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α-Adrenergic stimulation of glutamine metabolism in isolated rat hepatocytes

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The mechanisms by means of which phenylephrine stimulates glutamine metabolism were studied in isolated rat hepatocytes. In the first 2 min after phenylephrine addition there was a rapid fall in the concentrations of intracellular 2-oxoglutarate and glutamate, presumably owing to activation of 2-oxoglutarate dehydrogenase. This was followed 2–3 min later by activation of glutaminase and by increases in glutamate and 2-oxoglutarate. Activation of glutaminase by phenylephrine was due to direct stimulation of the enzyme rather than to reversal of inhibition by the decrease in 2-oxoglutarate and glutamate. The stimulation of glutaminase by phenylephrine is partly due to an increase in the affinity of the enzyme for ammonia, its essential activator. It is concluded that stimulation of steady-state flux through the pathway from glutamine to glucose and urea can only be achieved by stimulation of glutaminase, the first enzyme in the pathway.

The metabolism of glutamine in isolated hepatocytes is stimulated by various hormones, including glucagon (Joseph & McGivan, 1978), α-adrenergic agonists, vasopressin and angiotensin (Joseph *et al.*, 1981; Corvera & García-Saínz, 1983). These effects have been attributed to activation of mitochondrial glutaminase (EC 3.5.1.2), an enzyme that is considered to play an important role in controlling the rate of glutamine metabolism in liver. However, this view has been questioned by Ochs & Lardy (1983) and by Ochs (1984). These authors conclude that α-adrenergic agonists bring about an acceleration of the conversion of glutamate into aspartate rather than an activation of glutaminase; they consider the conversion of glutamate into aspartate to be the rate-limiting step in glutamine metabolism.

An important factor in the control of glutamine metabolism is probably the intramitochondrial concentration of ammonia, since at physiological pH values the activity of glutaminase is very sensitive to changes in the concentration of this compound, both in intact rat liver mitochondria (Verhoeven *et al.*, 1983) and in partially purified preparations (Patel & McGivan, 1984). Häussinger & Sies (1984) reported additive effects of phenylephrine and ammonia in the perfused rat liver; this result suggests that phenylephrine exerts its effect on glutaminase independently of the ammonia concentration.

In the present work, the effects of α-adrenergic agonists on the metabolism of glutamine in isolated hepatocytes were investigated in more detail, with emphasis on the question of the mechanisms by means of which flux through the first enzyme, glutaminase, is accelerated after hormone addition. The results demonstrate that α-adrenergic agonists stimulate glutamine metabolism by an effect on the activity of glutaminase itself, independently of other events occurring after hormone addition.

Experimental

Isolation and incubation of hepatocytes

Hepatocytes were isolated from male Wistar rats (200–250 g; starved for 20–24 h) by the method of Berry & Friend (1969), modified as described by Groen *et al.* (1982).

When incubated in flasks, hepatocytes (about 10 mg dry wt./ml) were suspended at 37°C in Krebs–Henseleit bicarbonate medium (pH 7.4) containing 1.3 mM-$CaCl_2$ and the additions indicated in the legends to the Figures. The gas atmosphere was O_2/CO_2 (19:1).

Samples of the cell suspension were centrifuged through a layer of silicone oil (AR 200/AR 20; 3:2, v/v) into $HClO_4$ (14%, w/v), and intracellular

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metabolites were measured in the neutralized protein-free extracts of the cells. Glucose and urea were measured in neutralized $HClO_4$ extracts of the supernatant obtained after centrifugation of the cells for 30s at 10000g.

When incubated in a perifusion system, hepatocytes (about 150mg dry wt./12ml) were suspended as described by Groen *et al.* (1982) in the same medium as described for incubations in flasks. After a steady state had been reached (within 45–60min), infusion of hormone was started. In order to establish the final concentration immediately, the appropriate amount of hormone was injected directly into the perifusion chamber at the start of infusion. For determination of metabolites, samples of the cell suspension were taken from the chamber without interrupting the flow. For this purpose a syringe was introduced into the chamber through a rubber septum, and a sample (0.8ml) was taken at a rate equal to the flow through the system (5ml/min). Samples were quenched with $HClO_4$ (final concn. 3.5%, w/v) and metabolites were measured in the protein-free extracts.

Isolation and incubation of mitochondria

Mitochondria were isolated from the livers of male Wistar rats (200–250g) as described in detail by Verhoeven *et al.* (1985) and incubated (3–4mg of protein/ml) at 30°C in a medium containing 100mM-KCl, 25mM-Mops, 10mM-succinate, 2μg of rotenone/ml, 10mM-potassium phosphate, 1mM-EGTA, 0.5mM-NH_4Cl, 15mM-glutamine and the additions indicated in the legends to the Figures. The pH of the medium was adjusted to 7.0 with Tris base. When the concentration of glutamate was varied in the medium, glutaminase activity was estimated from the production of ammonia. When 2-oxoglutarate was added to the medium, glutaminase activity was calculated from the production of glutamate, corrected for glutamate produced by glutamate dehydrogenase (EC 1.4.1.3). Under these conditions glutaminase activity is equal to the sum of the changes in the glutamate concentration and the ammonia concentration, divided by 2. Samples for determination of metabolites were treated as described above.

In the experiment of Table 2 a mitochondrial fraction was isolated from intact hepatocytes as described previously (Hensgens *et al.*, 1980), by using 250mM-mannitol, 2mM-Mops and 1mM-EGTA (pH7.0) as isolation medium and omitting the homogenization step. Subsequently the mitochondrial fraction was incubated as described above and glutaminase activity was estimated from the production of glutamate.

Metabolite assays

Urea was determined as described by Bernt & Bergmeyer (1970*b*) with urease (EC 3.5.1.5) and glutamate dehydrogenase. Glucose was measured spectrophotometrically as described by Bergmeyer *et al.* (1970). 2-Oxoglutarate was measured fluorimetrically as described by Wanders *et al.* (1983). Glutamate was measured either spectrophotometrically or fluorimetrically by the method of Bernt & Bergmeyer (1970*a*) with glutamate dehydrogenase and NAD^+. In samples obtained from mitochondria that had been incubated in the presence of 2-oxoglutarate, glutamate was determined fluorimetrically so that small amounts of sample could be used in order to decrease the concentration of inhibitory 2-oxoglutarate in the assay; in this way incomplete conversion of glutamate into 2-oxoglutarate could be avoided. Glutamate:oxaloacetate transaminase (EC 2.6.1.1) used for the determination of 2-oxoglutarate was dialysed against 10mM-potassium phosphate to remove 2-oxoglutarate present in the preparation.

Materials

Noradrenaline and phenylephrine were obtained from Sigma (St. Louis, MO, U.S.A.). For each experiment a freshly prepared solution of the hormone dissolved in Krebs–Henseleit bicarbonate medium was used. For perifusion experiments phenylephrine instead of noradrenaline was used, since solutions of noradrenaline were unstable when kept in the infusion syringe. Nucleotides and enzymes were obtained from Boehringer (Mannheim, Germany). All other reagents were of analytical grade.

Results

In our initial experiments we used the protocol of Ochs & Lardy (1983) to study the effect of noradrenaline on the metabolism of glutamine. The results are depicted in Table 1. Noradrenaline stimulated glucose and urea production from glutamine, in agreement with literature data (Joseph *et al.*, 1981; Ochs & Lardy, 1983). After 30min of incubation there was a small increase in the concentration of glutamate in the presence of noradrenaline ($P<0.05$, t-test for paired observations), with no apparent change after 60min. Ochs (1984) reported a small increase in the concentration of glutamate after 20min of incubation, with a subsequent decrease after 40min owing to the presence of the hormone.

In subsequent experiments, possible influences of the lag period observed in the production of glucose and urea from glutamine (Table 1; see also Joseph & McGivan, 1978) were avoided by adding the hormone after 20min of incubation, at which time the production of glucose and urea became

Table 1. *Effect of noradrenaline on glutamine metabolism in isolated rat hepatocytes*
Hepatocytes were incubated with 5mM-glutamine as described in the Experimental section in the absence or presence of noradrenaline (10 μM). Samples were taken after 30 and 60 min of incubation as described in the Experimental section. Values given are means ± S.E.M. for three experiments with separate batches of cells.

	Glucose production (μmol/g dry wt.)		Urea production (μmol/g dry wt.)		Glutamate (μmol/g dry wt.)	
Additions	At 30 min	At 60 min	At 30 min	At 60 min	At 30 min	At 60 min
None	30±5	76±9	68±9	202±9	28±4	29±7
Noradrenaline	46±6	113±9	102±6	251±21	34±2	29±5

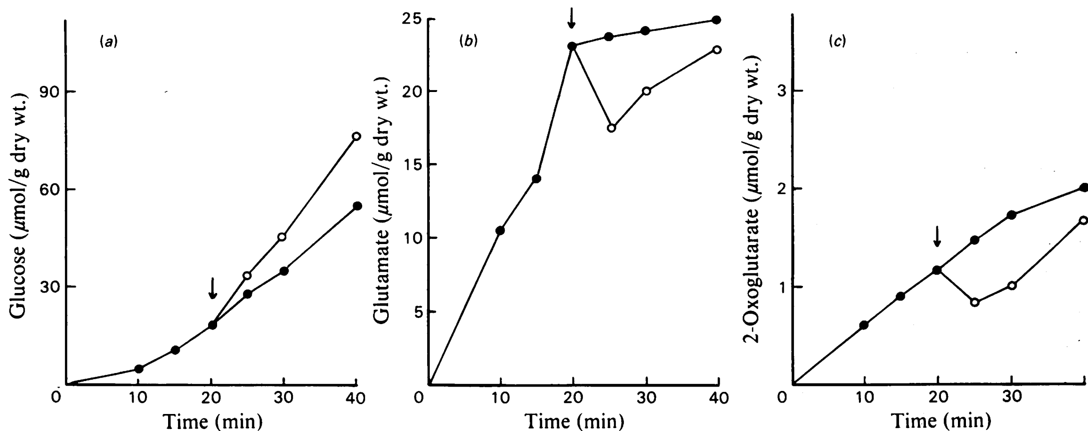

Fig. 1. *Effect of noradrenaline on (a) glucose production, (b) intracellular glutamate and (c) intracellular 2-oxoglutarate concentration in isolated hepatocytes incubated with glutamine*
Hepatocytes were incubated in flasks in the presence of 5mM-glutamine as described in the Experimental section. After 20 min of incubation (arrow), noradrenaline (final concn. 10 μM) or vehicle only was added. The results shown are means of two separate experiments: ●, control; ○, after hormone addition.

linear (Fig. 1*a*). With this protocol noradrenaline caused a transient fall in the steady-state concentration of intracellular glutamate, which was almost back to the control value 20 min after hormone addition (Fig. 1*b*). This result indicates activation by the hormone of an enzyme located after the conversion of glutamine into glutamate.

Several groups have provided evidence for stimulation of 2-oxoglutarate dehydrogenase (EC 1.2.4.2) by Ca^{2+}-dependent hormones (Taylor *et al.*, 1983; Sugden & Watts, 1983; Staddon & McGivan, 1984; Häussinger & Sies, 1984). This stimulation could explain the decrease in glutamate, since this amino acid and 2-oxoglutarate are in equilibrium via glutamate:oxaloacetate transaminase and glutamate dehydrogenase (Williamson *et al.*, 1967). Indeed, in hepatocytes metabolizing glutamine, noradrenaline also induced a decrease in the concentration of 2-oxoglutarate, which was transient in nature (Fig. 1*c*).

The metabolite changes occurring after hormone addition were also studied in perifused hepatocytes, in which a true steady state can be obtained with the possibility of continuous measurement of the metabolic rate and intracellular changes (Van der Meer & Tager, 1976). In this system it was found to be necessary to add ammonia, to ensure the catabolism of glutamine (Meijer *et al.*, 1983), and to add oleate, for as yet unknown reasons. The results of these experiments are depicted in Fig. 2. It is clear that, also, in perifused hepatocytes metabolizing glutamine, α-adrenergic stimulation led to a rapid, transient, fall in the intracellular concentrations of glutamate and 2-oxoglutarate. These changes were accompanied by a marked stimulation of glucose production, which was clearly not completely transient (Fig. 2).

The transient character of the changes in the concentrations of glutamate and 2-oxoglutarate after hormone addition indicates either that the stimulation of 2-oxoglutarate dehydrogenase is not stable (see, however, Häussinger & Sies, 1984) or that activation of the first enzyme in the pathway,

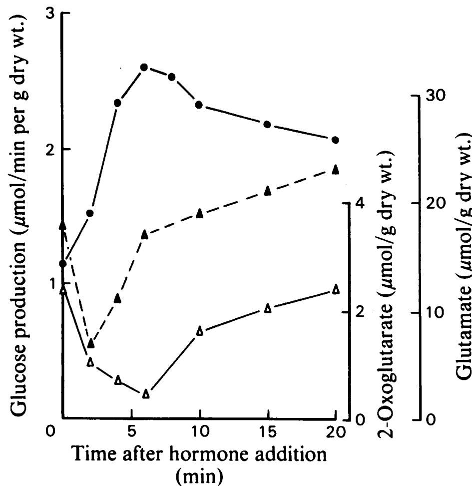

Fig. 2. *Metabolite changes after phenylephrine addition to hepatocytes perifused with glutamine as substrate*

Hepatocytes were incubated in a perifusion system as described in the Experimental section in the presence of 2.5mM-glutamine, 0.25mM-NH_4Cl, 0.1mM-ornithine and 0.1mM-oleate. After a steady state had been reached, phenylephrine (final concn. 10μM) was added and samples were taken at the times indicated in the Figure. The results shown are means of two separate experiments: ●, glucose in perifusate; ▲, intracellular glutamate; △, intracellular 2-oxoglutarate.

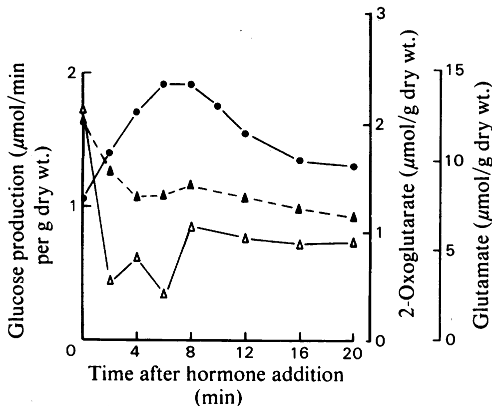

Fig. 3. *Metabolite changes after phenylephrine addition to hepatocytes perifused with proline as substrate*

Experimental details were as described in the legend to Fig. 2, except that 2.5mM-proline was added as substrate: ●, glucose in perifusate; ▲, intracellular glutamate; △, intracellular 2-oxoglutarate.

glutaminase, which is slower in onset than the activation of 2-oxoglutarate dehydrogenase, compensates for the fall in glutamate and 2-oxoglutarate concentrations. The latter possibility was

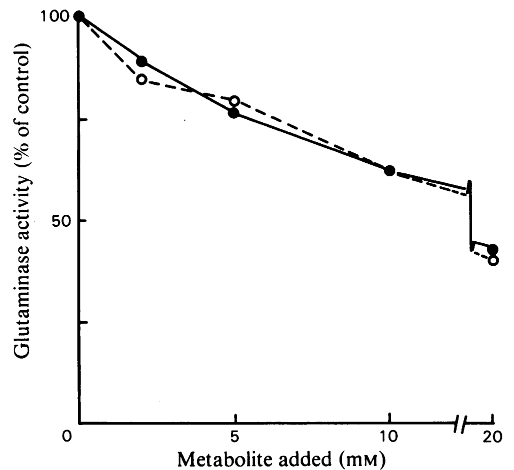

Fig. 4. *Glutaminase activity in isolated rat liver mitochondria in the presence of different concentrations of glutamate* (●) *or 2-oxoglutarate* (○)

Isolated mitochondria were incubated for 10min as described in the Experimental section. Glutaminase activity was expressed as a percentage of the activity measured in the absence of any addition (which was 89nmol/mg of mitochondrial protein). The results shown are means of two separate experiments.

tested by using proline instead of glutamate as substrate. Staddon & McGivan (1984) have shown that there is a rapid decrease in the concentration of intracellular glutamate and 2-oxoglutarate after the addition of vasopressin to hepatocytes metabolizing proline. In perifused hepatocytes with proline as substrate, the addition of phenylephrine had similar effects (Fig. 3). However, in contrast with the perifusions with glutamine as substrate, the concentrations of glutamate and 2-oxoglutarate did not return to the control values even 20min after phenylephrine addition. This result suggests that, with glutamine as substrate, activation of glutaminase is responsible for the transient character of the metabolite changes brought about by the hormone.

We have investigated the possibility that glutaminase is activated by changes in the concentrations of glutamate and 2-oxoglutarate. For this purpose isolated mitochondria were incubated in the presence of a subsaturating concentration of ammonia and different concentrations of glutamate or 2-oxoglutarate (Fig. 4).

Although glutamate is a much more potent inhibitor of kidney glutaminase than of the liver enzyme (Krebs, 1935), the results of Fig. 4 show that significant inhibition of liver glutaminase occurred at concentrations comparable with the cytosolic concentration of the amino acid found in hepatocytes metabolizing glutamine (Figs. 1 and 2;

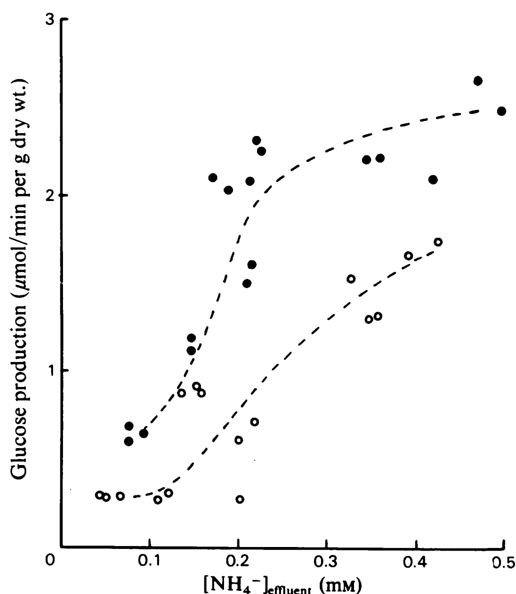

Fig. 5. *Effect of phenylephrine on the ammonia-dependence of glucose production from glutamine from perifused hepatocytes*

Hepatocytes were incubated in a perifusion system as described in the Experimental section in the presence of 2.5mM-glutamine, 0.1mM-ornithine, 0.1mM-oleate and different concentrations of ammonia. Rates of glucose production in the absence of the hormone were measured 45–50min after the addition of the substrates. Rates of glucose production in the presence of phenylephrine (10μM) were measured 15–20min after the addition of the hormone. Rates of glucose production were not corrected for endogenous glucose formation (0.26±0.02μmol/per min dry wt.; $n=3$). Points presented in the Figure were taken from three separate experiments: ○, control; ●, + phenylephrine.

see also the Discussion section). From the experiment depicted in Fig. 4 it can be concluded that 2-oxoglutarate does not regulate glutaminase activity, since inhibition occurred only at concentrations much higher than those found in hepatocytes metabolizing glutamine (Figs. 1 and 2).

As mentioned above, the metabolism of glutamine in isolated hepatocytes is fully dependent on the ammonia concentration. Therefore stimulation of glutaminase activity by hormone action could be brought about by a shift in the ammonia-dependence of the enzyme. This possibility was tested by incubating perifused hepatocytes with glutamine together with different concentrations of ammonia in the absence or presence of phenylephrine (Fig. 5). To obtain an approximation of the

Table 2. *Effect of pretreatment with noradrenaline on glutaminase activity in a mitochondrial fraction from hepatocytes, assayed in the presence of 5mM-NH_4Cl*

Hepatocytes were incubated with 5mM-glutamine as described in the Experimental section. After 20min of incubation, noradrenaline (final concn. 10μM) was added, or vehicle only. After subsequent incubation in the presence or absence of hormone for different time periods, the mitochondrial fraction was prepared as indicated in the Experimental section. Glutaminase activity in this fraction was determined as described in the Experimental section, with 5mM-NH_4Cl present. Values given are means of two experiments.

Addition during pretreatment	Duration of pretreatment (min)	Glutaminase activity (nmol/mg of protein in 10min)
None	5	236
Noradrenaline	5	360
None	10	258
Noradrenaline	10	391
None	20	251
Noradrenaline	20	347

flux through glutaminase in the presence of the hormone, glucose was measured 15–20min after the addition of phenylephrine, at which time the system was in a new steady state. The hormone did indeed shift the ammonia-dependence of glucose formation from glutamine to lower concentrations of ammonia. From Fig. 5 it is not quite clear whether the hormone would also activate the metabolism of glutamine in the presence of a saturating concentration of ammonia, because addition of high concentrations of ammonia to the perifusion system caused disintegration of the cells (results not shown). However, when hepatocytes were exposed to noradrenaline for different periods of time and glutaminase activity was subsequently measured in the mitochondrial fraction in the presence of 5mM-ammonia, a clear increase by the hormone treatment was observed (Table 2). In a separate experiment, no stimulation could be detected after treatment with the hormone for 30s (results not shown).

Discussion

According to Ochs & Lardy (1983) and Ochs (1984), production of aspartate from glutamate is rate-limiting for urea and glucose synthesis from glutamine. In their view the stimulatory effect of noradrenaline or NH_4Cl on glutamine metabolism can be explained by the changes in the concentrations of the metabolites involved in the transamination step rather than by activation of mitochondrial glutaminase. However, since in the

steady state the flux through an entire sequence of enzymes must proceed at the same rate, as also recognized by Ochs (1984), the flux through glutaminase must also have been increased by addition of noradrenaline or NH_4Cl. Indeed, one can calculate from the data of Ochs & Lardy (1983) and Ochs (1984) that flux through the enzyme (which is given by half of the sum of glutamate, aspartate and ammonia accumulated plus the amount of urea formed) increased on addition of NH_4Cl or the hormone. This increase in the flux through glutaminase was not discussed by those authors and therefore remained unexplained.

We have used the perifusion system for the study of glutamine metabolism because in this system true steady-state conditions can be obtained; moreover, this system allows one to analyse the sequence of events occurring after perturbation of the steady state by hormone addition.

In order to account for the activation of glutaminase by α-adrenergic agonists, we have considered the possibility that reversal of inhibition of the enzyme occurs owing to a fall in the concentration of glutamate or 2-oxoglutarate. On addition of phenylephrine to hepatocytes perifused with glutamine, intracellular glutamate and 2-oxoglutarate fell from 18 to 7 μmol/g dry wt. and from 2 to 0.4 μmol/g dry wt. respectively (Fig. 2). Upon digitonin fractionation (Zuurendonk *et al.*, 1976), about 80% of each of these metabolites was found in the extramitochondrial fraction (results not shown). Assuming that 1 g dry wt. of cells contains 2 ml of cytosolic water (Tischler *et al.*, 1977), the corresponding fall in the concentration of extramitochondrial glutamate was from 7.2 to 2.8 mM and that in the concentration of 2-oxoglutarate was 0.80 to 0.16 mM. According to Fig. 4 the decrease in extramitochondrial glutamate could, in principle, account for an activation of glutaminase of only about 20%. The concentration of 2-oxoglutarate was insufficient to influence the activity of glutaminase significantly (cf. Fig. 4). Moreover, the changes in the concentration of both glutamate and 2-oxoglutarate on addition of phenylephrine were transient in nature, but glucose production was stimulated during the entire experimental period (Fig. 2). We therefore conclude that acceleration of glutaminase flux by phenylephrine could not have been due to reversal of inhibition of the enzyme.

What, then, is the reason for the stimulatory effect of phenylephrine on flux through glutaminase? In our opinion, the reason is twofold. On the one hand, the affinity of glutaminase for ammonia, its essential activator (Verhoeven *et al.*, 1983; McGivan & Bradford, 1983), was increased (Fig. 5). In addition, however, phenylephrine increased the glutaminase activity in an ammonia-independent manner. This can be concluded from the additivity of the effects of phenylephrine and ammonia in stimulating glutamine degradation, as shown by Häussinger & Sies (1984). In further support of this dual effect, we observed an increase in glutaminase activity in mitochondria isolated from hormone-treated hepatocytes when assayed in the presence of saturating concentrations of ammonia (Table 2).

Similar conclusions can be drawn about the stimulatory effect of the hormone glucagon on glutamine metabolism. The only difference seems to be a smaller stimulation of 2-oxoglutarate dehydrogenase by glucagon compared with α-adrenergic agonists, resulting in a smaller change in the 2-oxoglutarate concentration (cf. Staddon & McGivan, 1984) and a more rapid increase in the glutamate concentration (results not shown).

The fact that glutaminase is almost insensitive to product inhibition by glutamate and is activated (rather than inhibited) by its other product, ammonia, implies that, in the metabolic pathway from glutamine to glucose, control of flux through the pathway in the steady state is completely confined to this step (see Westerhoff *et al.*, 1984). This is also evident from Fig. 5, which shows the effect of activation of glutaminase by ammonia on the pathway flux to glucose.

In summary, α-adrenergic stimulation of urea and glucose production from glutamine can be explained by a dual effect of the agonist. Initially, there is a rapid stimulation of 2-oxoglutarate oxidation, promoting aspartate formation. This is followed by direct activation of glutaminase, which is slower in onset. The activation of glutaminase compensates for the initial fall in the concentration of glutamate and hence also of 2-oxoglutarate.

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