino acid transport (Fig. 1) were calculated by subtracting the Na^+ -independent component in the choline assay at each concentration indicated. Computer analyses over the full range of α -aminoisobutyrate concentration (up to 250 mM) showed that saturation approached only at extremely high and non-physiological substrate concentration. The initial rates of Na^+ -dependent α -aminoisobutyrate uptake in hepatocytes from 150-day-old (adult) animals followed Michaelis-Menten kinetics of a single saturable system, since values obtained for a possible low- K_m component appeared to be negligible (Table 2). In contrast, kinetic evaluation of transport rates in hepatocytes from newborn and 5-, 10-, 20- and 30-day-old rats fit better to functions and curves characteristic for two saturable systems described by two different K_m values that indicate a transport process where two independent carriers transport the same substrate. The existence of this low- K_m component ranging from 0.819 to 1.036 mM was

Table 1. Comparison of body and liver weights, viable cell yield and viability index of hepatocyte suspensions from developing rats

The numbers of animals used in the experiments are given in parentheses.

Time after birth (days)	Body wt. (g)	Liver wt. (g)	$10^{-6} \times$ Viable cell yield (cells/g of liver)	Viability index (%)	Number of experiments
Newborn	5.91 ± 0.11 (75)	0.214 ± 0.021	207.8 ± 82.9	100	4
5	12.29 ± 0.20 (65)	0.446 ± 0.043	149.8 ± 17.6	100	6
10	25.40 ± 0.36 (42)	0.827 ± 0.035	97.9 ± 10.8	99.4	5
20	47.53 ± 3.10 (27)	1.65 ± 0.166	95.9 ± 22.9	98.3	7
30	72.28 ± 3.50 (21)	3.11 ± 0.433	41.3 ± 9.2	95.2	5

EXPLANATION OF PLATE 1

Hepatocytes from newborn (day 1) rats after 4 h in primary monolayer culture

Disaggregation of livers from newborn rats was performed enzymically by a non-perfusion technique (Fry *et al.*, 1976; Bellemann *et al.*, 1977), which yielded a sufficient number of cells to form 120–150 monolayers. After separation from erythropoietic cells hepatocytes were placed on culture dishes that were coated previously with collagen (C). The transmission electron micrograph shows the relatively small parenchymal cells with distinct cell membranes, unproportionally large nuclei (N) and lipid droplets (L), which are prominent especially during early phases of development (magnification $\times 11900$). The insert micrograph (magnification $\times 13500$) shows that mitochondria (M) are normal with regard to membrane integrity, size and form of cristae, and the rough endoplasmic reticulum (RER) is very well developed in large arrays even at the first day after birth.

Fig. 1. Initial rates of the Na^+ -dependent α -aminoisobutyrate uptake in neo- and post-natal hepatocytes. Observed and computer-analysed (lines) rates of the Na^+ -dependent α -aminoisobutyrate uptake into hepatocytes as a function of the extracellular α -aminoisobutyrate concentrations. Hepatocytes were isolated from newborn and 5-, 10-, 20-, 30- and 150-day-old (adult) animals. Transport studies were initiated 4 h after the cells were placed in monolayer culture. After subtracting the Na^+ -independent component at each concentration indicated, the rates of Na^+ -dependent α -aminoisobutyrate uptake, v , are expressed as nmol/mg of protein per 4 min. Each point represents the mean $\pm$ S.E.M. for three to five separate experiments with $n = 3-6$ plates in each. Two independent transport systems contributing to the Na^+ -dependent α -aminoisobutyrate uptake shown here are separated and described by a low- K_m and a high- K_m component (see Table 2).

observed throughout the entire postnatal age and contributed significantly (up to 80%) to the total Na^+ -dependent amino acid transport (Table 2). However, the contribution of the low- K_m system gradually declined with increasing extracellular α -aminoisobutyrate concentration in the area in which α -aminoisobutyrate mimics physiological range (hatched area in Fig. 1). In contrast, the high- K_m feature showed such a variety of values ranging between 33 and 422 mM that these data seem unlikely to characterize an appropriate transport system *in vivo*.

Fig. 2. Time dependence of Rb^+ uptake into hepatocytes from newborn and 5-, 10-, 20- and 30-day-old rats. Transport studies were initiated 4 h after cells have been placed on to culture dishes. $^{86}Rb^+$ was used as a tracer to measure the K^+ uptake; the K^+ concentration used was 5.8 mM. Each point is the mean $\pm$ S.E.M. for three separate experiments with $n = 6$ plates in each.

Table 2. Kinetic parameters of the Na^+ -dependent α -aminoisobutyrate transport in 4-h monolayer cultures of hepatocytes from developing rats

Values of K_m (mM) and V_{max} (nmol/mg of protein per 4 min) were obtained by computer analyses of the experimental plots shown in Fig. 1, as described in the Materials and methods section. The columns on the right represent the contribution (%) of the low- K_m component to the total Na^+ -dependent α -aminoisobutyrate transport in the physiological range of amino acid concentration (hatched area in Fig. 1).					
Time after birth (days)	Component I (low)		Component II (high)		Contribution of the high-affinity low- K_m component to the total amino acid transport (%)
	K_{m1}	$V_{max.1}$	K_{m2}	$V_{max.2}$	
α -Aminoisobutyrate (mM) ...					
Newborn	0.908	2.441	33.05	27.03	74.7
5	1.036	2.439	362.13	192.85	80.1
10	0.819	1.123	111.41	60.67	69.0
20	1.013	0.787	43.84	26.06	54.4
30	0.910	0.739	422.74	114.10	73.2
150 (adult)	0.080	0.033	28.74	13.46	28.2
					0.1 0.5 1.0 5.0
					74.7 68.3 61.7 36.8
					80.1 72.9 50.8 26.5
					69.0 61.0 53.4 27.0
					54.4 47.0 40.2 19.7
					73.2 66.0 59.0 31.9
					28.2 11.0 6.3 1.6

Fig. 3. Initial rates of Rb^+ uptake as a function of extracellular $[\text{K}^+]$ into hepatocytes from developing rats. Concentration dependence of Rb^+ uptake into hepatocytes from newborn and 5-, 10-, 20- and 30-day-old rats. Transport studies were initiated 4 h after cells have been placed into culture; $^{86}\text{Rb}^+$ was used as a tracer for K^+ . The rates of Rb^+ uptake were expressed as nmol/mg of protein per 4 min; each point represents the mean $\pm$ S.E.M. for three to four experiments with $n = 6$ plates in each.

Rb^+ transport

The results of the time-dependent uptake of Rb^+ in the presence of $5.8 \text{ mM } \text{K}^+$ are shown in Fig. 2. Steady-state values were achieved after 4–5 h, except when cells from newborns were used for investigations in which saturation was not reached within the time periods reported here. The cumulative influx into hepatocytes from early developing rats was elevated over that of the adults, but gradually declined with time after birth.

The concentration-dependent uptake of Rb^+ is shown in Fig. 3. The rates of Rb^+ transport were measured as a function of extracellular $[\text{K}^+]$. The initial uptake rates during the early postnatal phases were increased over those of young adults (30 days), but declined gradually with progressive time of development. Steady-state values were approached at 4.8 mM in hepatocytes from 10-, 20- and 30-day-old rats, but saturation was achieved at a much lower K^+ concentration (2.8 mM), reflecting faster transport when kinetics were performed on cells from 5-day-old animals. In contrast, transport rates of hepatocytes from newborn rats did not reach steady-state values at the concentrations indicated here.

Fig. 4. Kinetic analyses of the concentration dependence of Rb^+ uptake in neo- and post-natal hepatocytes. The initial rates of the enhanced Rb^+ uptake during early phases of postnatal age (Fig. 3) have been analysed by the Hill equation $\{\log[(v/V_{\max}) - v]\}$ versus $\log\{[\text{K}^+]\}$ (mM); v = initial rates of Rb^+ transport in nmol/mg of protein per 4 min. The observed values fit the linear plot of the Hill equation with a slope, $h = 1.60$, and $[\text{S}]_{50} = 0.95 \text{ mM}$.

Kinetic analysis of Rb^+ transport

The initial rates of the Rb^+ uptake in newborn hepatocytes follow first-order kinetics. In contrast, kinetic evaluation of the data reported in Fig. 3 revealed that the enhanced initial rates of the Rb^+ uptake during early stages of development did not follow Michaelis–Menten kinetics. However, experimental data (5-, 10-, 20- and 30-day-old rats) fit readily to a straight line in the linear plot of the Hill equation (Fig. 4). The evaluation suggests multiple and co-operative binding sites of the enzyme involved in the K^+ transport, and the transport system is positively co-operative (the Hill coefficient h is > 1.0).

Inhibition of the $(\text{Na}^+ + \text{K}^+)\text{-stimulated ATPase}$

The enhanced Rb^+ transport during early phases of postnatal age indicates increased activity of the

Table 3. *Ouabain sensitivity of Rb^+ transport in hepatocytes from developing rats*

Monolayer cultures of hepatocytes from newborn and 5-, 10-, 20- and 30-day-old rats were incubated for 10 min with Hanks/Hepes buffer containing K^+ (1 mM), $^{86}\text{Rb}^+$ (sp. radioactivity, $0.1 \mu\text{Ci}/0.1 \text{ mmol}$ of RbCl), and $\pm$ ouabain (1 mM). Transport studies in the presence of ouabain were preceded by a 10 min pre-incubation with Hanks/Hepes buffer containing K^+ (1 mM) and ouabain (1 mM), but no radioactive Rb^+ . Results are means $\pm$ S.E.M. for three experiments with 10 determinations in each.

Source of hepatocytes (days after birth)	Rb^+ uptake (nmol/mg of protein)		
	No ouabain	Ouabain at 1 mM	Inhibition (%)
Newborn	41.28 ± 0.92	4.70 ± 0.17	88.6
5	52.42 ± 1.73	3.88 ± 0.24	92.6
10	33.00 ± 0.93	2.54 ± 0.15	92.3
20	30.42 ± 0.93	2.91 ± 0.07	90.4
30	25.37 ± 0.53	2.90 ± 0.15	88.6

Na^+/K^+ -pump, which could be evaluated by studies using the inhibitor ouabain. When the Na^+/K^+ pump was inhibited by the heart glycoside, increasing extracellular K^+ failed to alter the $^{86}\text{Rb}^+$ uptake. The results (Table 3) demonstrate that the elevated rates of Rb^+ transport were mediated by a ouabain-sensitive ($\text{Na}^+ + \text{K}^+$)-stimulated ATPase activity.

Discussion

Numerous reports during the last decade have shown the retention of several liver biochemical functions when hepatocytes were enzymically isolated from the intact organ (for further references see Bellemann, 1980). The results presented here clearly demonstrate an increased Na^+ -dependent amino acid transport in rat hepatocytes during postnatal age. These enhanced initial rates of α -aminoisobutyrate uptake gradually declined with progressive development to uptake rates similar to those found in normal adult animals. The findings account qualitatively for results reported previously (Tews & Harper, 1967), which showed *in vivo* an increased α -aminoisobutyrate distribution ratio during early stages of development. Furthermore, similar results have been recently reported during early periods of compensatory growth in the regenerating liver *in vivo* (Wondergem & Potter, 1978) and *in vitro* (Short *et al.*, 1974; LeCam *et al.*, 1979; Wondergem *et al.*, 1979). The present study also reports no difference in the Na^+ -independent α -aminoisobutyrate transport during the entire postnatal age. This suggests that the enhanced amino acid transport into hepatocytes during growth and proliferation involves an amino acid transport system that is strictly dependent on the Na^+ flux into the cells. In addition, increased Na^+ ion flux seems necessary to initiate proliferation in rat hepatocytes (Koch & Leffert, 1979).

Kinetic evaluation of the Na^+ -dependent α -aminoisobutyrate transport (Fig. 1) in hepatocytes from

adult rats showed Michaelis–Menten properties characteristic for a single saturable system. In contrast, the initial rates of the amino acid transport in 4-h monolayers from neo- and post-natal hepatocytes followed a rather different kinetic pattern. The velocity data closely fit equations and characteristics of two overlapping saturable systems with clearly distinguishable K_m values (Table 2), e.g. a low- K_m and a high- K_m component. This low- K_m system (K_m 0.908–1.036 mM) exists (the concomitant V_{max} values are slightly declining) throughout the entire postnatal age and contributes considerably to the transport of amino acids, especially in low and ‘physiological’ ranges of concentration, e.g. up to 5 mM. The existence of a low- K_m system was described first in hepatocyte suspensions from starved rats by Fehlmann *et al.* (1979); LeCam *et al.* (1979) reported similar data in hepatocyte suspension from hepatectomized rats.

In hepatocyte suspensions alanine is actively accumulated (Joseph *et al.*, 1978) and its translocation across the plasma membrane seems to be rate-limiting for alanine metabolism in parenchymal cells (Sips *et al.*, 1980). For the transport of α -aminoisobutyrate (the non-metabolizable analogue of alanine) the significance of the high-affinity, low- K_m component versus the high- K_m system is emphasized by the fact that the physiological concentration of alanine in the portal serum ranges between 1 and 2 mM and the total amino acid concentration never exceeds 5 mM (Short *et al.*, 1974). Thus the velocity data of the Na^+ -dependent α -aminoisobutyrate transport in the range 0.10–5 mM actually represent the significance of the low- K_m contribution (up to 80%) to the total amino acid transport during development. On the other hand, the high- K_m component determined in hepatocyte monolayer cultures of neonatal, regenerating and starved animals (results not shown) revealed such a great variety that these values do not properly characterize a transport system of the cell.

Furthermore since the alanine concentration in the circulation is maintained in the order of 1–2 mM as described above, the magnitude of these high- K_m data (ranging between 29 and 10 000 mM; results not shown) indicates possibly artificial features, presumably resulting from the enzymic removal of the parenchymal cells from the organ. However, transport velocity data are preferentially expressed in terms of K_m and V_{max} values originated from rather sophisticated kinetic plots. Indeed, K_m and V_{max} values may be useful indicators to compare altered transport rates, since changes in V_{max} might reflect the number of sites involved in the carrier-mediated uptake, whereas alterations in K_m show qualitative differences in these sites. Therefore, alterations in V_{max} for α -aminoisobutyrate or sugar transport may reflect, as in many transformed cells (Isselbacher, 1972; Cecchini *et al.*, 1976), changes in the number of membrane sites involved in the uptake, whereas decreases in K_m (Hatanaka *et al.*, 1970; Hatanaka & Gilden, 1970) more specifically show alterations in the nature of the uptake sites. However, all discussions on the significance of these changes in K_m and V_{max} remain incomplete, when not correlated with changes in the membrane potential.

It has been well-documented in a variety of cell types that the transport of organic substrates requires Na^+ (Shultz & Curran, 1976; Lever, 1977) and that this contributes significantly to the active transport of amino acids, often against the concentration gradients. A complex of Na^+ , amino acid and a membrane carrier has been postulated to be involved in the molecular transport mechanism (Shultz & Curran, 1976; Lever, 1977), and therefore changes in any one parameter of this ternary complex directly affect the initial rates of amino acid movement into hepatocytes. However, despite the theoretical considerations of the kinetic interactions involved in these coupled transmembrane processes (Geck & Heinz, 1976), the molecular nature of this mechanism remains ambiguous.

The Na^+ -gradient across the cell membrane is maintained in part by the electrogenic pumping of Na^+ out of the cell and K^+ into the cell. Mammalian cells require K^+ during growth and they are able to concentrate K^+ from the surrounding medium, often against steep concentration gradients (Lubin, 1964; Ledbetter & Lubin, 1977). This requirement has been demonstrated by incubating cells in medium low in K^+ (Eagle, 1955) or by treating cells with the surface-active compound amphotericin B (Lubin, 1967). The increased entry of K^+ into hepatocytes during development reflects a greater activity of the electrogenic and ouabain-sensitive Na^+/K^+ -pump that may cause an increase in the electrochemical gradient for Na^+ , and along with this, changes in the electrical potential across the hepatocellular membrane. This conclusion is supported by new ob-

servations linking early ionic signalling events with membrane potential and intracellular pH changes that have been reported more recently by Koch & Leffert (1980).

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References

- Bellemann, P. (1980) *Arch. Toxicol.* **44**, 63–84
- Bellemann, P. & Potter, V. R. (1980) *Ann. N.Y. Acad. Sci.* **349**, 385–386
- Bellemann, P., Gebhardt, R. & Mecke, D. (1977) *Anal. Biochem.* **81**, 408–415
- Bellemann, P., Upovsky, K.-U. & Kummer, D. (1978) *Int. Congr. Pharmacol. (JUPHAR) 7th Paris*, 397
- Bhargava, P. M. (1977) *J. Theor. Biol.* **68**, 101–138
- Cecchini, G., Lee, M. & Oxender, D. L. (1976) *J. Supramol. Struct.* **4**, 441–447
- Christensen, H. N. (1969) *Adv. Enzymol. Relat. Areas Mol. Biol.* **32**, 1–20
- Christensen, H. N. (1975) *Biological Transport*, 2nd edn., pp. 107–213. W. A. Benjamin, Reading
- Cleland, W. W. (1967) *Adv. Enzymol. Relat. Areas Mol. Biol.* **29**, 1–31
- Cummings, H. (1970) in *Virology-Tissue Culture*, p. 91, E & S. Livingstone, Edinburgh
- Curthoys, N. P. & Bellemann, P. (1979) *Exp. Cell Res.* **121**, 31–45
- Eagle, H. (1955) *Science* **122**, 501–504
- Fehlmann, M., LeCam, A., Kitabgi, P., Rey, J.-F. & Freychet, P. (1979) *J. Biol. Chem.* **254**, 401–407
- Fry, J. R., Jones, C. A., Wiebkin, P., Bellemann, P. & Bridges, J. W. (1976) *Anal. Biochem.* **71**, 341–350
- Geck, P. & Heinz, E. (1976) *Biochim. Biophys. Acta* **443**, 49–53
- Hatanaka, M. & Gilden, R. V. (1970) *J. Natl. Cancer Inst.* **45**, 87–89
- Hatanaka, M., Augl, C. & Gilden, V. (1970) *J. Biol. Chem.* **245**, 714–717
- Holley, R. W. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 2840–2841
- Isselbacher, K. J. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 585–589
- Joseph, S. K., Bradford, N. M. & McGiven, J. D. (1978) *Biochem. J.* **176**, 827–836
- Koch, K. S. & Leffert, H. L. (1979) *Cell* **18**, 153–163
- Koch, K. S. & Leffert, H. L. (1980) *Ann. N.Y. Acad. Sci.* **349**, 111–127
- LeCam, A. & Freychet, P. (1977) *J. Biol. Chem.* **252**, 148–152
- LeCam, A., Rey, J. F., Fehlmann, M., Kitabgi, P. & Freychet, P. (1979) *Am. J. Physiol.* **236**, E594–E602

- Ledbetter, M. L. & Lubin, M. (1977) *Exp. Cell Res.* **105**, 223–236
- Ledbetter, M. L. & Lubin, M. (1979) *J. Cell Biol.* **80**, 150–165
- Lever, J. (1977) *J. Supramol. Struct.* **6**, 103–124
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Lubin, M. (1964) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **23**, 994–1001
- Lubin, M. (1967) *Nature (London)* **213**, 451–452
- MACC, Reference Manual for the 1110 (1972) Academic Computing Center, University of Wisconsin, Madison
- Mendelson, J., Skinner, S. A. & Kornfeld, S. (1971) *J. Clin. Invest.* **50**, 818–826
- Orkin, S. H., Harosi, F. J. & Leder, P. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 98–102
- Putney, J. W., Jr. (1978) *Pharmacol. Rev.* **30**, 209–245
- Rozengurt, E. & Heppel, L. A. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 4492–4495
- Sander, G. & Pardee, A. B. (1973) *J. Cell. Physiol.* **80**, 267–277
- Sattler, C. A., Michalopoulos, G., Sattler, G. L. & Pitot, H. C. (1978) *Cancer Res.* **38**, 1539–1549
- Segel, I. H. (1976) in *Biochemical Calculation*, 1st edn., pp. 381–385, J. Wiley and Sons, New York
- Short, H., Armstrong, N. B., Kolitsky, M. A., Mitchell, R. A., Zemel, R. & Lieberman, J. (1974) in *Control of Proliferation in Animal Cells* (Clarkson, R. & Baserga, R., eds.), pp. 37–48, Cold Spring Harbor Laboratory, New York
- Shultz, S. G. & Curran, P. F. (1976) *Physiol. Rev.* **50**, 657–718
- Sips, H. J., Groen, A. K. & Tager, J. M. (1980) *FEBS Lett.* **119**, 271–274
- Tews, J. K. & Harper, A. E. (1967) *Biochim. Biophys. Acta* **183**, 635–637
- Tramacere, M., Borghetti, A. F. & Guidotti, G. C. (1973) *J. Cell. Physiol.* **93**, 425–434
- Tupper, J. T., Zorngiotti, F. & Mills, B. (1977) *J. Cell. Physiol.* **91**, 429–440
- Villereal, M. L. & Cook, J. S. (1977) *J. Supramol. Struct.* **6**, 179–190
- Wondergem, R. & Potter, V. R. (1978) *Life Sci.* **23**, 1565–1576
- Wondergem, R., Bellemann, P., Ihlenfeldt, M. & Potter, V. R. (1979) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **38**, 119