GLUTAMINE METABOLISM IN THE RAT

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1. The enzymes of glutamine metabolism

1.1. *Glutaminase isoenzymes (EC 3.5.1.2)*

In his paper, entitled, 'The synthesis of glutamine from glutamic acid and ammonia, and the enzymic hydrolysis of glutamine in animal tissues' published 45 years ago [1], H. A. Krebs perceptively recognized:

> 'there are at least two types of glutaminase distinguishable by their pH optima and their inhibitions by glutamic acid ('brain type' and 'liver type')'.

The mitochondrial 'phosphate-dependent' isoenzymes are considered to be the true glutaminases. Liver, and possibly lung, possess the 'liver-type' isoenzyme; all other tissues, including brain and lung, have the 'kidney-type' [2]. The two are distinguishable on the basis of P_i requirement (the 'liver-type' requires a low concentration for activation, the 'kidney-type' a high concentration), pH optima, affinity for glutamine, reactions with activators and inhibitors [2] and inhibition by glutamate [1]. The tissue distribution of glutaminase activity reported by different workers is not identical, but highest activity is present in kidney, brain and possibly small intestine, with much lower activity in liver and other tissues [2,3].

The liver-type enzyme appears in rat liver during the 48 h before birth, reaching almost adult activity within 6 days [4]. Foetal liver contains the kidney-type enzyme. Its activity parallels the amount of haematopoietic, not the parenchymal, tissue. Although the phosphate requirement of the liver enzyme is low (K_m 2.8 mM) the K_m for glutamine is very high: 28 mM [5] to 42 mM [2]. Observations that low concentrations of glutamine are not metabolized by perfused liver [6] or hepatocytes [7] led to the suggestion that the liver enzyme does not degrade glutamine under physiological conditions [7]. Now a number of activators have been identified, and the picture is changed but by no means clear.

So far, increased flux through glutaminase has been observed in the presence of the following: NH_4^+ [8–13], HCO_3^- [13,14], glucagon [12], dibutryryl-cAMP [14], L-leucine [14], L-isoleucine, L-valine and NH_2OH [15]. The relative effectiveness of these compounds is very dependent on the conditions. In isolated hepatocytes their effects on glutamine removal decrease in the order $NH_4^+ > HCO_3^- >$ dibutyryl-cAMP > branched-chain amino acids [15], whereas in the perfused liver Haussinger et al. [16] find no effect of HCO_3^-, presumably because a short exposure to 3 mM NH_4Cl had pre-activated glutaminase in their experiments. Only HCO_3^-, NH_4^+ and ATP [13] are effective in mitochondrial preparations. However, activation by glucagon persists in mitochondria isolated from glucagon-treated rats [17]. Activation by NH_4^+ or HCO_3^- is greatest in the presence of phosphate and ATP [13], and both decrease the requirement for phosphate [18] and the K_m for glutamine (see [13]). The acceleration of glutamine metabolism by branched-chain amino acids in the intact cell may be an indirect effect on glutaminase. None of these activators has been tested with the solubilized enzyme. It is also worth remembering that the activity in liver [2,3] was determined before the allosteric nature of glutaminase was known. This basal activity is increased about 20-fold by the presence of HCO_3^-, NH_4^+ and ATP [13,15].

In surveying the literature on glutaminases it is very difficult to obtain a consistent picture. The 'phosphate-dependent' glutaminases of liver, kidney and brain described by Katunuma et al. [19] are all different, and the liver enzyme, in regard to heat inactivation, pH optimum and inhibition by mercurials, does not correspond with the liver enzyme described

Dedicated to Professor Sir Hans Krebs, FRS, on his eightieth birthday

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by Horowitz and Knox [2]. Recently, on the basis of inhibition by *p*-mercuribenzoate, two distinct phosphate-dependent mitochondrial glutaminases are reported to be present in both pig kidney cortex and rat brain [20], again contrary to the findings of Horowitz and Knox [2].

If one takes the view that the regulatory properties of an enzyme will reflect its role in the parent tissue, then it makes good physiological sense that the liver glutaminase should be unique and responsive to indicators of dietary state. There are possible explanations for some of the apparent differences in results from different laboratories:

(i) The liver glutaminase is associated with the inner mitochondrial membrane and exhibits the phenomenon of latency, in that activity is increased on incubation of mitochondria in hypotonic media [15,21,23].

(ii) The kinetic properties of the enzyme are altered by its association with the membrane; the K_m for glutamine of the membrane-bound form is lower (6 mM) than that of the solubilized form (21 mM) [18].

(iii) A factor that has not been taken into account has been the extent to which the various activators bring about mitochondrial swelling (see [22,23]).

(iv) The temperature at which the activators are studied affects the results [23].

Temperature can be ruled out as a variable in vivo, but variations in mitochondrial volume cannot. There seems to be no information available. It would be interesting to know whether the phenomenon of latency, in the context of mitochondrial swelling, has any meaning in the intracellular environment.

The 'kidney-type' glutaminase, as already mentioned, is widely distributed and has a very high phosphate requirement in vitro; K_m 74 mM [2]. The enzyme of brain and kidney has been most studied. The K_m for glutamine of the solubilized enzyme varies, depending on the phosphate concentration [24] and, as for the liver enzyme, is higher for the soluble than for the bound form. The lowest K_m is obtained in mitochondria in a hypotonic medium; 1.5 mM [25]. A characteristic of the kidney- or brain-type enzyme, first observed by Krebs [1], is that it is inhibited by glutamate. The enzyme is activated by riboflavin 5-phosphate and other phosphate esters [26], ATP, GTP, ITP, acetate, and di- and tricarboxylic acids [24,27]. Cyclic AMP and GMP are inhibitors [24]. The physiological function of these modifiers is not known.

1.2. *Glutamine 2-oxoacid aminotransferase (EC 2.6.1.15)*

The aminotransferase pathway for glutamine degradation may function in some tissues under certain conditions. The combined reactions of the aminotransferase and ω-amidase (EC 3.5.1.3) were formerly known as 'glutaminase II'. So far two forms of the aminotransferase have been described, a kidney-type and a liver-type, both of which exist as a mitochondrial and cytosolic isoenzyme. All four isoenzymes are found in different proportions in liver and kidney [28]. The specificity for the 2-oxoacid acceptor is different in the two tissues. In hepatocytes glutamine at physiological plasma concentration (0.5 mM) is readily metabolized in the presence of 2-oxo-4-methylthiobutyrate (the 2-oxoacid analogue of methionine) and to a lesser extent in the presence of phenylpyruvate, whereas pyruvate has no effect [7]. In kidney, which is reported to contain a higher activity of the aminotransferase than liver [29], neither phenylpyruvate nor 2-oxo-4-methylthiobutyrate is effective in increasing glutamine metabolism [30].

The aminotransferase is not specific for glutamine; methionine can replace glutamine [31]. We [32] find that this enzyme and the branched-chain 2-oxoacid dehydrogenase are involved in the degradation of methionine via the transamination pathway in the liver [33].

The glutamine aminotransferase is very important for the amination of 2-oxoacid analogues of essential amino acids [34] when administered clinically as protein-sparing agents (see [35]) in the treatment of uraemia or inborn errors of the urea cycle. Cooper and Meister [28] suggest that it may function under normal physiological conditions to re-aminate small amounts of 2-oxoacids formed during normal metabolism of essential amino acids. This would, in effect, be a salvage function.

1.3. *γ-Glutamyltranspeptidase (EC 2.3.2.2)*

Formerly known as 'phosphate-independent' glutaminase, it is not a true glutaminase. Its glutaminase activity is stimulated by maleate [36]. γ-Glutamyltransferase is the Enzyme Commission-recommended trivial name, but this activity is a property of several enzymes (see [37]). The transpeptidase was postulated as the amino acid-transporting enzyme in

the 'Meister cycle' [38]. Its location in outer cell membranes, such as the brush border membrane of kidney proximal tubules [39] and intestinal mucosa [40] and in the plasma membrane of liver [41] and other tissues, is in keeping with a transport function. However, recently an association with glutathione metabolism has been proposed in that glutathione in the glomerular filtrate is hydrolysed by the transpeptidase with recovery of the components [42–45]. In addition the enzyme catalyses the oxidation of glutathione to glutathione disulphide [46]. These functions do not rule out the possibility that the enzyme is also involved in amino acid transport. In lactating mammary gland, for example, which removes large amounts of amino acids from the circulation, and in which tissue there is a very large increase in γ-glutamyltranspeptidase activity during the lactogenic cycle [47].

1.4. *Glutamine synthetase (EC 6.3.1.2)*

The enzyme is present in most tissues, but in a separate compartment from glutaminase; in liver it is in soluble fraction, but in brain is largely membrane bound (see [48]). There are various reports on the tissue distribution [49–52], and on the distribution within heterogeneous tissues as, for example, the nephron of the rat [53]. Little is known of the regulation of the activity of the enzyme in situ. In the intact liver cell in vitro the high capacity for glutamine synthesis is not expressed [7,54]. On the other hand, perfused rat kidney forms glutamine [55] although the assayable enzyme activity is much lower than in liver. For details of the complex kinetic properties of glutamine synthetase, see Meister [56] and Deuel et al. [57].

1.5. *Other reactions involving glutamine*

Welbourne [58] has studied a γ-glutamyltransferase in rat kidney that is distinct from γ-glutamyltranspeptidase. Like glutamine synthetase, it is not present in dog or human kidney [59], which suggests a common identity. Alternatively it may be the γ-glutamyltransferase described by Herzfeld and Estes [52]. In either case its physiological function is not settled.

Other reactions involving glutamine which are not considered here include the formation of amino sugars (EC 5.3.1.19], asparagine (EC 6.3.5.4), purine (EC 2.4.2.14 and EC 6.3.5.3), pyrimidine (EC 6.3.5.5), NAD (EC 6.3.5.1) and incorporation into proteins.

2. Glutamine metabolism in vivo

2.1. *Utilization*

Under normal physiological conditions the small intestine is the main glutamine-utilizing tissue; others are quantitatively far less important (see section 3). For example, glutamine is an oxidative fuel of synaptosomes and a precursor of both excitatory (glutamate and aspartate) and of inhibitory (γ-aminobutyric acid) neurotransmitters [60], yet the metabolism of the brain, as measured by arteriovenous differences, appears to result in a net glutamine synthesis [54, 61, 62].

There are conditions (metabolic acidosis and lactation) during which the kidney and mammary gland, respectively, make extra demands on the glutamine supply. In normal acid–base balance there is no uptake of glutamine by rat kidney in vivo [7,63,64], essentially confirming earlier work of Pitts [65] who found that glutamine hydrolysis was almost exactly balanced by glutamine synthesis in the non-acidotic rat kidney. The uneven distribution of glutaminase and glutamine synthetase along the nephron [53] makes cycling feasible in the kidney of animals, like rat, that possess glutamine synthetase. By contrast, all rat kidney preparations in vitro, including the isolated perfused kidney, catabolize glutamine at the concentration found in plasma (0.5 mM). Therefore some important physiological regulatory mechanisms are lost on exposure of the tissue to an artificial 'plasma'. This should be borne in mind when using kidney preparations in vitro to study the mechanism of adaptation to metabolic acidosis. How glutamine degradation is 'switched-on' in acidosis is not settled, in spite of the vast amount of research. One simple explanation for many of the apparently conflicting observations is that an increase in mitochondrial volume somehow occurs in acidosis, leading to an activation of the latent glutaminase (see section 1.2). Many of the well-established metabolic events would then automatically occur. Ammonia is released from the normal rat kidney, mainly into renal venous blood, even though no net removal of glutamine or other amino acid is sufficient to account for it (see [64]). Possibly the components of glutathione, reabsorbed after hydrolysis by γ-glutamyltranspeptidase in the lumen (see section 1.3), are the precursors of this ammonia.

In the case of the lactating mammary gland large

amounts of amino acids are removed from the arterial plasma for milk protein synthesis. However, the amounts of glutamine removed are too great to be used only for casein synthesis [66], and glutamine appears to be used also as a metabolic fuel (see section 3). The oxidation of glutamine is puzzling in view of the report that glutaminase activity is extremely low [67]. On the other hand, γ-glutamyltranspeptidase appears and reaches high activity during the lactogenic cycle [47]. This raises again the question as to whether γ-glutamyltranspeptidase is involved in amino acid transport in certain tissues.

2.2. *Production*

Skeletal muscle has been taken to be the most important source of endogenous glutamine since the Cahill group [68] found that glutamine and alanine together account for about 50% of amino acids released into venous blood from the human forearm after an overnight fast. This discovery prompted research to determine whether the carbon skeleton of the alanine released is also derived from the catabolism of other amino acids (which would lead to a net synthesis of glucose in starvation) or from carbohydrate (which would not). As branched-chain amino acids constitute about 22% of the total amino acids of protein and as they, unlike other amino acids, are not metabolized by the liver, they have been the focus of attention. There is still no general agreement on the source of the carbon. A number of groups conclude that branched-chain amino acids are important precursors [69–73]; others that the pyruvate arises from glycolysis, while the carbon skeleton of glutamine is derived from other amino acids [74,75]. However, measurements of arteriovenous differences across the hindlimb of the rat show that large amounts of 2-oxoacids, formed by transamination of branched-chain amino acids are released from skeletal muscle, and removed by the liver [76]. This means that the contribution of the carbon skeleton of branched-chain amino acids to the formation of alanine or glutamine must be re-assessed, at least in the rat. The situation in man is different, because there is little or no release of branched-chain 2-oxoacids by skeletal muscle [77].

2.3. *Role of the liver*

The liver appears to function as either a glutamine-synthesizing or a glutamine-utilizing tissue, depending on the physiological state of the rat. In starved [78], high carbohydrate fed [79] or normal fed [7] there appears to be glutamine output, but livers of rats fed a high protein diet remove glutamine [79,80]. These data are based on arteriovenous difference measurements and should be interpreted with caution because of the problem of the relative contributions of the arterial and portal blood supplies. In general, assumptions have been made as to flow rate. The findings in vivo make more physiological sense than the experiments in vitro that suggested that neither glutaminase nor glutamine synthetase is functional in the intact liver cell (see section 1.2,1.5). The effect of the high protein diet can be explained by increased flux of ammonia and/or amino acids into the mitochondria, and the presence of glucagon which will serve to activate liver glutaminase (see section 1.2). The signal for glutamine synthesis is not known. Activation of liver glutaminase by ammonia provides an explanation for the observation, unexpected at the time, that injection of NH_4Cl into rats decreases the concentration of glutamine in the liver [81]. For the same reason NH_4Cl should not be used in future to induce metabolic acidosis for the study of how the extrarenal tissues meet the demands of the kidney for glutamine. Activation of liver glutaminase by NH_4^+ will override the normal response of the liver and, indirectly, possibly that of other tissues. These conclusions are, however, made on the assumption that increased flux of NH_4^+ into the mitochondrion is associated with an increased concentration at the site of glutaminase. While this is probably true after a load of NH_4Cl, the urea cycle may be active enough to prevent it occurring by dietary means. One should also remember that ammonia is continuously released into the portal vein in all physiological states as a result of glutamine metabolism in the small intestine (see section 3), so that there is continuous flux of ammonia into liver mitochondria.

3. Glutamine as a respiratory fuel

A number of tissues, including kidney [82], reticulocytes [83], synaptosomes [60], lactating mammary gland [84], small intestine [85,86], fibroblasts [87], lens [88], oocytes [89] and tumour cells (see [90]) can oxidize glutamine. In foetal rat liver, because of its haematopoietic tissue content and because of the properties of the foetal liver glutaminase [4], glutamine is probably a very important respiratory substrate. Thus the high phosphate requirement of the

'kidney-type' glutaminase (see section 1.1) does not seem to be necessary for the enzyme in situ.

The quantitative contribution of glutamine oxidation in vivo has been established only for the small intestine, in which the mucosa was identified as the site of oxidation, and arterial blood (as opposed to the lumen) as the main source of glutamine [91]. In this tissue arterial glutamine is a more important respiratory fuel than is glucose in both fed and starved rats [92,93]. In the fed state oxidation of arterial glucose accounts for only about 10% of the O_2 uptake, and 97% of luminal glucose is transported across the tissue without undergoing metabolism. Arterial glutamine, on the other hand, can account for 38% of the total O_2 uptake, and luminal glutamine, glutamate and aspartate a further 39%, a total of 77% [93]. Major products other than CO_2 and ammonia, are alanine, citrulline, and proline [91]. The synthesis of citrulline from glutamine is interesting from the nutritional point of view, because it provides an explanation as to why arginine is not an essential amino acid in the adult. The amounts of alanine and ammonia formed from glutamine, glutamate and aspartate are so great that they can account for about 60% of the urea nitrogen excreted [93]. These experiments in vivo illustrate the limitations of other intestinal preparations, especially of the isolated enterocyte, which can provide no information on the relative contributions of luminal and arterial substrates. In isolated enterocytes glucose is the most readily oxidized substrate, there is a very high rate of aerobic glycolysis, glutamate is a major endproduct of glutamine metabolism, and the amounts of tissue used are necessarily small, so that citrulline synthesis is too low to be detectable [94]. The work of Windmueller and Spaeth has now dispelled the myth of the pre-eminence of glucose in the energy metabolism of the small intestine.

For glutamine to be completely oxidized, acetyl-CoA must be formed via pyruvate from malate or oxaloacetate. Possible reactions are oxaloacetate decarboxylase (EC 4.1.1.3), phosphoenolpyruvate carboxykinase (EC 4.1.1.32) plus pyruvate kinase (EC 2.7.1.40), NADP-linked malic enzyme (EC 1.1.1.40) or NAD(P)-linked malic enzyme. Of these PEP carboxykinase plus pyruvate kinase is least likely because mercaptopicolinate, an inhibitor of PEP carboxykinase [95,96] has no effect on the oxidation of glutamine in the small intestine or in rat kidney tubules [97,98]. Watford et al. [98] came to the conclusion that the cytosolic NADP-linked malic enzyme was the most likely pathway. A mitochondrial route would make more physiological sense because it would eliminate shuttling of intermediates to and from the cytosol; because of this the NAD-linked malic enzyme is an attractive possibility. It has been reported to be present in mitochondria of rat liver and kidney [99], small intestinal mucosa [100] and heart [101]. It is interesting that, in spite of the presence of all these enzymes in rat liver, complete oxidation of glutamine does not appear to occur as glutamine removed by hepatocytes can be accounted for as glutamate and glucose [14].

4. Turnover of glutamine

In order to minimize interconversions, L-[1-^{14}C]-glutamine has been used to measure turnover. Results obtained by the single injection technique agree very closely for normal fed rats: 282 ± 33 μmol . h^{-1} . 100 g body wt^{-1} [62] and 235 ± 12 μmol . h^{-1} . 100 g body wt^{-1} [102]. The rate obtained by the constant infusion technique is lower: 134 ± 8 μmol . h^{-1} . 100 g body wt^{-1} [62]. Whichever rate is taken, turnover is extremely high compared with the glutamine requirements of the main utilizing tissue, the small intestine (1.8 mmol/day in a 280 g rat starved overnight) [91]. The discrepancy cannot be explained by cycling between glutamine and glutamate because cycling would involve no loss of ^{14}C. Protein turnover, which amounts to about 4.4 g/day in 100 g rats [103], provides the most likely explanation.

Glutamine turnover is not increased in metabolic acidosis [62], which suggests that the demands of the kidney are met by decreased utilization in extra-renal tissues. In support of this, with one exception [104], there is reported to be no increased release of glutamine from skeletal muscle in acidosis [7,62,105]. But the small arteriovenous differences for the normal controls make it doubtful whether skeletal muscle can supply the glutamine requirement of the small intestine. Similar measurements by Ruderman and Berger [106], on the other hand, suggest that glutamine release is more than adequate. Neither the quantitative importance of skeletal muscle as a source of glutamine in the rat, nor how the animal copes with the increased demand for glutamine in metabolic acidosis or lactation, can be taken as settled. It is rather surprising to realise that the body pool of

glutamine is maintained largely by endogenous synthesis because, as a result of intestinal metabolism, the glutamine concentration in the portal vein, even in the fed state, is generally lower than the arterial concentration (see [7,62,79,93]). This is also true in lactating rats [102] although food intake is increased by 300%. The liver may yet prove to play a fundamental regulatory role in glutamine metabolism in the rat.

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