A quantitative analysis of the control of glutamine catabolism in rat liver cells

Use of selective inhibitors

Sylvia Y. LOW,*‡ Mark SALTER,† Richard G. KNOWLES,† Christopher I. POGSON† and Michael J. RENNIE*

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

1. At a physiological concentration of glutamine (0.5 mM), 87% of the total transport across the plasma membrane of liver cells isolated from fed rats involved the Na^+-dependent system N; this was substantially inhibited by L-histidine. The residual Na^+-independent component was attributed to system L on the basis of inhibition by 2-amino-2-norbornanecarboxylate and L-tryptophan. 2. Catabolism of glutamine by intact liver cells or by isolated mitochondria was inhibited by glutamate γ-hydrazide with IC_{50} values of 13.7 ± 3.5 μM and 22.6 ± 3.8 μM respectively and a maximal inhibition of approx. 75%. The site of inhibition was identified as glutaminase; glutamate γ-hydrazide inhibited this enzyme in cell-free extracts (IC_{50} 37.8 ± 7.7 μM) but had no activity against glutamate dehydrogenase or transport of glutamine, whether across mitochondrial or plasma membranes. 3. The major control site in cells from fed animals incubated with 0.5 mM L-glutamine was glutaminase (flux control coefficient 0.96). Appreciable control also resided in both plasma membrane transport systems, with coefficients of 0.51 for system N and -0.46 for system L, such that both interacted to provide a fine control of the intracellular concentration of the amino acid. Similar values were obtained by computer simulation based on theoretical determination of elasticities. 4. Previous controversy about the locus of regulation of hepatic glutamine metabolism is resolved by this distribution of control.

INTRODUCTION

Glutamine is the most abundant free amino acid in mammalian plasma and in the cytosol of liver and skeletal muscle cells. It is a major carrier between tissues of α-amino nitrogen, a major fuel of rapidly dividing cells such as intestinal mucosa, lymphocytes and tumour cells, and a nitrogen donor in reactions involved in a variety of cell functions (for reviews, see Abumrad et al., 1989; Rennie et al., 1989; Christensen, 1990; Häussinger, 1990). In the liver, glutamine is a key metabolite involved in ureagenesis, gluconeogenesis and acid–base balance.

Glutamine is transported against a concentration gradient into liver exclusively by a Na^+-coupled transport mechanism designated system N (Kilberg et al., 1980), although other systems may also contribute to flux across the plasma membrane. Unlike the other major neutral amino acid transport systems found in mammalian cells, i.e. systems A, ASC (both Na^+-coupled) and L (for review, see Christensen, 1990), system N has a narrow substrate specificity, accepting only asparagine, glutamine and histidine among the natural amino acids. This specificity of system N indicates an unusual degree of selectivity at the presumed binding site for substrate amino acids on the transport protein (Low et al., 1991). Glutamine is transported from the cytosol into the inner mitochondrial space (Soboll et al., 1991), where glutaminase (EC 3.5.1.2) and glutamate dehydrogenase (EC 1.4.1.2) are located and convert glutamine carbon to 2-oxoglutarate and subsequent citric acid cycle metabolites.

The relative importance of the various steps in glutamine degradation has been the subject of some controversy (Häussinger et al., 1985; Verhoeven et al., 1985; Ochs, 1986). Häussinger et al. (1985), working with the perfused liver, showed that histidine, which inhibits glutamine transport across the plasma membrane, inhibited glutamine breakdown, and concluded that transport could be the major site of regulation of the pathway. In contrast, Verhoeven et al. (1985), studying the adrenergic stimulation of glutamine catabolism in isolated liver cells, pinpointed glutaminase as the locus of control. The purpose of the present work was to use selective inhibitors and the principles of the Metabolic Control Theory (Kacser, 1987; Fell, 1992) to measure the distribution of control between the early steps of glutamine degradation in the liver (see Scheme 1), and thereby to reconcile these apparently conflicting views. A preliminary account of this work has been published elsewhere (Pogson et al., 1991).

MATERIALS AND METHODS

Animals

Male Wistar rats (200–250 g; Charles River, U.K.), deprived of food for 18 h (overnight), were used throughout. Before liver perfusion, rats were anaesthetized with sodium pentobarbital (60 mg/kg intraperitoneal; May & Baker, Dagenham, U.K.) containing heparin (5 mg/ml).

Chemicals

Chemicals and enzymes were obtained from either Sigma, Poole, Dorset, U.K. or BDH, Poole, Dorset, U.K. except where stated in the text. All radiochemicals were from Amersham International (Amersham, Bucks., U.K.). L-[2-^{3}H]Glutamine was purified before use by chromatography on Dowex 1 (to remove glutamate); any labelled water was removed by freeze drying.

Experiments with cells

Cells were isolated from livers of fed rats as described previously

Abbreviation used: BCH, 2-amino-2-norbornanecarboxylate.

‡ To whom correspondence should be addressed.

(Salter et al., 1986a). All incubations were performed in Krebs–Henseleit buffer containing 4–5 mg dry weight of cells/ml and appropriate substrates and inhibitors; the final volume was 1.5 or 2 ml. Cell viability was assessed from ATP content measured with luciferase (Stanley and Williams, 1969).

Glutamine transport

Suspensions of liver cells were preincubated for 10 min at 37 °C, under O_2/CO_2 (19:1) and with shaking (100 oscillations/min), before addition of L-[U-^{14}C]glutamine (0.5 mM final; 0.74 kBq per assay). At appropriate times, 1 ml portions of cell suspensions were layered on to 0.5 ml of silicone oil (Dow Corning 550/dinonyl phthalate, 2:1, v/v) over 20 μl of $HClO_4$ (12%, v/v), and were centrifuged (30 s, 12000 *g*) in a Sarstedt MH2 microcentrifuge with a swing-out rotor (Salter et al., 1986a; Knowles et al., 1987). After aspiration of the top aqueous and silicone oil layers, a further 180 μl of $HClO_4$ was added and the pellets were resuspended. After recentrifugation, portions were removed and radioactivity was counted by liquid scintillation spectrometry. The intracellular and contaminating extracellular spaces were determined simultaneously by measurement of the ^3H_2O- and [U-^{14}C]sucrose-permeable spaces (Salter et al., 1986a). The intracellular space was 1.92 ± 0.23 μl/mg dry wt. (mean ± S.E.M. for three independent observations); this value is similar to that reported by Siess et al. (1982).

Glutamine catabolism

Incubations were as described above, except that the substrate was 0.5 mM L-[2-^{3}H]glutamine (1.48 kBq per assay) and reactions were terminated by addition of 0.1 vol. of $HClO_4$ (20%, v/v). Portions of 1.5 ml were removed and centrifuged (1 min, 12000 *g*) in a Sarstedt MHK-2 fixed-angle cooled Microfuge. Samples of the supernatants were removed for analysis. Labelled glutamine and water in neutralized samples were separated by ion-exchange chromatography essentially as described in Pishak and Phillips (1979). Recoveries throughout were close to 100%. Counts in the fraction containing glutamate were small (< 5–10% of those in ^3H_2O) and did not change significantly with length of incubation; label was otherwise retained only in glutamine and water. Release of ^{3}H as ^3H_2O was linear during the period of incubation.

Experiments with mitochondria

Mitochondria, isolated and characterized as described in Rickwood et al. (1987), were resuspended in 2–4 ml of homogenization medium. Preliminary experiments showed that mitochondria remained fully coupled (as judged by the maintenance of the respiratory control ratio with malate and glutamate as substrates) during the time courses subsequently employed.

Glutamine transport

The incubation volume was 0.2 ml, containing 20 μl of mitochondrial suspension and L-[2-^{3}H]glutamine (3.5 mM final; 2.22 kBq per assay); experiments with [^{14}C]glutamine gave similar results. Transport was terminated as described in Curthoys and Shapiro (1978) after quenching with 0.5 ml of ice-cold incubation buffer.

Glutamine catabolism

A portion of mitochondrial suspension (20 μl) was added to 480 μl of buffer (pre-incubated at 37 °C) containing 130 mM KCl, 20 mM Hepes, 0.2 mM $MgSO_4$, 2.5 mM potassium phosphate, 2 mM ADP (K^+ salt), 5 mM pyruvate, pH 7.5, with 3.5 mM L-[2-^{3}H]glutamine (2.22 kBq per assay) and inhibitors as appropriate. Incubations were terminated by addition of 0.05 ml of ice-cold $HClO_4$ (12%, v/v). Subsequent analysis was as described above for cell experiments. Release of ^{3}H as ^3H_2O was linear during the period of incubation.

Assays

Protein concentration was determined using the bicinchoninic acid method (Smith et al., 1985). The intracellular concentrations of glutamine and histidine were determined with a Biotronik LC6001 amino acid analyser (Biotronik, München, Germany) and a Biotronik cation-exchange resin (Taylor et al., 1989). Glutamate was determined as described by Lund (1983). L-Glutamate γ-hydrazide concentrations in extracts were determined as follows. Portions of extracts containing the hydrazide were added to liver cell incubations containing 0.5 mM L-[2-^{3}H]glutamine; the release of ^3H_2O was measured as described above. The degree of inhibition was compared with standard curves generated by addition of various concentrations of the hydrazide to parallel cell incubations. Appropriate controls corrected for glutaminase in the 'unknown' extracts.

The activities of glutaminase in freeze–thawed mitochondria and of glutamate dehydrogenase were measured as described by McGivan and Bradford (1983) and Low et al. (1990) respectively. Under these conditions (without added ammonia), glutaminase remains associated with the membrane and exhibits a K_m value for glutamine of 6 mM (McGivan et al., 1980; Kovacevic and McGivan, 1983). Aspartate aminotransferase (EC 2.6.1.1) was measured in crude extracts (Herzfeld and Greengard, 1971) as described by Cooper (1985). Glutamine transaminases K (EC 2.6.1.64) and L (EC 2.6.1.15) were assayed in crude extracts (Cooper, 1988) as described by Cooper and Meister (1985a) and Cooper and Meister (1985b) respectively.

Calculation and expression of results

All values (including those in the text) are means ± S.E.M. from at least three independent observations. The significances of differences between means were assessed by means of Student's *t* test.

The transport and metabolism of glutamine were modelled from theoretical calculations of elasticities and solution of the control matrix as described by Sauro et al. (1987) using the 'MetaModel' program, version 2.0 (Cornish-Bowden and Hofmeyr, 1991).

RESULTS AND DISCUSSION

Plan of experiments

In the context of the Metabolic Control Theory, flux control coefficients (C^J) for individual components of a pathway indicate the extent to which those components contribute to the overall control under a given set of conditions. Flux control coefficients can be measured in a number of ways (Salter et al., 1986b, 1993; Fell, 1992); in this work we chose to use selective inhibitors and compare the effect of each on their target steps and on overall flux at a concentration of glutamine similar to that found in portal blood *in vivo* (0.5 mM; Häussinger et al., 1985; Salter et al., 1986b).

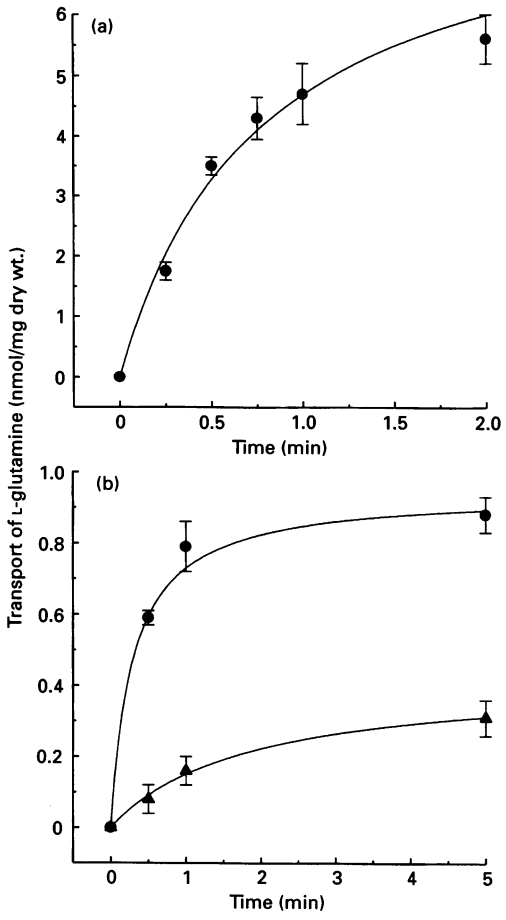


Figure 1 Time courses of the uptake of 0.5 mM L-glutamine into rat liver cells

(**a**) In Krebs–Henseleit buffer (presence of Na^+); (**b**) with NaCl and $NaHCO_3$ in the buffer replaced by choline chloride and choline bicarbonate respectively. ●, Control; ▲, plus 25 mM BCH. Transport was determined as described in the Materials and methods section. Data represent means ± S.E.M. from three independent determinations, with individual experiments performed in triplicate.

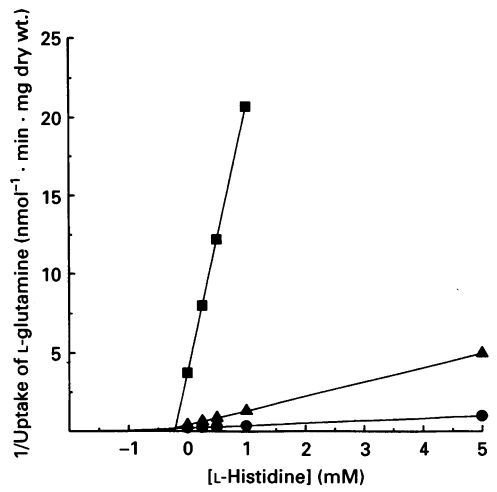


Figure 2 Inhibition by L-histidine of L-glutamine transport into rat liver cells through system N

Transport of L-glutamine into cells over a 30 s time period was measured as described in the Materials and methods section. L-Tryptophan (25 mM) was present throughout to inhibit Na^+-independent transport of L-glutamine mediated by system L. Results are means of triplicate determinations for three separate preparations. Glutamine concentrations: ■, 0.05 mM; ▲, 0.5 mM; ●, 2 mM.

Table 1 Effects of glutamine analogues on glutamine transport into isolated rat liver cells

Transport into cells was measured as described in the Materials and methods section. The concentration of glutamine was 0.5 mM and that of analogues was 5 mM. The control rate of glutamine uptake was 8.12 ± 0.5 nmol/min per mg dry wt. for three independent observations. Differences from control (Student's *t* test): $^*P < 0.05$, $^{**}P < 0.005$.

Additions (5 mM)	Transport (% of control)
None	100 ± 6
Acivicin	87 ± 7
Azaserine	78 ± 6*
6-Diazo-5-oxo-L-norleucine	94 ± 8
N-Acetylglutamine	110 ± 10
L-Glutamate t-butyl ester	108 ± 6
L-Glutamate γ-hydrazide	97 ± 11
L-Glutamate γ-hydroxamate	35 ± 4**
L-Glutamate γ-methyl ester	108 ± 8
L-Histidine	21 ± 5**

Transport of glutamine across the liver cell plasma membrane

In the presence of Na^+, the time course of glutamine transport was curvilinear, with the initial rate of transport approximating to linearity only over the first 30 s (Figure 1a). All measurements were therefore made over this 30 s period.

The time course of glutamine transport in the absence of Na^+ was similarly non-linear, and reached a plateau at about 1 min. In the presence of 25 mM 2-amino-2-norbornanecarboxylate (BCH) or 25 mM L-tryptophan, the kinetics followed a similar pattern but the rate of glutamine transport was decreased to 12.5 ± 9.5% (BCH) and approx. 15% (tryptophan; results not shown) of the control value at 0.5 min (Figure 1b).

Na^+-dependent uptake accounted for 87% of the total uptake of L-glutamine, with the remaining uptake being inhibited by BCH and L-tryptophan. L-Histidine inhibited overall glutamine transport with an IC_{50} for the overall process of 0.57 ± 0.09 mM. This is consistent with the K_i values for systems N and L of 0.21 mM (Figure 2) and 3 mM (Kilberg et al., 1983) respectively.

A number of glutamine and glutamate analogues were examined for their ability to inhibit glutamine transport into liver cells. Of these, only L-glutamate γ-hydroxamate inhibited appreciably, although azaserine was weakly active at 5 mM (22% inhibition); glutamate γ-hydrazide had no significant effect (Table 1). The IC_{50} for L-glutamate γ-hydroxamate was 0.38 ± 0.03 mM; at 5 mM, this inhibitor decreased the uptake of glutamine from 8.40 ± 0.28 to 2.94 ± 0.37 nmol/min per mg dry wt. (results not shown). These results confirm observations with sinusoidal membrane vesicles in which L-glutamate γ-hydroxamate was shown to be selective for system N (Low et al., 1991). In preliminary experiments, however, L-glutamate γ-hydroxamate proved also to be an inhibitor of glutaminase (see Table 2); because of this it was not used further in these studies.

Transport of glutamine across the mitochondrial membrane

The concentration of glutamine used routinely in these experiments was 3.5 mM, similar to values reported for liver cell cytosol (Joseph et al., 1978; Fafournoux et al., 1983). At this

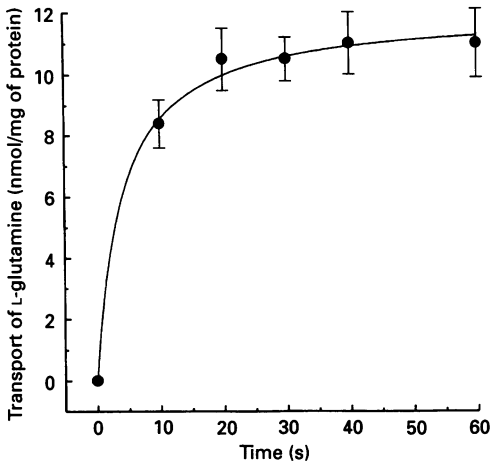


Figure 3 Time course of the uptake of 3.5 mM L-glutamine into rat liver mitochondria

Transport was determined as described in the Materials and methods section. Data represent means ± S.E.M. from three independent determinations, with individual experiments performed in triplicate.

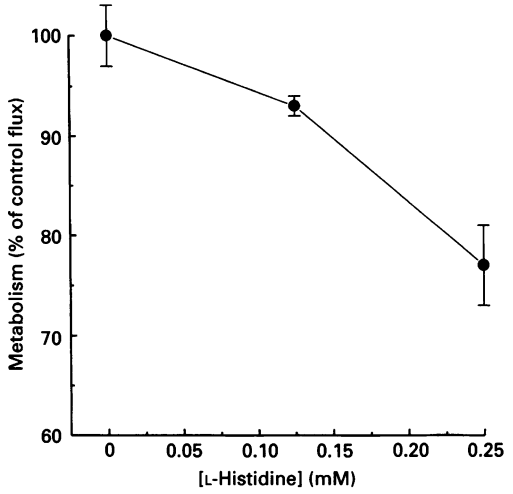


Figure 4 Inhibition of the metabolism of L-glutamine in rat liver cells by L-histidine

Metabolism of 0.5 mM L-glutamine was measured over a 10 min period as described in the Materials and methods section. Note that the ordinate begins at 60%. Results are means ± S.E.M. for three separate preparations. The control rate was 1.12 ± 0.11 nmol/min per mg dry wt.

concentration, glutamine was rapidly taken up into mitochondria, although again in a non-linear fashion (Figure 3). Preliminary studies showed uptake to be approximately linear with time at 5 and 10 s; a period of 10 s was chosen for subsequent experiments. No inhibitors used in this study (at concentrations up to 10 mM) had any significant inhibitory effect on this process (results not shown). The calculated rate of uptake of glutamine into mitochondria at 3.5 mM (52.5 ± 6.9 nmol/min per mg of mitochondrial protein) was at least 4-fold higher than the rate of mitochondrial metabolism of glutamine at this concentration, suggesting that transport is unlikely to exert significant control on the catabolism of glutamine.

Glutamine metabolism in cells

Metabolism of 0.5 mM glutamine by whole cells was linear for at least 20 min; in the presence of the inhibitors used in this study the time course remained linear over at least 10 min (results not shown). The time period adopted for further experiments was 10 min.

Metabolic Control Analysis requires that the concentrations of agents added to perturb flux should be sufficiently low that the effect produced should be substantially linear with concentration. The effect of 0.125 mM L-histidine (well below the K_m for system N) on glutamine metabolism (measured as the release of ^3H_2O from [2-^{3}H]glutamine) is shown in Figure 4; histidine inhibited flux by 8%.

We used tryptophan as a selective inhibitor of Na^+-independent glutamine transport, and measured the effect of this amino acid (again at a concentration well below the K_m) on glutamine metabolism. Apparently paradoxically, tryptophan enhanced the rate of glutamine metabolism (Figure 5).

Glutamate γ-hydrazide proved to be a useful and selective inhibitor of the release of ^3H_2O from [^{3}H]glutamine. It was inactive, at 10 mM, against membrane transport (by either system N or L; see above), glutamate dehydrogenase, aspartate aminotransferase and glutamine transaminase L; glutamine transaminase K was activated by approx. 35% by glutamate γ-hydrazide at 10 mM but was unaffected by lower concentrations. Inhibition of glutamine catabolism was concentration-depen-

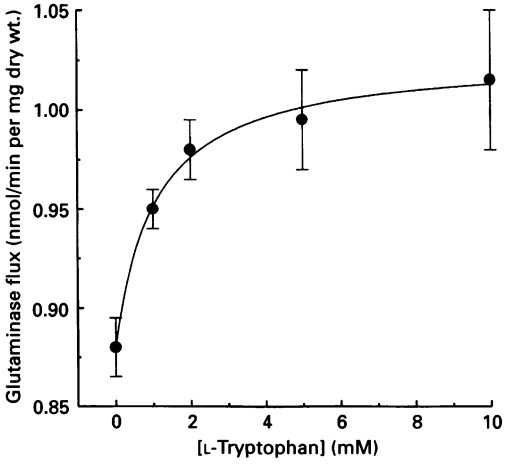


Figure 5 Stimulation of the metabolism of L-glutamine in rat liver cells by L-tryptophan

Metabolism of 0.5 mM L-glutamine was measured over a 10 min period as described in the Materials and methods section. Note that the ordinate begins at 0.85 nmol/min per mg dry wt. Results are means ± S.E.M. for three separate preparations.

dent, with an IC_{50} of 13.7 ± 3.5 μM; maximal inhibition (77%) occurred at 0.1 mM (Figure 6). Why inhibition of glutaminase by this compound is less than total at higher concentrations remains unclear. One possibility is that the residual activity is associated with transamination of glutamine (which would also lead to loss of ^{3}H from the substrate). Such activities are, however, cytosolic and could not explain the incomplete inhibition in mitochondria. Even if some transaminase activity is mitochondrial, as suggested by Kovacevic and McGivan (1983), it is unlikely that this could contribute to any measurable extent in extracts not supplemented with oxo-acid substrates.

Metabolism of glutamine in mitochondria

In a soluble extract obtained by freeze–thawing of rat liver mitochondria (McGivan et al., 1980), glutaminase was

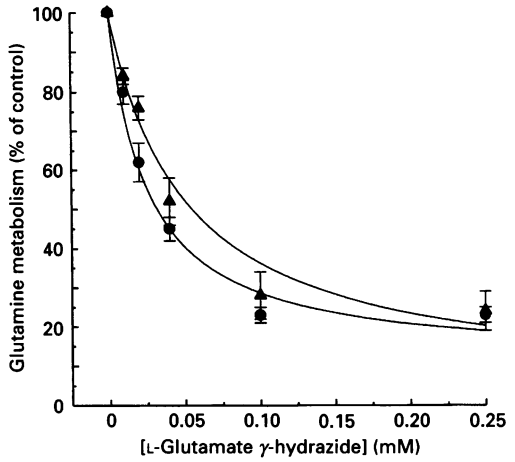


Figure 6 Effect of glutamate γ-hydrazide on L-glutamine metabolism

Rates of metabolism were determined as described in the Materials and methods section. Data represent means ± S.E.M. from three independent determinations, with individual experiments performed in triplicate. ●, Intact liver cells with 0.5 mM glutamine; ▲, isolated mitochondria with 3.5 mM glutamine. The rates of metabolism in the absence of glutamate γ-hydrazide were 1.18 ± 0.07 nmol/min per mg dry wt. of cells and 13.60 ± 1.42 nmol/min per mg of mitochondrial protein.

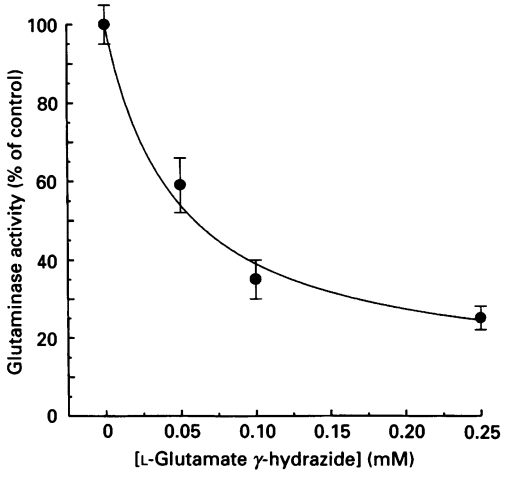


Figure 7 Effect of glutamate γ-hydrazide on glutaminase activity in suspensions of disrupted rat liver mitochondria

Glutaminase activity was measured as described in the Materials and methods section. Data represent means ± S.E.M. from three independent determinations, with individual experiments performed in triplicate. Enzyme activity in the absence of glutamate γ-hydrazide was 22.70 ± 2.04 nmol/min per mg of protein.

inhibited by glutamate γ-hydrazide to an extent similar (IC_{50} 37.8 ± 7.7 μM) to that found for glutamine catabolism in isolated mitochondria (Figure 7).

Metabolism of 3.5 mM glutamine by rat liver mitochondria was linear for at least 10 min. Glutamate γ-hydrazide (0.25 mM) decreased flux from 11.5 ± 0.8 to 3.8 ± 0.4 nmol/min per mg of mitochondrial protein. Inhibition was concentration-dependent (Figure 6), with an IC_{50} of 22.6 ± 3.8 μM; inhibition was maximal (approx. 75%) at concentrations above 0.1 mM. Mitochondrial metabolism of 3.5 mM glutamine was unaffected by L-histidine (up to 5 mM) (Table 2).

Figure 8 shows a comparison of the effects of low concentrations of glutamate γ-hydrazide on glutamine metabolism in

Table 2 Effect of glutamine analogues on glutamine metabolism in liver cells and mitochondria

The concentrations of glutamine in incubations of cells and mitochondria were respectively 0.5 mM and 3.5 mM. Analogues were added at 5 mM. Procedures were as described in the Materials and methods section. Results are means ± S.E.M. from three independent observations; n.d., not determined. The control values were 1.18 ± 0.07 nmol/min per mg dry wt. and 13.60 ± 1.42 nmol/min per mg of protein for whole cells and mitochondria respectively. Differences from control (Student's t test): $^*P < 0.005$.

	Glutamine metabolism (% of control)	
Additions (5 mM)	Whole cells	Mitochondria
None	100 ± 7	100 ± 10
L-Histidine	27 ± 6*	98 ± 4
L-Glutamate γ-hydroxamate	33 ± 9*	45 ± 6*
L-Glutamate γ-hydrazide	20 ± 7*	18 ± 6*
N-Acetylglutamine	97 ± 9	n.d.
L-Glutamate t-butyl ester	92 ± 4	n.d.
L-Glutamate γ-methyl ester	86 ± 9	n.d.

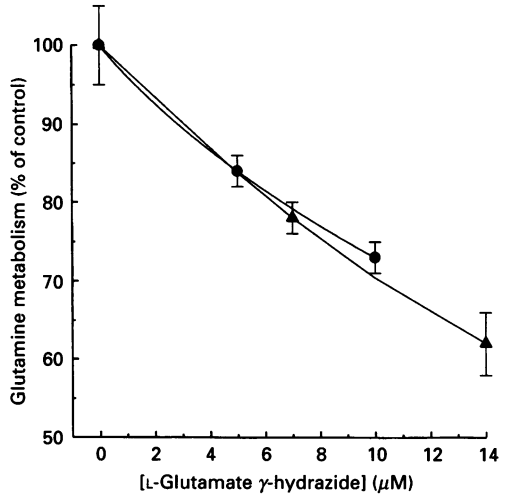


Figure 8 Inhibition of glutamine metabolism in whole rat liver cells and in isolated mitochondria

Metabolism of glutamine was measured in cells (▲) and isolated mitochondria (●) as described in the Materials and methods section. Catabolic product analysis was as described in the Materials and methods section. The concentrations of L-glutamate γ-hydrazide in cell experiments have been corrected (multiplied by 1.4) to allow for the gradient across the plasma membrane. The concentration of L-glutamine in the medium was 0.5 mM (cells) or 3.5 mM (mitochondria; Soboll et al., 1988). Note that the ordinate begins at 50%. Results are means ± S.E.M. for three separate preparations. The uninhibited rates were: cells, 1.18 ± 0.07 nmol/min per mg dry wt. of cells; mitochondria, 13.60 ± 1.42 nmol/min per mg of mitochondrial protein.

whole cells and in isolated mitochondria. Independent measurements showed that the inhibitor was concentrated by cells and that the ratio of the concentrations in the cytoplasmic (0.35 ± 0.03 mM) and extracellular (0.25 ± 0.02 mM) compartments was 1.4; the kinetics of inhibition in both cell-free extracts and isolated organelles indicated that there was no concentration gradient of the inhibitor between cytoplasm and mitochondria. When this factor was taken into account, as in Figure 8, the gradients of the two slopes were very similar (2.5%/μM and 2.6%/μM in mitochondria and cells respectively).

Calculations of flux control coefficients

The concentration of L-glutamine in cells incubated with an extracellular concentration of 0.5 mM was found to be equivalent to 3.58 mM, assuming that there was a single pool. It is known, however, that glutamine is more concentrated in the mitochondrial matrix. When due allowance was made for this, using values for the cytosolic and mitochondrial spaces of 1.57 and 0.19 ml of H_2O/g dry wt. respectively (Siess et al., 1982) and of 2.9 for the mitochondria/cytoplasm concentration ratio (Soboll et al., 1988), we calculated that the cytoplasmic concentration ($[Gln]_i$) was 2.96 mM.

The maximum rate of Na^+-independent (system L) transport of glutamine is 8.1 nmol/min per mg dry wt. (calculated from data in Figure 1b); the K_m of system L for glutamine is 4 mM (Fafournoux et al., 1983). The net rates (v) of glutamine uptake by system L in cell incubations with and without L-tryptophan (which is inactive against system N; Kilberg et al., 1980) were obtained from equation (1), where $[Gln]_o = 0.5$ mM, $[Gln]_i = 2.96$ mM, $[Trp]_o = 0.25$ mM, $[Trp]_i = 0.20$ mM (calculated from data in Salter et al., 1986b) and the K_i for tryptophan was assumed to be the same as the K_m (1.65 mM; Salter et al., 1986a).

$$v = \frac{V_{max.} \times [Gln]_o}{[Gln]_o + K_m^{Gln}\left(\frac{[Trp]_o}{K_i^{Trp}} + 1\right)} - \frac{V_{max.} \times [Gln]_i}{[Gln]_i + K_m^{Gln}\left(\frac{[Trp]_i}{K_i^{Trp}} + 1\right)} \quad (1)$$

The control (i.e. uninhibited) net rate was −2.54 and that with tryptophan was −2.43 nmol/min per mg dry wt. From the data in Figure 5 we calculated that flux was increased by 2.0% by 0.25 mM L-tryptophan. The flux control coefficient of system L for glutamine metabolism (C^J_L) was calculated as follows:

$$C^J_L = \frac{\%\text{ inhibition of flux}}{\%\text{ inhibition of net uptake}} = \frac{-2}{1-(-2.43/-2.54)\times 100} = -0.46 \quad (2)$$

Because system N is Na^+-dependent, differences in intracellular and extracellular $[Na^+]$ have to be taken into account. Substitution in equation (3)

$$V_{max.} = \left(\frac{[Na^+]_o + K_m^{Na^+}}{[Na^+]_o}\right)\left(\frac{[Gln]_o + K_m^{Gln}}{[Gln]_o}\right) \times 6.92 \quad (3)$$

gives a value for the maximal rate of inward transport of glutamine at saturating $[Na^+]$ ($V_{max.}$) of 24.77 nmol/min per mg dry wt., where $[Na^+]_o = 143.5$ mM (Cantarow and Trumper, 1975), $K_m^{Na^+} = 17$ mM (Kilberg et al., 1980), the external glutamine concentration ($[Gln]_o$) = 0.5 mM and $K_m^{Gln} = 1.1$ mM (Kilberg et al., 1980); 6.92 nmol/min per mg dry wt. is the inward rate of Na^+-dependent uptake of 0.5 mM L-glutamine in this study.

With the calculated values for $V_{max.}$ (24.77 nmol/min per mg dry wt.) and $[Gln]_i$ (2.96 mM), the known intracellular Na^+ concentration ($[Na^+]_i$) of 34.2 mM (Berry et al., 1991), and with the assumption that $V_{max.}$ and K_m^{Gln} are the same for outward as for inward transport, we could use an equation formally similar to eqn. (3) to obtain a value for the actual rate of efflux of 12.06 nmol/min per mg dry wt. System N is, however, electrogenic, glutamine entry being stimulated by the charge gradient (M. J. Rennie, unpublished work). The value for $V_{max.}$ in the outward direction will therefore be reduced by a factor determined by the Nernst equation. With a membrane potential of −33.5 mV (within the range given by Moule and McGivan, 1990), this factor is 3.51. The net inward rate through system N is therefore $6.92-(12.06/3.51) = 3.48$ nmol/min per mg dry wt.

The rate of unidirectional inward transport (i.e. the 'forward' rate, v_f) of glutamine in the presence of 0.125 mM L-histidine was obtained from eqn. (4), with the K_i as determined in this study (0.21 mM; Figure 2).

$$v_f = 6.92\left(\frac{[Gln]_o + K_m^{Gln}}{[Gln]_o + K_m^{Gln}\left(\frac{[His]_o}{K_i^{His}} + 1\right)}\right) \quad (4)$$

This gave a value of 4.91 nmol/min per mg dry wt. The rate of efflux of glutamine in the presence of the same concentration of extracellular histidine was obtained by substitution in a similar equation in which $[His]_o$ was replaced by $[His]_i$, the intracellular concentration of histidine (0.41 ± 0.05 mM; mean ± S.D. for three determinations with a single cell preparation). The efflux rate was calculated to be 2.25 nmol/min per mg dry wt. The net inward rate with histidine present was thus $4.91-2.25 = 2.66$ nmol/min per mg dry wt.

At the low concentrations of histidine used in the experiments in Figure 4, the inhibitor effect of histidine on transport was essentially linear with concentration. These concentrations of histidine will, however, also have an inhibitory effect on transport of glutamine through system L (Fafournoux et al., 1983); this can be appreciable because of the ratio of the concentrations of the amino acid across the plasma membrane. A calculation similar to that above with a K_m value for glutamine of 4 mM (Fafournoux et al., 1983) and a K_i for histidine of 3 mM (Kilberg et al., 1983) shows that the net rate of glutamine transport by system L is inhibited by 8.5%. An equal decrease (calculation not shown) is produced by the system L-selective inhibitor, L-tryptophan, at a concentration (0.5 mM) that produces an increase of 4% in the flux (loss of ^3H from labelled glutamine; see Figure 5). Inhibition of system N gave an apparent 8% inhibition of flux (see above); this must be corrected for the impact of histidine on system L (8% minus −4%), giving 12% as the actual decrease of the flux through glutaminase due to inhibition of system N. The flux control coefficient for the N system for glutamine uptake (C^J_N) was therefore calculated as follows (cf. eqn. 2):

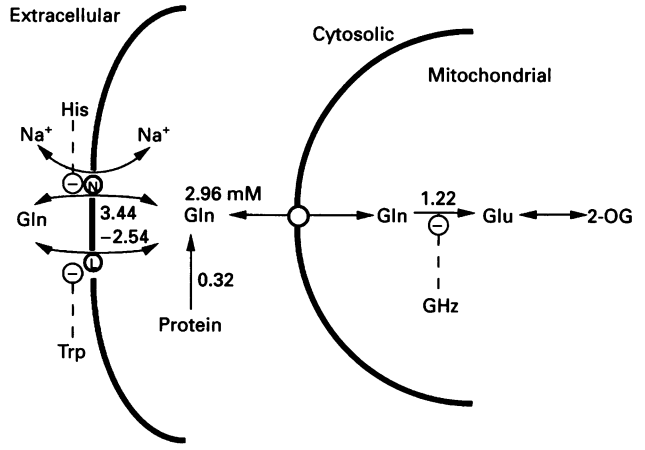
$$C^J_N = \frac{12}{1-(2.66/3.48)\times 100} = 0.51 \quad (5)$$

For any given change in the concentration of an inhibitor, the flux control coefficient of a particular enzymic reaction is obtained by dividing $\partial J/J$ by $\partial v/v$ (where J is the flux and v is the net rate catalysed by that enzyme and measured under conditions where the enzyme is 'isolated' from other flux-controlling reactions in the pathway). For glutamine metabolism (see above, Figure 8), the flux control coefficient of glutaminase (C^J_{Glnase}) is, therefore, equal to $2.5/2.6 = 0.96$.

In addition to these experimental studies, the pathway was modelled using similar assumptions for systems N and L, and a value of 6 mM for the K_m of glutaminase for its substrate (McGivan et al., 1980, 1984).

General discussion

Our experiments were designed to investigate the control structure of glutamine metabolism under a defined set of conditions.



Scheme 1 Pathway of glutamine metabolism in liver: experimentally derived fluxes in incubations of rat liver cells with 0.5 mM L-glutamine

'N' and 'L' represent systems N and L for amino acid transport across the plasma membrane. Glutaminase catalyses the conversion of glutamine to glutamate; ^3H from [2-^3H]glutamine is lost as ^3H_2O during the conversion of glutamate to 2-oxoglutarate (2-OG) by transamination or action of glutamate dehydrogenase. GHz, glutamate γ-hydrazide. Numbers are the net rates of specific reactions, expressed as nmol/min per mg dry wt. of cells.

These results show that: (1) histidine is a selective inhibitor of transport system N; (2) tryptophan is a specific inhibitor of transport system L; and (3) glutamate γ-hydrazide selectively inhibits glutaminase. Because none of these compounds has, at low concentrations, any appreciable action on other processes in this pathway, their identification permits the determination of those steps that are important in the control of glutamine metabolism.

In using one fixed concentration of glutamine, we have avoided possible effects of volume changes (Häussinger et al., 1990; Häussinger and Lang, 1991), and have also taken no account of metabolic differences between cells from different parts of the liver (Gebhardt, 1992). Perivenous cells contain glutamine synthetase (Gebhardt and Mecke, 1983) and a relatively higher concentration of system L than do periportal cells (Burger et al., 1989), but the fact that the overall percentage of perivenous cells is small suggests that errors due to 'averaging' are not crucial.

Our calculations also involve assumptions and acceptance of literature values for some parameters. Specifically, we have assumed that, firstly, the rates of forward and backward transport are independent (as in eqn. 4), and, secondly, the kinetic constants of the transporter systems are the same on both sides of the plasma membrane.

Cellular protein contains approx. 10% glutamine (Orr and Watt, 1957), and liver cells incubated in simple media exhibit a net proteolytic rate of 4%/h (Seglen, 1975). From these data we calculated a net rate of production of glutamine from protein of 0.32 nmol/min per mg dry wt. of cells. When the dilution of the cytoplasmic glutamine pool was taken into account, the true flux through glutaminase was 1.22 (as opposed to an uncorrected value of 1.18) nmol/min per mg dry wt. of cells; this value is incorporated into Scheme 1, which shows the balance between the various fluxes under the conditions of this study. The computer model gave steady-state values for the various parameters of the pathway that are very close to those determined and calculated as described in this paper. Table 3 shows the flux control coefficients obtained by both methods.

According to the Summation Theorem (Kacser and Burns, 1973; Fell, 1992), the sum of all flux control coefficients in a pathway is 1.0. Under our conditions the sum of the flux control

Table 3 Flux control coefficients for glutamine metabolism

Values were obtained as discussed in the text; n.d., not determined.

	Flux control coefficient	
Component	Experimental	Theoretical (MetaModel)
System N	0.51	0.41
System L	−0.46	−0.30
Glutaminase	0.96	0.85
Proteolysis	n.d.	0.04

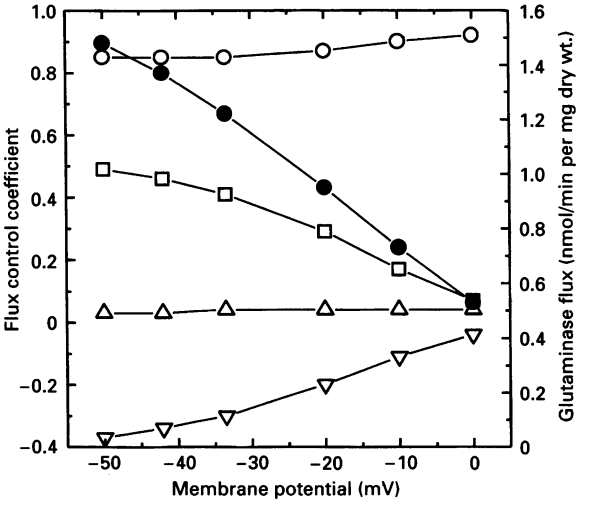


Figure 9 Modelling of the effect of membrane potential on flux control coefficients for glutamine metabolism and flux through glutaminase

Modelling was performed with 'MetaModel', version 2.0, as described in the text. Flux control coefficients (C^J): ○, glutaminase; □, system N; ▽, system L; △, proteolysis. Flux through glutaminase is shown by ●.

coefficients (see Table 3) for system N, system L and glutaminase is 1.01. It is plausible in theory that transport across the mitochondrial inner membrane plays a part in the control of the pathway. In preliminary experiments, however, we found that the unidirectional rate of this step was fast (7.7 ± 0.7 nmol/min per mg dry wt.) in comparison with that catalysed by glutaminase, so that a significant role is unlikely. From the Summation Theorem the sum of the control coefficients of glutamate dehydrogenase, glutamine aminotransferase, protein synthesis and degradation and other reactions in which glutamine is a participant must be small.

While the numerical values we report for the control coefficients are consistent both internally and with theoretical prediction (see Table 3), they are not precise, and may vary according to the correctness or otherwise of the mechanisms assumed and parameter values used. They do, however, clearly indicate how glutamine metabolism may be controlled in liver and help to reconcile the competing views expressed in earlier literature, in that a significant degree of control is exerted both by glutaminase (see Verhoeven et al., 1985) and by uptake across the plasma membrane (see Häussinger et al., 1985).

Glutamine metabolism is known to be sensitive to certain hormones (Joseph and McGivan, 1978) that also affect the membrane potential (Moule and McGivan, 1990). We have

therefore modelled the effect of varying the membrane potential acting on system N on both the rate of glutamine metabolism and the distribution of control (Figure 9). The predicted effect of changes in membrane potential on flux through glutaminase is consistent with the known effect of glucagon on these parameters (Joseph and McGivan, 1978). As can be seen in Figure 9, the positive and negative control values for systems N and L respectively decline markedly and reciprocally as the cell membrane is depolarized. This is a consequence of a progressive decline in the rate at which glutamine 'cycles' between medium and cytoplasm through the two transport systems (in through system N, out through system L; Fell and Sauro, 1985), and of the associated decrease in the intracellular steady-state glutamine concentration.

Where there are concentrative and non-concentrative amino acid uptake systems for transport of a given substrate, these systems in principle will, under most circumstances, have flux control coefficients of opposite sign; this arises from the internal accumulation of the shared substrate and therefore net efflux through the non-concentrative transporter. If both transporter systems exert appreciable control, such a combination permits fine regulation of the intracellular concentrations of amino acids, with retention of sensitivity to fluctuations in the external medium. This is of particular significance for glutamine, which is at the 'cross-roads' of several pathways in most tissues.

S.Y.L. was the holder of a Science and Engineering Research Council CASE studentship supported by Wellcome Research Laboratories.

REFERENCES

Abumrad, N. N., Williams, P., Frexes-Steed, M., Geer, R., Flakoll, P., Cersosmio, E., Brown, L. L., Melki, I., Bulus, N., Hourani, H., Hubbard, M. and Ghishan, F. (1989) Diabetes Metab. Rev. **5**, 213–226

Berry, M. N., Edwards, A. M. and Barritt, G. J. (1991) Isolated Hepatocytes: Preparation, Properties and Applications in Laboratory Techniques in Biochemistry and Molecular Biology (Burdon, R. H. and van Knippenberg, P. H., eds.), Elsevier, Amsterdam, New York and Oxford

Burger, H. J., Gebhardt, R., Mayer, C. and Mecke, D. (1989) Hepatology **9**, 22–28

Cantarow, A. and Trumper, M. (1975) in Clinical Biochemistry, 7th edn. (Latner, A. L., ed.), pp. 339–367, W. B. Saunders Co., Philadelphia

Christensen, H. N. (1990) Physiol. Rev. **70**, 43–77

Cooper, A. J. L. (1985) Methods Enzymol. **113**, 66–69

Cooper, A. J. L. (1988) in Glutamine and Glutamate in Mammals (Kvamme, E., ed.), vol. 1, pp. 33–52, CRC Press, Boca Raton, FL

Cooper, A. J. L. and Meister, A. (1985a) Methods Enzymol. **113**, 338–343

Cooper, A. J. L. and Meister, A. (1985b) Methods Enzymol. **113**, 344–349

Cornish-Bowden, A. and Hofmeyr, J.-H. S. (1991) Comp. Appl. Biosci. **7**, 89–93

Curthoys, N. P. and Shapiro, R. A. (1978) J. Biol. Chem. **253**, 63–68

Fafournoux, P., Demigné, C., Rémésy, C. and Le Cam, A. (1983) Biochem. J. **216**, 401–408

Fell, D. A. (1992) Biochem. J. **286**, 313–330

Fell, D. A. and Sauro, H. M. (1985) Eur. J. Biochem. **148**, 555–561

Gebhardt, R. (1992) Pharmacol. Ther. **53**, 275–354

Gebhardt, R. and Mecke, D. (1983) EMBO J. **2**, 567–570

Häussinger, D. (1990) Biochem. J. **267**, 281–290

Häussinger, D. and Lang, F. (1991) Biochim. Biophys. Acta **1071**, 331–350

Häussinger, D., Soboll, S., Meijer, A. J., Gerok, W., Tager, J. M. and Sies, H. (1985) Eur. J. Biochem. **152**, 597–603

Häussinger, D., Lang, F., Bauers, K. and Gerok, W. (1990) Eur. J. Biochem. **193**, 891–898

Herzfeld, A. and Greengard, O. (1971) Biochim. Biophys. Acta **237**, 88–98

Joseph, S. K. and McGivan, J. D. (1978) Biochim. Biophys. Acta **543**, 16–28

Joseph, S. K., Bradford, N. M. and McGivan, J. D. (1978) Biochem. J. **176**, 827–836

Kacser, H. (1987) Biochem. Plants **11**, 39–67

Kacser, H. and Burns, J. A. (1973) Symp. Soc. Exp. Biol. **32**, 65–104

Kilberg, M. S., Handlogten, M. E. and Christensen, H. N. (1980) J. Biol. Chem. **255**, 4011–4019

Kilberg, M. S., Weissbach, L. and Barber, E. F. (1983) in Isolation, Characterization and Use of Hepatocytes (Harris, R. A. and Cornell, N. W., eds.), pp. 227–232, Elsevier, Amsterdam, New York and Oxford

Knowles, R. G., McCabe, J. P., Beevers, S. J. and Pogson, C. I. (1987) Biochem. J. **242**, 721–728

Kovacevic, Z. and McGivan, J. D. (1983) Physiol. Rev. **63**, 547–605

Low, S. Y., Salter, M., Knowles, R. G., Rennie, M. J. and Pogson, C. I. (1990) Biochem. Soc. Trans. **18**, 1239–1240

Low, S. Y., Taylor, P. M., Ahmed, A., Pogson, C. I. and Rennie, M. J. (1991) Biochem. J. **278**, 105–111

Lund, P. (1983) in Methods in Enzymatic Analysis (Bergmeyer, H.-U., ed.), vol. 2, pp. 357–363, Verlag Chemie, Weinheim

McGivan, J. D. and Bradford, N. M. (1983) Biochim. Biophys. Acta **759**, 296–302

McGivan, J. D., Lacey, J. H. and Joseph, S. K. (1980) Biochem. J. **192**, 537–542

McGivan, J. D., Bradford, N. M., Verhoeven, A. J. and Meijer, A. J. (1984) in Glutamine Metabolism in Mammalian Tissues (Häussinger, D. and Sies, H., eds.), pp. 122–137, Springer Verlag, Berlin

Moule, S. K. and McGivan, J. D. (1990) Biochim. Biophys. Acta **1031**, 383–397

Ochs, R. S. (1986) Trends Biochem. Sci. **11**, 235–236

Orr, M. L. and Watt, B. K. (1957) The Amino Acid Composition of Foods. U.S. Dept. Agr. Home Econ. Res. Rep.

Pishak, M. R. and Phillips, A. T. (1979) Anal. Biochem. **94**, 82–88

Pogson, C. I., Low, S. Y., Knowles, R. G., Salter, M. and Rennie, M. J. (1991) Alfred Benzon Symp. **30**, 262–272

Rennie, M. J., MacLennan, P., Hundal, H. S., Weryk, B., Smith, K., Taylor, P. M., Egan, C. J. and Watt, P. W. (1989) Metab. Clin. Exp. **38**, 47–51

Rickwood, D., Wilson, M. T. and Darley-Usmar, V. M. (1987) in Mitochondria: A Practical Approach (Darley-Usmar, V. M., Rickwood, D. and Wilson, M. T., eds.), pp. 1–16, IRL Press, Oxford and Washington, DC

Salter, M., Knowles, R. G. and Pogson, C. I. (1986a) Biochem. J. **233**, 499–506

Salter, M., Knowles, R. G. and Pogson, C. I. (1986b) Biochem. J. **234**, 635–647

Salter, M., Knowles, R. G. and Pogson, C. I. (1993) Essays Biochem., in the press

Sauro, H. M., Small, J. R. and Fell, D. A. (1987) Eur. J. Biochem. **165**, 215–221

Seglen, P. O. (1975) Biochem. Biophys. Res. Commun. **66**, 44–52

Siess, E. A., Brocks, D. G. and Wieland, O. H. (1982) in Metabolic Compartmentation (Sies, H., ed.), pp. 234–257, Academic Press, London and New York

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, P. K., Gartner, F. T., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. (1985) Anal. Biochem. **150**, 76–85

Soboll, S., Lenzen, C. and Häussinger, D. (1988) in Advances in Ammonia Metabolism and Hepatic Encephalopathy (Soeters, P. B., Wilson, J. H. P., Meijer, A. J. and Holm, E., eds.), pp. 37–44, Elsevier, Amsterdam

Soboll, S., Lenzen, C., Rettich, D., Gründel, S. and Ziegler, B. (1991) Eur. J. Biochem. **197**, 113–117

Stanley, P. E. and Williams, S. G. (1969) Anal. Biochem. **29**, 381–392

Taylor, P. M., Egan, C. J. and Rennie, M. J. (1989) Am. J. Physiol. **256**, E550–E558

Verhoeven, A. J., Estrela, J. M. and Meijer, A. J. (1985) Biochem. J. **230**, 457–463

Received 11 February 1993/11 June 1993; accepted 23 June 1993