Bidirectional transport of glutamine across the cell membrane in rat liver

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1. Hepatocytes isolated from fed rats were used to investigate glutamine transport. 2. Glutamine transport appears as a composite process involving at least two saturable components. The Na^+ -dependent component probably represents the entry through the N system. The Na^+ -independent component was also inhibited by histidine and exhibited *trans*-stimulation, suggestive of a facilitated diffusion process. 3. Kinetic parameters for both systems suggest that facilitated diffusion only plays a minor role in glutamine influx. In contrast, the K_m for glutamine efflux was consistent with a physiological role of the facilitated-diffusion component in glutamine release. 4. In Na^+ medium, relatively constant distribution ratios (about 8) between intra- and extra-cellular concentrations were observed, with external glutamine ranging from 0.5 to 5 mM. 5. The present observations suggest that glutamine influx might largely be mediated by the N system, whereas facilitated diffusion allows hepatocytes to release glutamine when intracellular concentrations are elevated. 6. The physiological consequences of this bidirectional transfer of glutamine across the liver cell membrane is discussed.

Glutamine is one of the main amino acids involved in inter-organ relationships, besides alanine (Christensen, 1982). Both amino acids can be released by muscles, but, unlike alanine, glutamine is extensively metabolized by the digestive tract (Windmueller & Spaeth, 1978). Hepatic utilization of glutamine has also been widely investigated, owing to its contribution to urea synthesis or nitrogen cycling, and its possible function in the regulation of intracellular proteolysis (Mortimore, 1982). In contrast with alanine, hepatic concentrations of glutamine are always very high; thus, during starvation or acidosis, glutamine is released by the liver, and is essentially used for renal gluconeogenesis and elimination of NH_4^+ . However, in rats fed on high-protein diets, glutamine is efficiently taken up by the liver and may thus constitute an important substrate for ureogenesis (Rémésy *et al.*, 1978).

Thus the hepatic balance of glutamine may result in a net uptake or a net release, and the possibility that both processes of influx and efflux across the liver cell membrane could be carrier-mediated have to be examined. There is an apparent discrepancy between the release of glutamine frequently observed *in vivo* and data showing that glutamine is the most readily transported amino acid into liver cells (Pardridge, 1977). Glutamine uptake has been reported to be mediated by a Na^+ -dependent system

(N system; Kilberg *et al.*, 1980), but overall glutamine transport might be less closely Na^+ -dependent than is alanine transport (Joseph *et al.*, 1978). The apparent K_m for glutamine, estimated from 1 mM (Kilberg *et al.*, 1980) to 4.5 mM (Joseph *et al.*, 1978), is markedly higher than physiological concentrations in blood. The present study was undertaken to determine the relative importance of the processes of influx and efflux across the cell membrane in the fluctuations of glutamine balance across the liver. We suggest that glutamine may be transported by two systems: the N system, which allows glutamine accumulation into liver cells, and a Na^+ -independent system which may offer the possibility of significant glutamine efflux. The activity of these systems might give rise to a futile cycle for glutamine transport across the liver cell membrane.

Materials and methods

Chemicals

L-[G- ^3H]Glutamine (46 Ci/mmol) and [^{14}C]polyethylene glycol (22 mCi/g) were purchased from The Radiochemical Centre (Amersham, Bucks., U.K.); $^3\text{H}_2\text{O}$ (1 mCi/ml) was obtained from the Commissariat à l'Energie Atomique (Gif-sur-Yvette, France). Amino-oxyacetic acid, albumin and unlabelled amino acids were from Sigma (St. Louis,

MO, U.S.A.), collagenase was from Boehringer (Meylan, France) and Percoll was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden).

Preparation of isolated hepatocytes

Hepatocytes were isolated at 14:00h from rats fed *ad libitum*. Collagenase dissociation was performed by the method of Berry & Friend (1969) as modified by Krebs *et al.* (1974). Cells were purified by centrifugation on a Percoll gradient (Dalet *et al.*, 1982). The gradient was preformed by centrifugation of a 30% Percoll solution in Krebs–Henseleit (1932) physiological buffer (pH 7.4, saturated with CO₂/O₂, 1:19) at 20 000 g for 20 min. The cell suspension obtained after collagenase dissociation was then put on the top of the gradient and centrifuged at 1000 g for 5 min, in a swinging rotor. The pellets of viable cells were suspended in physiological buffer and centrifuged twice (200 g, 1 min) to eliminate the Percoll. Cell viability estimated by cell-membrane refractoriness in phase-contrast microscopy ranged from 97 to 99%. Incubations were performed in the physiological buffer containing 10 mM-Hepes [4-(2-hydroxy-ethyl)-1-piperazine-ethanesulphonic acid] and 2% (w/v) albumin dialysed against the same medium.

Transport experiments

Hepatocytes were first preincubated for 5 min at 37°C in the presence of 2 mM-amino-oxyacetic acid. Joseph *et al.* (1978) did not observe a loss of label from 0.5 mM-glutamine, even in the absence of inhibitor, which is in keeping with the fact that low concentrations of glutamine are not utilized by hepatocytes (Lund & Watford, 1976). However, amino acid metabolism could be incompletely inhibited when high concentrations (>2 mM) are used. In the present experiments, relatively high concentrations of inhibitor have been used. Furthermore, at high concentrations of glutamine, the specific radioactivity of glutamine is markedly lowered; as a result, possible labelling of end products of glutamine metabolism should be quite limited. Transport was initiated in 1.5 ml Eppendorf Microfuge tubes containing L-[³H]glutamine (0.5 µCi) by addition of 300 µl of hepatocytes suspension (about 30–40 mg fresh wt. of cells/ml). For time-course experiments on glutamine uptake, 2 ml of hepatocyte suspension was incubated at 37°C with radioactive glutamine (1 µCi) with appropriate agitation and gassed with CO₂/O₂ (1:19). Samples (300 µl) of hepatocytes were taken at the times required and the reaction was stopped by addition of 1 ml of ice-cold Krebs buffer containing albumin to the Microfuge tubes, which were immediately centrifuged for 5 s at about 1000 g; the supernatant was discarded and the cell pellets were resuspended in 1 ml of the same ice-cold buffer. The

same procedure was used to collect the cell pellets, which were transferred into counting vials with 3 ml of scintillation liquid (Instagel; Packard, Paris, France).

The validity of the washing procedure was carefully assessed: contamination by extracellular radioactivity did not exceed 3% of total uptake, and efflux at 0°C was not significant during the washing procedure. To measure glutamine distribution ratio (ratio of intracellular to extracellular concentrations), cells were incubated in the presence of [¹⁴C]polyethylene glycol and ³H₂O for 2 min. After centrifugation (about 1000 g, 5 s) the supernatant was carefully discarded. Measured values of intracellular water ($3.46 \pm 0.15 \mu\text{l}/10^6$ cells) were identical for batches of cells incubated with or without Na⁺.

The time course of glutamine efflux was studied after a 20 min preincubation of hepatocytes with [³H]glutamine. The cell suspension (700 µl) was then quickly centrifuged and the preincubation medium discarded. The cells were rapidly resuspended in Krebs medium containing albumin (1400 µl), either glutamine-free or containing 50 mM-glutamine, and further incubated; collection of samples of the suspension (300 µl) was started exactly 10 s after the addition of the efflux medium. Samples were briefly centrifuged (3 s at about 1000 g) and 200 µl of supernatant was collected for determination of glutamine efflux. The intracellular concentration of glutamine was determined after rinsing the cell pellets with ice-cold Krebs buffer containing albumin.

Results

Time course of glutamine uptake

Uptake of glutamine was studied at 0.3 mM external glutamine. Fig. 1 shows that uptake increased with time and reached a plateau after 25–30 min. The distribution ratio remained relatively constant (about 8) for a wide range of external concentrations, from 0.5 to 5 mM. At the highest concentrations, glutamine accumulation reached very high values, in the range 40–50 mM. In Na⁺-free medium, glutamine uptake was far from being negligible and the distribution ratio was about 1.6 at all the concentrations used between 0.5 and 5 mM.

Characteristics of glutamine transport

Kinetic analysis of influx by graphical plots (Fig. 2a) shows that the initial rate of glutamine uptake was saturable in media containing Na⁺ or choline. The diffusion rate constant was estimated from the slope of the curve at the highest concentrations (>10 mM) of substrate, for which the carrier is saturated. In choline medium, the existence of a saturable component, besides the diffusion process,

Fig. 1. Glutamine uptake and accumulation into liver cells

Amino acid transport was initiated by the addition of 0.3 mM-glutamine to cells incubated either in a normal Krebs-Henseleit bicarbonate buffer (O) or in a buffer in which Na^+ was replaced by choline (●). The results for time course of glutamine uptake are the averages of three determinations on three separate batches of cells. The distribution ratio (ratio of intracellular to extracellular concentrations) of glutamine after 35 min incubation was constant (7.7), up to 5 mM external glutamine in Na^+ medium, and approx. 1.6, up to 3 mM external glutamine, in choline medium.

indicated that glutamine could also be transferred by a Na^+ -independent carrier. The hypothesis of two classes of carrier for glutamine transport was supported by the curvilinearity of the plot of v against v/s , in Na^+ -containing medium (Fig. 2b). The same representation in a Na^+ -free medium yielded a straight line, which suggests that under such conditions glutamine transport might be mediated by one carrier or by carriers with similar affinities. The K_m for glutamine influx mediated by the Na^+ -dependent component (broken line) was estimated as 1.25 mM, against 4 mM in the Na^+ -free medium. Furthermore, differences appear in the $V_{\max}$ of glutamine carriers, the Na^+ -dependent system having the highest capacity (1.3 nmol/min per mg of cells), against 0.65 for the Na^+ -independent component.

Inhibition of glutamine transport

Table 1 shows that glutamine transport was not affected by *N*-methylaminoisobutyrate, cysteine and cycloleucine, but was strongly inhibited by addition of histidine, regardless of the presence of Na^+ in the medium. However, the observed K_i values were different: 0.30 ± 0.05 mM in Na^+ medium, compared with 1.40 ± 0.10 mM in choline medium (Fig. 3a and 3b). This appears in keeping with the existence of two distinct carriers. Theoretically the plot of $1/v$

Fig. 2. Effect of external concentrations on glutamine influx

(a) Effects of increasing concentrations of glutamine in the medium on glutamine influx in Na^+ buffer (O), and in Na^+ -free Krebs-Henseleit bicarbonate buffer (●). Cells were incubated over 2 min with glutamine concentrations ranging from 0.3 to 30 mM. The broken lines represent the saturable component in Krebs-Henseleit bicarbonate medium containing Na^+ (----) and in Na^+ -free Krebs-Henseleit bicarbonate medium (-.-.-). (b) Woolf-Augustinson-Hofstee plot of saturable glutamine transport in Na^+ -containing Krebs-Henseleit bicarbonate medium (O) and in Na^+ -free Krebs-Henseleit bicarbonate medium (●). The broken line represents the Na^+ -dependent component, which is obtained by subtracting the Na^+ -insensitive uptake.

against [histidine] for a two-component system should yield a curvilinear relation. However, in Na^+ medium, since glutamine is essentially transported by the N system, the inhibitory effects of histidine on the facilitated-diffusion component were almost completely masked.

Fig. 3. Dixon plot of the inhibition of glutamine transport by histidine

The initial rate of transport was measured after 2 min of incubation for different concentrations of substrate (glutamine) and inhibitor. Each experiment was performed in either Na^+ (a) or choline (b) medium. The results are means of two determinations for two batches of cells.

Table 1. Effects of natural L-amino acids and analogues on L-glutamine uptake by isolated hepatocytes

Experimental details are described in the text. Transport of L-glutamine (1 mM, $1 \mu\text{Ci}$ of $[^3\text{H}]\text{glutamine}$) was measured in the presence or the absence of the inhibitor. The data are the means $\pm$ S.E.M. for three determinations; nd, not determined.

Inhibitor	Uptake (% of control)	
	Na^+ medium	Choline medium
10 mM-Cycloleucine	94 ± 5	118 ± 10
50 mM-Histidine	5 ± 3	7 ± 4
50 mM-Cysteine	85 ± 4	nd
50 mM-Methylamino-isobutyrate	95 ± 4	nd

Fig. 4. Influence of pH on glutamine uptake

To determine the time course of glutamine uptake at various pH values, the cells were gently centrifuged and then resuspended in Krebs-Henseleit bicarbonate buffer containing 20 mM-Hepes adjusted to pH 7.45 ($\bullet$), 7.05 ($\circ$) or 6.60 ($\blacktriangle$). After 40 min incubation, the pH of the control batch was lowered to 6.5. The changes in initial velocity (expressed as percentages of the velocity at pH 7.5) were determined in a separate experiment: -20% (pH 7.14) and -37% (pH 6.8). The results presented are the average for two determinations.

Influence of pH on glutamine uptake

Lowering external pH (from 7.5 to 6.8) diminished by 37% the initial rate of glutamine entry (Fig. 4). External acidosis also affected the distribution ratio of glutamine, which was substantially decreased; in addition, intracellular glutamine was markedly depleted when hepatocytes previously loaded at physiological pH were further incubated in acidic conditions. As a result, it appears that when external pH is lowered, decreased influx processes could no longer ensure high distribution ratios, owing to the importance of efflux processes. Thus it was decided to examine further the characteristics of glutamine efflux.

Analysis of processes of exchange-diffusion

The influx of glutamine was compared in cells loaded with unlabelled glutamine (50 mM in the external medium, for 30 min) and in unloaded cells.

Fig. 5. *Trans-stimulation of glutamine uptake by intracellular glutamine*

Hepatocytes were preincubated for 30 min at 37°C either in Krebs–Henseleit bicarbonate medium (○, ●) or in Krebs–Henseleit bicarbonate medium supplemented with 50 mM unlabelled glutamine (△, ▲). Thereafter, the cells were quickly washed, then resuspended in 2 vol. of Krebs–Henseleit bicarbonate medium containing (a) Na⁺ or (b) choline. Uptake was measured for 1 min at 37°C. The passive diffusion has not been subtracted. The results are the means for triplicate assays with the same batch of cells.

Fig. 5 shows that, in the presence of high intracellular concentrations of glutamine, an enhanced influx of labelled glutamine was obtained, this process of *trans*-stimulation being consistent with the existence of a facilitated-diffusion component. This was also observed with a Na⁺-free medium, provided that the cells had been loaded in a Na⁺ medium. Furthermore, prior incubations with histidine (2 mM) did not elicit an enhanced influx of glutamine at 0.3 mM (result not shown).

Analysis of glutamine efflux

This was studied after loading of the hepatocytes with labelled glutamine. Preliminary assays indicated that rapid kinetic analyses of glutamine efflux were required (Fig. 6), since glutamine efflux was very high at 37°C, and readily deviated from

Fig. 6. *Time course of glutamine efflux*

Cells were incubated with medium containing 1 mM [³H]glutamine for 30 min. The cells were then gently centrifuged and resuspended in Krebs–Henseleit bicarbonate buffer, pH 7.4 at 0°C (●) or 37°C (■), or in Krebs–Henseleit bicarbonate medium containing 50 mM unlabelled glutamine (▼). The results are the means for triplicate assays with the same batch of cells.

linearity. Thus the time course was studied over no more than 20 s after the start of efflux, in order to operate with relatively constant intracellular concentrations. Efflux was also tested in two media, either glutamine-free or containing 50 mM-glutamine. High external glutamine brought about a substantial increase in the rate of glutamine efflux (2-fold increase), in keeping with the hypothesis of facilitated transport. The plot of the rate of glutamine efflux against intracellular concentrations of glutamine yielded a curve showing a decreasing slope with high cellular glutamine (Fig. 7a). However, a plateau was not actually reached, since cells could not be sufficiently loaded and since the K_m for glutamine efflux appears high. The Lineweaver–Burk plot (Fig. 7b), in which the passive diffusion component has not been subtracted, indicates that the K_m might be around 10 mM. In contrast with influx, glutamine efflux was not affected by short-term lowering of external pH to 6.5.

Discussion

Characteristics of Na⁺-dependent glutamine uptake

The most conspicuous characteristic of glutamine transport is its very high rate for entry, and, in agreement with previous investigations (Pardridge, 1977; Kilberg *et al.*, 1980), we have observed a higher initial rate of transport of glutamine than for

Fig. 7. Efflux of glutamine; effect of prior loading of the cells

The rate of efflux and the intracellular concentrations of glutamine were determined as described in the Materials and methods section. The efflux medium contained either 0 (□) or 50 mM (■) unlabelled glutamine. Efflux was plotted according to Michaelis-Menten (a) and Lineweaver-Burk (b) representations. The results are means ($\pm$ S.E.M.) of five determinations for two batches of cells.

alanine. When metabolism is inhibited, the distribution ratio of glutamine remains relatively constant and efficiently regulated even at unphysiological glutamine concentrations, in contrast, for instance, with the accumulation ratio for alanine, which falls when external concentrations are very high (Joseph *et al.*, 1978). It must be noticed that, in spite of the high rate of glutamine entry, maximal distribution ratios are obtained only after about 25 min. The distribution ratios observed *in vitro*

(about 8) are roughly similar to the values reported *in vivo* in either starved or high-carbohydrate-fed rats (Rémésy *et al.*, 1978; Peret *et al.*, 1981), situations that correspond to a net release of glutamine by the liver. Paradoxically, in spite of this highly efficient entry of glutamine mediated by the N system, hepatic balance of glutamine is seldom positive and may frequently be practically nil. In contrast, alanine may be still substantially taken up in the presence of high liver/plasma gradients (about 5) (Fafournoux *et al.*, 1983), as observed with protein-deficient diets (Rémésy & Demigné, 1982).

It seems unlikely that glutamine uptake is mediated by the A and L systems, since their respective inhibitors were ineffective. However, it must be noted that a slight inhibitory effect of cysteine was observed in Na⁺ medium, in keeping with the data of Kilberg *et al.* (1980). These observations raise the question as to whether cysteine could, to some extent, inhibit the N system or whether a small part of glutamine uptake is mediated by the ASC system. However, the effects of cysteine might be unspecific, since this substrate has been shown to deplete glutathione and elicit extensive membrane damage (Viña *et al.*, 1983).

Histidine appears to be the most potent competitive inhibitor for glutamine uptake, its effect being higher than that of more closely related analogues such as L-asparagine or L-glutamate γ -hydroxamate (Kilberg *et al.*, 1980). However, this inhibition has probably no physiological significance, since circulating histidine concentrations *in vivo* are always very low (Rémésy *et al.*, 1978).

Role of the Na⁺-independent components

The present work suggests that, besides the N system, a facilitated component is also operative for glutamine influx *in vitro*. This component is also efficiently inhibited by histidine, but the K_i is markedly higher. The same percentage of passive diffusion has been obtained, either by total inhibition of glutamine transport by histidine, or by measurement of the slope (Michaelis-Menten plot) with saturating concentrations of glutamine for the carriers: this supports the view that no glutamine is transported by a histidine-insensitive carrier. The comparison of kinetic parameters for the N system ($K_m = 1.25$ mM; $V_{max} = 1.3$ nmol/min per mg of cells) and the Na⁺-independent component ($K_m = 4$ mM; $V_{max} = 0.65$ nmol/min per mg of cells) shows that the role of this last system in the overall entry of glutamine is relatively minor under physiological conditions. This system exhibited characteristics of facilitated diffusion since glutamine uptake was accelerated in cells previously loaded with this amino acid; reciprocally, the efflux of [³H]glutamine was enhanced at high external concentration.

Glutamine efflux

Since the facilitated-diffusion component seems to play no significant role in glutamine influx against a concentration gradient, it was interesting to examine further its possible contribution to glutamine efflux. The determination of the kinetic parameters of efflux, although relatively imprecise owing to the high rate of depletion of cellular glutamine and to the inaccuracy of efflux measurements with slightly loaded cells, yielded values of approx. 10 mM for K_m and 1.3 nmol/min per mg of cells for V_{max} .

The activities of the N system and of facilitated diffusion give rise to a relatively constant distribution ratio when cellular metabolism is inhibited, even with large changes in extracellular concentrations. This suggests that capacities for efflux may keep pace with the high activity of the N system when intracellular glutamine reaches high enough concentrations.

The question arises as to whether facilitated diffusion is mediated by a system definitely distinct from the N system, or, if only one carrier is involved in glutamine transport, that this carrier could exist in two forms with distinct kinetic characteristics. Indeed histidine inhibited both systems, but we failed to observe *trans*-acceleration of glutamine entry in Na^+ -free medium after prior loading of the cells with histidine. Thus histidine seems to inhibit the facilitated-diffusion component, but possibly without being transported by this system. The problem of the carrier involved could be solved if more specific inhibitors of the N system were available.

Influence of pH

From a physiological point of view, it was interesting that acidosis affected these processes. We observed that acidic external pH depressed the rate of glutamine entry, as for 3-aminoisobutyrate transport (Kilberg *et al.*, 1980). At acidic pH the distribution ratio was decreased, but this could hardly be ascribed to stimulation of glutamine efflux, since rapid changes in external pH failed to affect the efflux rate. However, substantial changes in cellular pH might affect facilitated transport. In the same way, acidosis and bicarbonate depletion have been reported to decrease the activity of glutaminase (Baverel & Lund, 1979) and the hepatic extraction of glutamine (Fine, 1982).

Physiological role of bidirectional transport

The present results suggest that the concept of a futile cycle, frequently mentioned in metabolic pathways (Häussinger *et al.*, 1983), could also be extended to transport processes (Hacking & Eddy, 1981). In fact, in the liver cell membrane there coexist a concentrative system (N), very active for glutamine entry, and a facilitated-diffusion com-

ponent, essentially involved in glutamine efflux, since the K_m for efflux (10 mM) is relatively close to physiological glutamine concentrations in the liver (approx. 5 mM) and since the concentration gradient is highly favourable for glutamine output. The existence of considerable inward and outward fluxes for glutamine could make unnecessary induction of specific components in response to hormonal or metabolic stimuli, in contrast with alanine (Fehlmann *et al.*, 1979; Fafournoux *et al.*, 1982). Accordingly, attempts to induce the N system by starvation have been unsuccessful (Hayes & McGivan, 1982) but, *in vitro*, Handlogten *et al.* (1982) showed that stimuli such as amino acids might affect, in a different pattern, both A and N systems. These systems provide the liver cells with high intracellular concentrations of glutamine, even at relatively low blood concentrations. This contrasts with hepatic concentrations of alanine, which undergo dramatic changes (about 10-fold) according to metabolic status (Peret *et al.*, 1981; Rémésy & Demigné, 1982). The apparent lack of induction of glutamine transport systems suggests that changes in hepatic metabolism are essentially governed by the respective activities of glutamine synthetase and glutaminase. However, the fact that hepatic glutamine is generally maintained at high concentrations might be related to its numerous roles in hepatocyte metabolism, besides amino-group cycling and ureogenesis: control of lysosomal proteolysis (Mortimore, 1982), involvement in synthesis of purines, pyrimidines and glucosamine (Lund, 1980) or glycogen (Katz *et al.*, 1979). Moderate changes in hepatic glutamine could affect glutamine catabolism, since the K_m of mitochondrial glutaminase (membrane-bound form) is close to physiological concentrations of glutamine in the liver (McGivan *et al.*, 1980). However, glutaminase activity probably plays an essential role in the regulation of glutamine utilization rather than the cellular concentration. As a result, extensive utilization by the liver is generally concomitant with depleted cellular glutamine. This might arise from changes in glutaminase effectors or in mitochondrial transport of glutamine. This is illustrated with high-protein diets, which elicit an enhanced catabolism of glutamine concomitant with decrease in liver glutamine: the rise of portal concentrations then allows a substantial uptake of glutamine (Rémésy *et al.*, 1978).

Glutamine may constitute either an efficient substrate for ureogenesis when nitrogen availability is high or an important vehicle for nitrogen recycling when the supply of amino acids is low (Lund & Watford, 1976). In this view, the systems of bidirectional transport described in the present paper might contribute to the regulation of glutamine exchange between liver and blood.

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