Transport of L-glutamine and L-glutamate across sinusoidal membranes of rat liver

Effects of starvation, diabetes and corticosteroid treatment

Sylvia Y. LOW,* Peter M. TAYLOR,*† Harinder S. HUNDAL,* Christopher I. POGSON† and Michael J. RENNIE*

* Department of Anatomy and Physiology, University of Dundee, Dundee DD1 4HN, and † Wellcome Research Laboratories, Langley Court, Beckenham, Kent BR3 3BS, U.K.

There is increasing evidence that membrane transporters for glutamine and glutamate are involved in control of liver metabolism in health and disease. We therefore investigated the effects of three catabolic states [starvation (60 h), diabetes (4 days after streptozotocin treatment) and corticosteroid (8-day dexamethasone) treatment] associated with altered hepatic amino acid metabolism on the activity of glutamine and glutamate transporters in sinusoidal membrane vesicles from livers of treated rats. In control preparations, L-[¹⁴C]glutamine uptake was largely Na⁺-dependent, but L-[¹⁴C]glutamate uptake was largely Na⁺-independent. *V*_{max} values for Na⁺-dependent uptake of glutamine and/or glutamate exceeded control values (by about 2- and 12-fold respectively) in liver membrane vesicles from starved (glutamine), diabetic (glutamate) or steroid-treated (glutamine and glutamate) rats. The *K*_m values for Na⁺-dependent transport of glutamine or glutamate and the rates of their Na⁺-independent uptake were not significantly altered by any treatment. Na⁺-independent glutamate uptake appeared to include a dicarboxylate-exchange component. The patterns of inhibition of glutamine and glutamate uptake by other amino acids indicated that the apparent induction of Na⁺-dependent amino acid transport in catabolic states included increased functional expression of systems A, N (both for glutamine) and X⁻_{ag} (for glutamate). The results demonstrate that conditions resulting in increased secretion of catabolic hormones (e.g. corticosteroid, glucagon) are associated with increased capacity for Na⁺-dependent transport of amino acids into liver cells from the blood. The modulation of hepatic permeability to glutamine and glutamate in these situations may control the availability of amino acids for intrahepatic metabolic processes such as ureagenesis, ammonia detoxification and gluconeogenesis.

INTRODUCTION

Glutamine and glutamate are important intermediary metabolites involved in ammonia detoxification (including ureagenesis), gluconeogenesis and acid–base balance, and the liver plays an important role in these processes; transport of these amino acids across the liver sinusoidal membrane is now recognized to be important in controlling hepatic nitrogen flux, e.g. glutamine for periportal ureagenesis and glutamate for perivenous glutamine synthesis (Häussinger, 1990).

Glutamine is reported to be transported in the liver by Na⁺-dependent transport systems A and N (Kilberg *et al.*, 1980; Low *et al.*, 1991) and also by Na⁺-independent transport L (Low *et al.*, 1991). There is recent evidence indicating that the pattern of distribution of transporters on the sinusoidal face of the cells changes along the length of the sinusoid, i.e. from periportal to perivenous regions (Burger *et al.*, 1989). In particular, the uptake of glutamate (a precursor of glutamine synthesis) is much higher in perivenous than in periportal hepatocytes (Burger *et al.*, 1989; Stoll & Häussinger, 1989). An Na⁺-dependent component of glutamate uptake across the liver cell membrane has been identified in isolated hepatocytes and in perfused-liver studies (Gebhardt & Mecke, 1983; Taylor & Rennie, 1987; Burger *et al.*, 1989) and in isolated membrane vesicles (Sips *et al.*, 1982; Ballatori *et al.*, 1986) but there is no general agreement about how much of this transport activity (designated system X⁻ or G by different authors) is localized in sinusoidal or bile-canalicular membranes.

Adaptive up-regulation of amino acid transport systems A and

N occurs in cultured rat hepatocytes deprived of exogenous amino acids (Kelley & Potter, 1978; Kilberg *et al.*, 1980; Gebhardt & Kleemann, 1987). *N*-Methylaminoisobutyric acid (MeAIB), the paradigm substrate for system A, is neither a substrate nor an inhibitor of system N, but has been found to have a repressive effect on the stimulation of system N by deprivation of exogenous amino acids in cultured hepatocytes (Kelley & Potter, 1978; Handlogten *et al.*, 1982). There are conflicting reports as to the effects of starvation *in vivo* on hepatic amino acid transport activity (Pardridge, 1977; Hayes & McGivan, 1982), and for this reason we have investigated the relative importance of systems A and N for liver glutamine transport in sinusoidal membrane vesicles isolated from 60 h-starved and post-absorptive control rats. Liver glutamine transport may also be modulated pathophysiologically. Glucagon and dexamethasone (a synthetic glucocorticoid) have additive stimulatory effects on the uptake of L-glutamine in cultured cells (Gebhardt & Kleemann, 1987), indicating a likely effect of the counter-regulatory hormones *in vivo*. Nevertheless, induction of diabetes (with alloxan) did not significantly affect the rate of L-glutamine uptake into membrane vesicles isolated from rat liver (Jacob *et al.*, 1986), although the rate of alanine uptake (through systems A and/or ASC) was increased significantly (Rosenthal *et al.*, 1985). In the present study we have re-investigated the effect of diabetes on liver glutamine transport using streptozotocin-diabetic rats.

In freshly cultured hepatocytes Na⁺-dependent glutamate transport appears to be expressed only in perivenous cells, but transporter induction by dexamethasone (Gebhardt, 1989) ap-

Abbreviation used: MeAIB, *N*-methylaminoisobutyric acid.

† To whom reprint requests should be addressed.

pears to occur in both perivenous and periportal cells (Burger *et al.*, 1989). Insulin suppresses the induction of Na⁺-dependent glutamate transport by dexamethasone in cultured hepatocytes (Gebhardt, 1989). We have therefore investigated the effects on liver glutamate transport of extremes of hormone secretion *in vivo* under physiological and pathophysiological circumstances (starvation and diabetes respectively).

Since increases in glucocorticoid concentrations are associated with many catabolic states, including starvation and diabetes (Martin, 1987), we have also investigated the effects of a synthetic glucocorticoid, dexamethasone, on liver glutamine and glutamate transport.

EXPERIMENTAL

Materials

L-[U-¹⁴C]Glutamine and L-[U-¹⁴C]glutamate (sp. radioactivity ~ 10 GBq/mmol) were obtained from Amersham International (Aylesbury, Bucks., U.K.). [1-¹⁴C]MeAIB (sp. radioactivity 1.8 GBq/mmol) was obtained from NEN Products (Boston, MA, U.S.A.). All other chemicals were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.) unless otherwise stated.

Animals and experimental procedures

Female Wistar rats (Bantin and Kingman, Hull, U.K.) weighing 200–250 g were used. In the first study, a group of rats was starved from 21:30 h for 60 h [controls were post-absorptive (overnight-fasted) rats]. In the second study, one group of rats was given daily (for 8 days) subcutaneous injection of 0.44 mg of dexamethasone/kg rat (dexamethasone 21-acetate) in saline (controls received saline only). In the third study, rats were made diabetic by a single subcutaneous injection of streptozotocin (85 mg of streptozotocin/kg body wt.) in 0.05 M-citrate-buffered saline (pH 4.5) and were used 4 days later; during the first 24 h they were provided with 5% glucose in their drinking water (controls were given a citrate-buffered saline injection and were not provided with glucose in drinking water). Induction of diabetes was assessed by estimation of urinary pH, protein and glucose with BM-Test-5L strips (Boehringer-Mannheim). Rats in the second and third studies were not starved over the night before tissue removal. In a single experiment, one diabetic rat was treated with CCl₄ [2.5 ml/kg, 1:1 (v/v) in olive oil, via an intragastric tube 20 h before study] to induce perivenous liver-cell necrosis (Häussinger & Gerok, 1983).

Tissue and blood sampling

Tissue was obtained from rats under terminal pentobarbitone anaesthesia [Sagatal (May and Baker); 60 mg/kg body wt. intraperitoneally]. Liver for membrane isolation was excised and transferred rapidly to 0.25 M-sucrose homogenization buffer (Low *et al.*, 1991) for processing. Tissue samples for amino acid analysis were obtained by freeze-clamping and stored in liquid nitrogen. Blood samples (0.2 ml) were obtained from the hepatic portal vein and the hepatic vein by using 23-gauge needles.

Preparation of liver sinusoidal membrane vesicles

Liver tissue was homogenized and fractionated by differential and discontinuous sucrose-density-gradient centrifugations to prepare purified sinusoidal and bile-canalicular membranes (obtained from 26/34% and 10/26% sucrose interfaces respectively) as described previously (Low *et al.*, 1991). Membranes were resuspended in buffer (0.4 M-sucrose, 5 mM-MgCl₂, 0.2 mM-CaCl₂, 5 mM-Tris, 10 mM-Hepes, pH 7.4) at 1 mg of membrane protein/ml.

Assessment of membrane purity

Protein was assayed by the biconchonic acid method (Smith *et al.*, 1985) and membrane purity was assessed by assay of marker enzyme activities: Na⁺-K⁺-ATPase, 5'-nucleotidase and glucose-6-phosphatase activities were measured by standard methods as described previously (Ahmed *et al.*, 1990) and γ -glutamyltransferase activity was measured with a Sigma diagnostic kit.

Measurement of amino acid uptake in membrane vesicles

Uptake of L-[¹⁴C]glutamine, L-[¹⁴C]glutamate and [¹⁴C]MeAIB into vesicles was measured by a rapid gel-filtration method as described previously (Low *et al.*, 1991). The quantities of tracer used were (per 20 μ g sample of membrane protein): glutamine 1.5 kBq, glutamate 2 kBq, MeAIB 7 kBq. The uptake buffer included (final concns.) 100 mM-NaCl or choline chloride, 5 mM-MgCl₂, 0.2 mM-CaCl₂, 5 mM-Tris and 10 mM-Hepes, pH 7.4.

Blood and tissue analysis

Blood and tissue amino acid concentrations were measured from deproteinized samples by ion-exchange chromatography (Biotronik amino acid analyser), and extracellular-space volume of the liver was estimated from the [¹⁴C]sucrose space, both by methods described previously (Taylor *et al.*, 1989).

Data analysis

All measurements were performed in triplicate unless stated. Statistical analysis was performed by Student's unpaired *t* test. Kinetic characteristics (*K_m*, *V_{max}*) of glutamine and glutamate transport processes were estimated from lines fitted by least squares to linear-transformed data (Hanes plot) by using commercial software on an Apple IIe microcomputer (Barlow, 1982).

Results are presented as means $\pm$ S.E.M. for the indicated number of vesicle preparations (*n*) (or number of rats for amino acid analysis).

RESULTS

Experimental treatments

The 60 h-starved rats lost 27.5 ± 2.4 g of weight from 228 ± 3.2 g (i.e. ~ 12% weight loss). The final weight of control (overnight-starved) rats did not differ significantly from the initial weight.

In the dexamethasone study, control rats gained 14.5 ± 1.0 g (*n* = 6) in weight over the 8-day period, whereas the dexamethasone-treated rats lost 16.5 ± 1.2 g (*n* = 6) over the same period from 233.8 ± 2.6 g (*n* = 12) (i.e. 6.5% weight gain and 7.1% weight loss in control and dexamethasone-treated rats respectively).

In the streptozotocin-diabetes study, control rats gained 6.7 ± 1.2 g in weight over the 4-day period, whereas diabetic rats lost 13.5 ± 1.6 g (*n* = 6) over the same period from 238 ± 2.8 g (*n* = 12) (i.e. 2.8% weight gain and 5.7% weight loss in control and diabetic rats respectively). Streptozotocin-diabetes was confirmed in treated rats by positive tests for urinary glucose (> 55 mM), protein (> 1 g/l) and urinary acid (pH < 5).

Blood and liver concentrations of amino acids

There was no significant change in the concentrations of glutamine, glutamate or total amino acids in the blood or liver after 60 h starvation (compared with post-absorptive controls; Table 1), although the liver alanine concentration was decreased by 66%. In diabetic rats the blood alanine concentration was significantly lower, and the liver glutamate concentration significantly higher, than in controls (Table 1), but other amino acid concentrations were unaffected. In dexamethasone-treated rats the liver glutamate concentration increased significantly (Table

Table 1. Tissue and blood amino acid concentrations in livers of control, 60 h-starved, streptozotocin-diabetic and dexamethasone-treated rats

All values represent means $\pm$ S.E.M. from 6 or 7 rats. Liver blood amino acid concentrations were calculated as the mean of hepatic portal and hepatic venous concentration for the same rat. * $P < 0.05$, ** $P < 0.01$.

	L-Glutamine	L-Glutamate	L-Alanine	Total amino acid
Liver tissue ($\mu\text{mol/g}$)				
Control	4.6 ± 0.4	1.15 ± 0.15	1.20 ± 0.15	11.8 ± 0.9
60 h-starved	3.7 ± 0.9	1.06 ± 0.05	$0.40 \pm 0.05^{**}$	9.9 ± 0.9
Control	4.37 ± 0.65	0.88 ± 0.04	2.49 ± 0.39	13.4 ± 0.7
Diabetic	4.25 ± 0.43	$1.90 \pm 0.36^{**}$	3.02 ± 0.48	16.9 ± 2.5
Dexamethasone	4.14 ± 0.36	$1.48 \pm 0.26^*$	2.62 ± 0.38	15.7 ± 1.7
Liver blood ($\mu\text{mol/ml}$)				
Control	0.39 ± 0.03	0.16 ± 0.02	0.34 ± 0.04	3.72 ± 0.34
60 h-starved	0.46 ± 0.02	0.19 ± 0.01	0.36 ± 0.03	3.17 ± 0.22
Control	0.51 ± 0.04	0.19 ± 0.03	0.56 ± 0.05	4.12 ± 0.48
Diabetic	0.48 ± 0.03	0.22 ± 0.02	$0.42 \pm 0.04^*$	3.72 ± 0.24
Dexamethasone	0.44 ± 0.05	0.15 ± 0.02	0.48 ± 0.06	4.26 ± 0.48

Table 2. Marker enzyme analysis of liver sinusoidal membrane preparations

Each value represents the mean $\pm$ S.E.M. for 3 preparations with individual experiments performed in duplicate.

Preparation	Enzyme activity					
	Na ⁺ -K ⁺ -ATPase ($\mu\text{mol/h per mg of protein}$)		Glucose-6-phosphatase ($\mu\text{mol/h per mg of protein}$)		γ -Glutamyl transferase ($\text{nmol/h per mg of protein}$)	
	Homogenate	Membranes	Homogenate	Membranes	Homogenate	Membranes
Control	0.027 ± 0.013	0.675 ± 0.043	0.021 ± 0.008	0.044 ± 0.006	0.361 ± 0.065	1.98 ± 0.19
60 h-starved	0.032 ± 0.011	0.800 ± 0.083	0.027 ± 0.011	0.056 ± 0.013	0.402 ± 0.090	2.48 ± 0.21
Control	0.014 ± 0.005	0.480 ± 0.060	0.036 ± 0.007	0.074 ± 0.012	0.320 ± 0.019	2.59 ± 0.86
Diabetic	0.017 ± 0.008	0.530 ± 0.090	0.050 ± 0.015	0.090 ± 0.016	0.360 ± 0.060	2.52 ± 0.36
Control	0.019 ± 0.004	0.551 ± 0.062	0.034 ± 0.010	0.065 ± 0.012	0.390 ± 0.036	2.26 ± 0.32
Dexamethasone	0.021 ± 0.007	0.627 ± 0.075	0.042 ± 0.015	0.092 ± 0.023	0.482 ± 0.056	2.59 ± 0.24

Fig. 1. Time course of 0.05 mM-L-glutamine uptake into sinusoidal membrane vesicles from livers of (a) sham-injected control and (b) dexamethasone-treated rats

Membrane vesicles were prepared from livers of control and dexamethasone-treated rats. Uptake was initiated by addition of vesicle suspension ($\sim 20 \mu\text{g}$ of protein in $20 \mu\text{l}$ of 0.4 M -sucrose buffer) to $20 \mu\text{l}$ of double-strength transport buffer containing L-[^{14}C]glutamine. Uptake was terminated at the times shown by dilution of the reaction mixture with 0.4 ml of ice-cold stopping buffer. The transport buffer included 0.05 mM -L-glutamine and 100 mM -NaCl ($\bullet$) or -choline chloride ($\blacktriangle$). Each point represents the mean $\pm$ S.E.M. for 4 preparations with individual experiments performed in duplicate.

1) but the total concentrations of amino acids in the blood and liver were unaltered from control values (Table 1).

Purity of liver membrane vesicles

There was no significant difference between the marker enzyme profiles of liver membranes isolated from 60 h-starved and control rats (Table 2). Similarly, the marker enzyme profiles of

membranes isolated from dexamethasone- or streptozotocin-treated rat livers were not significantly different from those of respective control preparations for the major surface-membrane markers (Table 2). The sinusoidal-membrane marker enzyme Na⁺-K⁺-ATPase was enriched ~ 30 -fold in activity in the sinusoidal-membrane fractions used in the present studies (reticular and bile-canalicular markers showed a substantial relative

Fig. 2. Time course of 0.05 mM-L-glutamate uptake into sinusoidal membrane vesicles from livers of (a) sham-injected control and (b) dexamethasone-treated rats

Membrane vesicles were prepared from livers of control and dexamethasone-treated rats. Uptake of L-glutamate was measured at the times shown. The transport buffer included 0.05 mM-L-[14 C]glutamate and 100 mM-NaCl (●) or -choline chloride (▲). Each point represents the mean $\pm$ S.E.M. for 4 preparations with individual experiments performed in duplicate.

Fig. 3. Effect of osmolarity of the medium on equilibrium uptake of L-[14 C]glutamine (0.05 mM) in sinusoidal membrane vesicles from rat liver

Membrane vesicles were incubated for 1 h in transport buffer containing [14 C]glutamine and 80 mM-NaCl at osmolarities adjusted with sucrose. Sample osmolarity was measured with a Gonotec Osmomat 030 instrument. Values are means $\pm$ S.E.M. for triplicate measurements in a single membrane preparation.

Fig. 4. Effect of MeAIB on 0.05 mM-L-glutamine uptake into sinusoidal membrane vesicles from rat liver

Membrane vesicles were prepared from the liver of a 60 h-starved rat. Uptake of L-glutamine was measured over 30 s and the transport buffer contained 0.05 mM-L-[14 C]glutamine, MeAIB at 0–10 mM (inset shows values at 0–0.1 mM-MeAIB), 100 mM-NaCl (●) or -choline chloride (▲). Each point represents the mean $\pm$ S.E.M. for triplicate measurements.

Table 3. Effect of amino acids on MeAIB uptake into liver sinusoidal membrane vesicles

Membrane vesicles were prepared from the liver of a 60 h-starved rat. Uptake was measured over 30 s (initial rate) and the transport buffer included 0.1 mM-L-[14 C]MeAIB, 100 mM-NaCl or 100 mM-choline chloride (ChoCl) and 1 mM-glutamine or -alanine where appropriate. Each point represents the mean $\pm$ S.E.M. for triplicate measurements: * significantly different from control ($P < 0.01$).

Experimental conditions		0.1 mM-MeAIB uptake (nmol/min per mg of protein)	Inhibition by amino acid (%)
NaCl	Control	0.172 $\pm$ 0.039	
	+ 1 mM-Glutamine	0.026 $\pm$ 0.010*	85
	+ 1 mM-Alanine	0.024 $\pm$ 0.012*	86
ChoCl	Control	0.069 $\pm$ 0.024	
	+ 1 mM-Glutamine	0.045 $\pm$ 0.029	35
	+ 1 mM-Alanine	0.043 $\pm$ 0.023	38

Time course of 14 C-labelled amino acid uptake into sinusoidal membrane vesicles: effect of experimental treatments

The time course of tracer glutamine and glutamate uptake in the presence of Na⁺ or choline⁺ was measured in preliminary studies using membrane vesicles isolated from control, starved, diabetic and dexamethasone-treated rats. Representative results (Figs. 1 and 2) demonstrate that tracer uptake remained linear for > 30 s; this was the case for all studies. Intravesicular volume estimated from equilibrium uptake of both tracers was ~ 1.15 μ l/mg of protein for all treatments. This volume was shown to be an osmotically active space, at least for glutamine (Fig. 3).

Kinetic and competition characteristics of L-glutamine and L-glutamate uptake into sinusoidal membrane vesicles

L-Glutamine uptake into liver membrane vesicles included Na⁺-dependent and Na⁺-independent components. The system-A substrate MeAIB inhibited a small but significant component of Na⁺-dependent L-glutamine uptake in liver membrane vesicles in a concentration-dependent manner (Fig. 4; $K_i \approx 0.1$ mM-MeAIB); this transport component was largest in membrane vesicles isolated from starved or dexamethasone-treated rats (Table 3). Na⁺-dependent uptake of MeAIB (0.1 mM) in liver membrane vesicles was strongly inhibited by glutamine or alanine at 1 mM (Table 4). The mutual inhibition between Na⁺-dependent

depletion in these fractions). The activity of the canalicular-marker enzyme γ -glutamyltransferase in bile-canalicular membrane fractions obtained from the same preparations was enriched 50-fold (homogenate 0.36 ± 0.02 , bile membrane 17.9 ± 0.9 nmol/h per mg of protein).

Table 4. Effect of 1 mM-MeAIB on Na⁺-dependent glutamine uptake into liver sinusoidal membrane vesicles

Membrane vesicles were prepared from the livers of control, 60 h-starved, streptozotocin-diabetic and dexamethasone-treated rats. Uptake was measured over 30 s and the transport buffer included 0.05 mM-L-[¹⁴C]glutamine, 100 mM-NaCl and 1 mM-MeAIB where appropriate. Each point represents the mean $\pm$ S.E.M. for 3 or 4 preparations with individual experiments performed in duplicate: *significant difference between values with and without MeAIB ($P < 0.05$).

	Na ⁺ -dependent 0.05 mM-glutamine uptake (nmol/min per mg of protein)		Inhibition by MeAIB (%)
	No MeAIB	+ MeAIB	
Control	0.268 $\pm$ 0.014	0.222 $\pm$ 0.014	17
60 h starvation	0.656 $\pm$ 0.048	0.446 $\pm$ 0.034*	32
Control	0.298 $\pm$ 0.022	0.263 $\pm$ 0.020	12
Diabetic	0.311 $\pm$ 0.023	0.238 $\pm$ 0.016*	23
Control	0.358 $\pm$ 0.036	0.310 $\pm$ 0.044	13
Dexamethasone	0.854 $\pm$ 0.054	0.570 $\pm$ 0.034*	33

transport of glutamine and MeAIB indicates that the two amino acids share a common transport pathway in the liver membrane, presumably system A, as reported previously (Handlogten *et al.*, 1982) for hepatocytes. In our experiments 1 mM-MeAIB is sufficient to produce maximum MeAIB inhibition of glutamine transport in liver membrane vesicles; therefore we have ascribed Na⁺-dependent glutamine uptake inhibited by 1 mM-MeAIB to system A and the balance to system N.

L-Glutamine uptake was measured at various external L-glutamine concentrations in the presence of NaCl or choline chloride. In membrane vesicles isolated from 60 h-starved and dexamethasone-treated (but not streptozotocin-diabetic) rats, Na⁺-dependent L-glutamine uptake was increased compared with control values (Table 3, Fig. 5). The mean values for Na⁺-independent glutamine uptake after starvation or dexamethasone treatment were $\sim 50\%$ higher than control values (Table 5), but the increases were not statistically significant.

Kinetic characteristics (i.e. K_m and V_{max}) for systems N and A were estimated from experimental data by using the Hanes plot (Table 6). The K_m values of systems A and N did not appear to change significantly after any of the experimental treatments

Table 5. Na⁺-independent glutamine uptake into sinusoidal membrane vesicles from livers of control, starved, diabetic and dexamethasone-treated rats

Uptake was measured over 30 s, and the transport buffer included 0.5 mM-L-[¹⁴C]glutamine and 100 mM-choline chloride. Each point represents the mean $\pm$ S.E.M. for 3 or 4 preparations with individual experiments performed in duplicate: there were no significant differences between control and treated values.

	Na ⁺ -independent 0.5 mM-glutamine uptake (nmol/min per mg of protein)
Control	0.57 $\pm$ 0.11
60 h starvation	0.82 $\pm$ 0.12
Control	0.57 $\pm$ 0.10
Diabetic	0.58 $\pm$ 0.09
Control	0.56 $\pm$ 0.10
Dexamethasone	0.83 $\pm$ 0.16

(although estimates of K_m for system A are approximate). In contrast, the V_{max} for systems A and N were raised significantly after starvation and dexamethasone treatment, as was the V_{max} for system A (but not system N) in diabetes. We have assumed that glutamine and MeAIB are competitive substrates of system A, and on this basis the K_i for MeAIB inhibition of glutamine transport should equal the K_m for MeAIB transport by system A; Na⁺-dependent uptake of 0.1 mM-MeAIB in membrane vesicles from a 60 h-starved rat was about 0.1 nmol/min per mg of protein (Table 3) and therefore, assuming a K_m of 0.1 mM, V_{max} for MeAIB transport by system A is of the order of 0.2 nmol/min per mg of protein. This value is of similar magnitude to the calculated V_{max} for glutamine transport by system A after 60 h starvation (0.20 ± 0.01 nmol/min per mg of protein; Table 6). The equivalence of these V_{max} values provides a further indication that glutamine is transported by a low-capacity (and high-affinity; $K_m \leq 0.02$ mM-glutamine) system A transporter in rat liver. A system A transporter with similar affinity for amino acid substrates has also been identified in isolated hepatocytes (Handlogten *et al.*, 1982) and in human liver membrane vesicles (Mailliard & Kilberg, 1990).

Glutamate uptake in control liver membrane vesicle preparations was predominantly Na⁺-independent (Fig. 2a). Na⁺-dependent uptake of glutamate (0.05 mM) was increased in liver membrane vesicles from dexamethasone-treated (Fig. 6, Table 7)

Fig. 5. (a) Effect of external L-glutamine concentration on Na⁺-dependent L-glutamine uptake into sinusoidal membranes from livers of control and dexamethasone-treated rats; (b) semi-logarithmic plot of tracer uptake data

Membrane vesicles were prepared from livers of control (Δ) and dexamethasone-treated ($\bullet$) rats. Uptake of L-glutamine was measured over 30 s, and the transport buffer contained L-[¹⁴C]glutamine, L-glutamine at the concentrations shown, 100 mM-NaCl and 1 mM-MeAIB. Uptake in the presence of 100 mM-choline chloride was deducted for each glutamine concentration; therefore transport data reflect system-N activity (i.e. Na⁺-dependent glutamine uptake not inhibited by MeAIB). The line shown in the semi-logarithmic plot (b) represents the predicted tracer uptake calculated by using nominal specific radioactivities and kinetic characteristics estimated from a Hanes plot of transport data ($K_m = 0.75$ mM, $V_{max} = 8.7$ nmol/min per mg of protein). Each point represents the mean $\pm$ S.E.M. for 4 preparations with individual experiments performed in duplicate.

Table 6. Effect of 60 h starvation, diabetes and dexamethasone treatment on K_m and V_{max} values of transport systems A and N in liver sinusoidal membrane vesicles

Membrane vesicles were prepared from livers of control, 60 h-starved, streptozotocin-diabetic and dexamethasone-treated rats. L-[14 C]Glutamine uptake was measured in transport buffer containing different external L-glutamine concentrations (0.025–5 mM), 100 mM-NaCl or -choline chloride and 1 mM-MeAIB where appropriate. Kinetic parameters for transport were obtained from Hanes plots of (a) MeAIB-inhibitable Na^+ -dependent glutamine uptake (system A transport) and (b) Na^+ -dependent glutamine uptake measured in the presence of 1 mM-MeAIB (system N transport). Each value represents the mean $\pm$ S.E.M. for 3 or 4 preparations; no S.E.M. is attached to estimates of system-A K_m , because a large error is inherent to individual measurements, since the K_m values obtained (~ 0.01 mM) are lower than experimental glutamine concentrations (≥ 0.025 mM). Values significantly different from control: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

	K_m (mM)	V_{max} (nmol/min per mg of protein)
(a) System A		
Control	0.014	0.05 ± 0.01
60 h starvation	0.007	0.20 ± 0.01 ***
Control	0.009	0.04 ± 0.005
Diabetic	0.014	0.10 ± 0.01 *
Control	0.011	0.05 ± 0.005
Dexamethasone	0.010	0.35 ± 0.04 ***
(b) System N		
Control	1.20 ± 0.14	5.30 ± 0.40
60 h starvation	0.87 ± 0.25	8.00 ± 0.82 *
Control	1.09 ± 0.19	5.52 ± 0.40
Diabetic	1.37 ± 0.09	5.63 ± 0.33
Control	1.02 ± 0.17	4.06 ± 0.35
Dexamethasone	0.75 ± 0.10	8.72 ± 0.20 **

Fig. 6. Effect of external L-glutamate concentration on Na^+ -dependent L-glutamate uptake into sinusoidal membranes from livers of control and dexamethasone-treated rats

Membrane vesicles were prepared from livers of control ($\blacktriangle$) and dexamethasone-treated ($\bullet$) rats. Uptake of L-glutamate was measured over 30 s at the concentrations of L-glutamate shown, and the transport buffer included L-[14 C]glutamate and 100 mM-NaCl (uptake in the presence of 100 mM-choline chloride was deducted for each glutamate concentration). Each point represents the mean $\pm$ S.E.M. for 4 preparations with individual experiments performed in duplicate.

or diabetic (Table 7) rats, but not from 60 h-starved rats (Na^+ -dependent uptake of 0.05 mM-glutamate was about 0.005 nmol/min per mg of protein under control and starvation conditions; results not shown). This increased glutamate uptake reflected a large (11-fold) increase in the V_{max} for Na^+ -dependent glutamate transport in sinusoidal membrane vesicles from the

Table 7. Kinetic parameters of glutamate uptake into sinusoidal membrane vesicles isolated from control, diabetic and dexamethasone-treated rats

Membrane vesicles were prepared from the livers of control, dexamethasone-treated and streptozotocin-diabetic rats. L-[14 C]-Glutamate uptake was measured in transport buffer containing different external L-glutamate concentrations (0.05–5 mM) and 100 mM-NaCl or -choline chloride. The kinetic parameters K_m and V_{max} were estimated by the Hanes plot. Each value represents the mean $\pm$ S.E.M. for 3 or 4 preparations: * significantly different from control ($P < 0.001$). There were no significant differences between K_m values for glutamate transport.

	K_m (mM)	V_{max} (nmol/min per mg of protein)
Control	0.25 ± 0.14	0.046 ± 0.008
Dexamethasone	0.18 ± 0.04	0.523 ± 0.021 *
Control	0.39 ± 0.13	0.023 ± 0.002
Diabetic	0.15 ± 0.02	0.330 ± 0.070 *

Table 8. Effect of perivenous liver-cell necrosis (CCl_4 pretreatment *in vivo*) on glutamate uptake into sinusoidal membrane vesicles from diabetic-rat liver

Membrane vesicles were prepared from the livers of one control, one streptozotocin-diabetic and one diabetic/ CCl_4 -treated rat. Uptake was measured over 30 s, and the transport buffer included 0.05 mM-L-[14 C]glutamate and 100 mM-NaCl or -choline chloride (ChoCl). Each result represents the mean $\pm$ S.E.M. for experiments performed in triplicate: * significantly different from control ($P < 0.01$).

Experimental medium	Glutamate uptake (nmol/min per mg of protein)		
	Control	Diabetic	Diabetic/ CCl_4 -treated
NaCl	0.049 ± 0.005	0.122 ± 0.007 *	0.035 ± 0.009
ChoCl	0.037 ± 0.003	0.051 ± 0.004	0.029 ± 0.002

dexamethasone-treated rats as compared with those from controls, with no significant change in transport K_m (Table 7). Similarly, in vesicles from streptozotocin-diabetic rat liver, transport V_{max} was increased markedly (equivalent to a 14-fold induction of Na^+ -dependent glutamate transport), but the K_m value was not affected significantly. Na^+ -independent L-glutamate uptake into membrane vesicles was unaltered by diabetes (0.1 mM-L-glutamate uptake of 0.054 ± 0.010 and 0.058 ± 0.008 nmol/min per mg of protein in control and diabetic respectively; $n = 3$ preparations each), dexamethasone treatment (0.1 mM-L-glutamate uptake of 0.088 ± 0.014 and 0.104 ± 0.018 nmol/min per mg of protein for control and dexamethasone treatment respectively; $n = 4$ preparations each) or 60 h starvation (0.05 mM-L-glutamate uptake of 0.03 ± 0.003 and 0.033 ± 0.004 nmol/min per mg of protein in control and starved respectively; $n = 3$ preparations each). Uptake of 0.05 mM-L-glutamate into bile-canalicular membranes was not altered after induction of streptozotocin-diabetes [0.031 ± 0.012 and 0.027 ± 0.008 nmol/min per mg of protein in controls and diabetics respectively; the control values were similar to those reported previously by other workers (Ballatori *et al.*, 1986)].

In a separate study the effect of perivenous-liver-cell necrosis (CCl_4 -treatment) on liver glutamate transport was investigated. Sinusoidal membrane vesicles isolated from control, diabetic and

Table 9. Effect of amino acids and other carboxylates on glutamate uptake into sinusoidal membrane vesicles

Membrane vesicles were prepared from the livers of streptozotocin-diabetic and dexamethasone-treated rats. Uptake was measured over 30 s and the transport buffer included 0.05 mM-L-[¹⁴C]glutamate, 100 mM-NaCl or choline chloride (ChoCl), with or without 1 mM test compound. Results are expressed as percentages of control 0.05 mM-L-[¹⁴C]glutamate uptake. Each point represents the mean $\pm$ S.E.M. for $n = 3$ or 4 preparations with individual experiments performed in duplicate: * uptake significantly different from control value ($P < 0.05$). Control values of glutamate uptake for the dexamethasone experiments were 0.133 ± 0.025 and 0.076 ± 0.012 nmol/min per mg of protein in Na⁺ and choline media respectively, and for the diabetic-rat experiments were 0.128 ± 0.020 and 0.047 ± 0.010 nmol/min per mg of protein in Na⁺ and choline media respectively. Abbreviations: 2OG, 2-oxoglutarate; OAA, oxaloacetate; β -HB, β -hydroxybutyrate.

	L-Glutamate uptake (% of control)						
	D-Aspartate	L-Aspartate	D-Glutamate	L-Glutamate	2OG	OAA	β -HB
Dexamethasone							
+ NaCl	49 $\pm$ 4*	38 $\pm$ 5*	125 $\pm$ 15	27 $\pm$ 6*	76 $\pm$ 4*	79 $\pm$ 13	–
+ cholineCl	116 $\pm$ 15	107 $\pm$ 19	105 $\pm$ 9	43 $\pm$ 8*	69 $\pm$ 10	82 $\pm$ 7	–
Diabetic							
+ NaCl	44 $\pm$ 8*	42 $\pm$ 4*	83 $\pm$ 5	25 $\pm$ 9*	83 $\pm$ 6	84 $\pm$ 10	103 $\pm$ 4
+ cholineCl	98 $\pm$ 22	81 $\pm$ 7	107 $\pm$ 7	46 $\pm$ 9*	65 $\pm$ 8	89 $\pm$ 12	120 $\pm$ 18

diabetic/CCl₄-treated rats had similar marker enzyme profiles (results not shown), indicating that the experimental treatment had not caused significant damage to liver membranes. The induction of Na⁺-dependent glutamate transport seen in diabetic rat liver membrane was abolished by pre-treatment with CCl₄ (Table 8), a result which is consistent with previous reports (Taylor & Rennie, 1987; Burger *et al.*, 1989) that Na⁺-dependent glutamate transport is expressed largely in perivenous cells in rat liver.

Effect of amino acids and other carboxylates on L-glutamate uptake into sinusoidal membrane vesicles

Na⁺-independent glutamate uptake (choline chloride medium) in liver membrane vesicles was saturable ($\sim 55\%$ inhibition of 0.05 mM-glutamate tracer uptake by 1 mM-L-glutamate: Table 9), but was not greatly affected by 1 mM-D-glutamate (i.e. uptake was stereospecific), -L-aspartate, -D-aspartate, - β -hydroxybutyrate or -oxaloacetate, although 1 mM-2-oxoglutarate consistently inhibited uptake by $\sim 33\%$. The pattern of inhibition of glutamate uptake in NaCl medium (with liver membrane vesicles isolated from dexamethasone-treated or diabetic rats) differed from that in choline chloride medium (Table 9), as it reflected inhibition of both Na⁺-dependent and Na⁺-independent glutamate uptake. Thus 1 mM-L-glutamate inhibited 0.05 mM-glutamate tracer uptake by $\sim 74\%$ and, although D-glutamate had no significant effect, both L- and D-aspartate inhibited glutamate tracer uptake (by ~ 60 and 54% respectively). These results demonstrate that the Na⁺-dependent component of glutamate uptake in liver sinusoidal membranes is stereospecific and (unlike Na⁺-independent uptake) is strongly inhibited by L- and D-aspartate.

DISCUSSION

The present results show that 60 h starvation induces systems A and N in rat liver sinusoidal membranes. Pardridge (1977) has reported an increased hepatic capacity for glutamine extraction *in vivo* for rats starved for 48 h, but Hayes & McGivan (1982) could find no increase in glutamine uptake via system N in hepatocytes isolated from 48 h-starved rats. This apparent conflict is explicable if system A is induced before system N and significant increases in system N activity occur only when starvation exceeds 48 h.

Blood and intracellular amino acid pools were not significantly decreased by starvation, so it seems unlikely that amino

acid deprivation itself caused induction of liver glutamine transport *in vivo*, in contrast with the behaviour of cultured hepatocytes (Handlogten *et al.*, 1982). Transporter induction is more likely to be due to the action of counter-regulatory hormones such as glucagon and glucocorticoids, whose concentrations are known to rise during starvation (Gebhardt & Kleemann, 1987; Martin, 1987; Gebhardt, 1989). The effects of glucagon on membrane transport may be short-term (protein-synthesis-independent; Moule *et al.*, 1987) or long-term (protein-synthesis-dependent; Edmondson & Lumeng, 1980), but the effects of corticosteroid are likely to occur largely over a longer term as the result of increases in net synthesis of transport proteins (see below). Induction of system N transporters during prolonged starvation is likely to be important for maintenance of the intrahepatic glutamine cycle (Häussinger, 1990) under conditions in which both glutamine utilization and glutamine synthesis may be increased (Sies & Häussinger, 1984). Furthermore, starvation is associated with a depolarization of the hepatocyte membrane (Fitz & Scharschmidt, 1987) and, since Na⁺-dependent glutamine transport is voltage-regulated (Jacob *et al.*, 1986), the driving force for hepatic glutamine uptake could be lessened. Such a decrease could be counterbalanced by an increase in the $V_{\max}$ for glutamine transport, as is seen in the liver of starved rats.

Treatment of rats with dexamethasone increased the capacity of Na⁺-dependent glutamine transport via systems A and N without significant effects on the Na⁺-independent component of L-glutamine uptake. Dexamethasone is thought to cause an increase in the net synthesis of transporters, because its induction of system N was prevented by cycloheximide (Gebhardt & Kleemann, 1987). An alternative possibility is that the transporter occurs in the membrane in an inactive form, with the stimulus of dexamethasone causing activation of the transporter via synthesis of a derepressor protein (e.g. Handlogten & Kilberg, 1984).

The K_m for Na⁺-dependent glutamate uptake in sinusoidal membrane vesicles from control, diabetic and dexamethasone-treated rats (~ 0.24 mM) was of the same order as those reported by Taylor & Rennie (1987) in perfused rat liver and by Gebhardt (1989) in cultured hepatocytes. Na⁺-dependent glutamate uptake was stereospecific (the D-isomer had negligible inhibitory effect), whereas both L- and D-aspartate inhibited L-glutamate uptake to a similar extent. These features are characteristic of the Na⁺-dependent glutamate transport system X_{ag}, which has previously been identified in perfused-liver studies (Taylor & Rennie, 1987). This transporter appears to be expressed largely in perivenous cells of rat liver (Taylor & Rennie, 1987; Burger *et al.*, 1989).

There was a significant increase in the intracellular concentration of glutamate in dexamethasone-treated rat livers which may reflect their increased capacity for Na⁺-dependent glutamate transport. Gebhardt (1989) also observed that dexamethasone treatment induced Na⁺-dependent glutamate uptake in perivenous (glutamine-synthesizing) hepatocytes and suggested that transporter induction was linked to an increased demand for glutamate by glutamine synthetase.

In streptozotocin-diabetic rat liver there was no significant increase in the amount of L-glutamine transport via system N, but there was increased transport via system A, which corroborates the results of a previous study (Jacob *et al.*, 1986) using plasma-membrane vesicles isolated from alloxan-diabetic rats. It should be stressed that, despite the 3-fold increase in the $V_{\max}$ for system-A glutamine uptake, most of the L-glutamine uptake at prevailing blood glutamine concentrations (0.5–1 mM) should still be via system N in the diabetic state. Burger *et al.* (1989) showed that in cultured hepatocytes (periportal or perivenous) the stimulation of glutamine transport by dexamethasone required insulin, which may explain the lack of any significant stimulation of hepatic system N in diabetic rats from our study. Handlogten *et al.* (1982), however, found that both systems A and N were induced in hepatocytes cultured from streptozotocin-diabetic rats, although this may reflect differential activation of glutamine transport at basolateral and apical surfaces of the liver cell.

In contrast with the effect on glutamine transport, streptozotocin-diabetes causes a marked increase in the Na⁺-dependent component of L-glutamate transport in sinusoidal (but not bile-canalicular) membrane vesicles from rat liver, with no significant change in the magnitude of the Na⁺-independent component. The increase in Na⁺-dependent glutamate transport appears to be largely due to a $V_{\max}$ effect, indicative of an increase in the effective number of transporters in the membranes. An increased capacity for hepatic glutamate uptake may be adaptive to the diabetic rat, because it could increase the provision of glutamate for glutamine synthesis in perivenous cells, which would help to promote renal H⁺ excretion during ketoacidosis (Häussinger, 1986; Gebhardt, 1989). We observed an increase in the intracellular concentration of glutamate in diabetic-rat livers, which could have resulted from the increased Na⁺-dependent transport of glutamate in liver sinusoidal membranes. The saturable component of Na⁺-independent glutamate uptake may reflect activity of a dicarboxylate exchange mechanism (Stoll & Häussinger, 1989), because 2-oxoglutarate was a relatively strong inhibitor of glutamate uptake in choline chloride medium. Our results indicate that monocarboxylates such as β -hydroxybutyrate (the plasma concentration of which is elevated during starvation and diabetes) do not interact directly with liver glutamate transport.

We have demonstrated that the activity of glutamine and glutamate transporters in liver sinusoidal membranes is influenced by a variety of factors *in vivo*, which is consistent with the idea that modulation of trans-membrane flux of amino acids could contribute to control of nitrogen flux within the liver. It is important to note that *in vivo* the significance of any regulatory role would be affected by factors such as changes in plasma amino acid composition or Na⁺ electrochemical gradient across the sinusoidal membrane. There would be advantages to the animal if modulation of both membrane transport and intra-

cellular enzyme activity were co-ordinated to control glutamate and glutamine metabolism in liver cells, and there is some evidence that this may occur, e.g. corticosteroid promotes simultaneous activation of glutamate transport and glutamine synthetase in cultured hepatocytes (Gebhardt, 1989). Modulation of both the degree and the distribution of amino acid transporter expression within the liver may enhance the range of hepatic response to a variety of physiological and pathophysiological challenges to whole-body nitrogen metabolism.

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