

ON THE METABOLIC FUNCTION OF GLUTAMATE DEHYDROGENASE IN RAT LIVER

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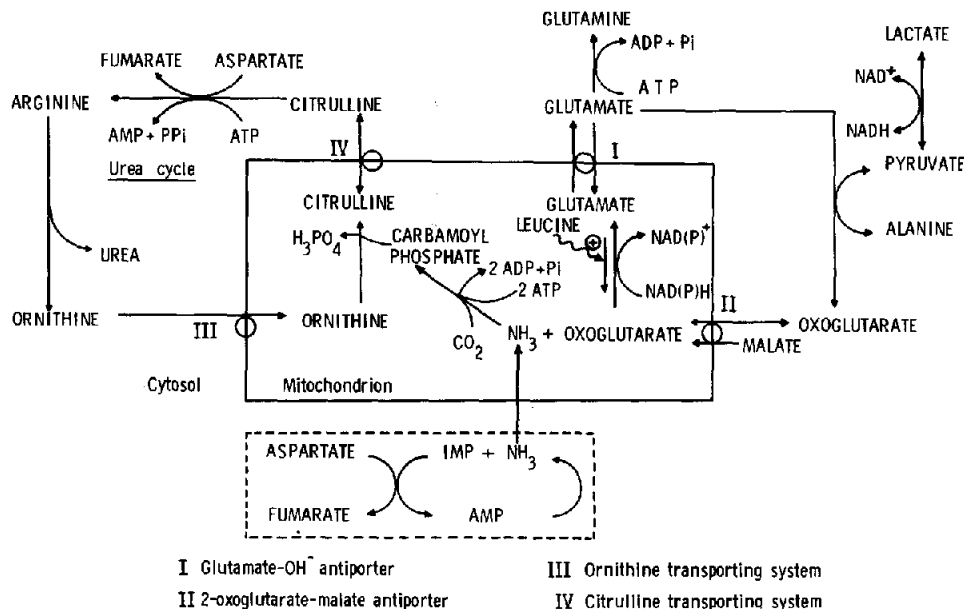
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1. Introduction

Glutamate dehydrogenase (EC 1.4.1.2) catalyzes the reaction: $\text{L-glutarate}^- + \text{NAD(P)}^+ + \text{H}_2\text{O} = 2\text{-oxo-glutarate}^{2-} + \text{NAD(P)H} + \text{NH}_4^+ + \text{H}^+$. The enzyme from mammalian liver reacts with either nicotinamide nucleotide with comparable facility. The equilibrium is in favour of glutamate synthesis and the equilibrium constant, which has been determined by several workers, is in the range $1-10 \times 10^{-14} \text{ M}^2$ [1], which corresponds to values of $1-10 \times 10^{-7} \text{ M}$ at pH 7.

The purified bovine liver enzyme is activated by ADP and is inhibited by GDP and GTP. The molecule has a weight of 350 000 daltons and consists of six sub-units, each of which has binding sites for substrates,

nicotinamide- and purine-nucleotides. At higher concentrations the bovine liver enzyme undergoes aggregation, an aggregation which is promoted by ADP binding and is inhibited by the binding of GDP and GTP. The activation and inhibition of glutamate dehydrogenase has been attributed to this aggregation-disaggregation process [see 2]. However the rat liver enzyme, the activity of which is affected by purine nucleotides in a similar fashion to that of the bovine enzyme, either does not apparently undergo polymeric changes [3] or shows only minor changes [3a]. The purified bovine liver enzyme has also been shown to be activated, to a small extent, by leucine and some other amino acids [4,5].



Scheme 1. Compartmentation of nitrogen metabolism in liver. From [19]

It is generally considered that a primary metabolic function of glutamate dehydrogenase is to furnish the NH_3 required for the production of carbamoyl phosphate, which in turn is used to provide one of the N atoms of urea (Scheme 1) (see [5a]). Since the amino groups of the majority of amino acids may be transferred to oxoglutarate to form glutamate, it is considered that glutamate and its dehydrogenase represent the major pathway of NH_3 production in liver. This view of the function of glutamate dehydrogenase seems to us to be based on the following considerations:

- 1) Its plausibility.
- 2) The finding that glutamate dehydrogenase activity of *extracts* of those tissues which catalyze urea synthesis (liver) or some part of the urea cycle (kidney) is high, whereas in those tissues which do not (e.g. brain and muscle) glutamate dehydrogenase activity is low [see 6].
- 3) The apparent absence of any alternative source capable of producing NH_3 at rates compatible with the observed rate of urea synthesis.
- 4) The concept that the activity of glutamate dehydrogenase is so high *in vivo* that the enzyme is able to maintain a 'thermodynamic equilibrium' under a wide variety of metabolic conditions [7]. If this concept is correct then it follows that NH_3 may be produced at any rate at which it may be required.

It is the purpose of this review to show that the interpretations which have been made from points 2) to 4) are incorrect in some very important respects and therefore that point 1) is in error. It is suggested that the primary function of glutamate dehydrogenase is the synthesis of glutamate and consequently of glutamine; glutamate dehydrogenase is primarily involved in N storage. It is suggested that the NH_3 for carbamoyl phosphate synthesis is derived from elsewhere, probably the deamination of AMP.

We have been led to question the accepted dogma with regard to the role of glutamate dehydrogenase because of the effects of leucine on soluble glutamate dehydrogenase and on the expression of the activity of this enzyme in mitochondria and in isolated rat liver cells.

2. Compartmentation and communication

It is now widely accepted that glutamate dehydro-

genase is located exclusively in the mitochondria, and most probably in the matrix compartment, of rat liver [3]. A potent aspartate aminotransferase occurs in the same location as glutamate dehydrogenase; moreover another iso-enzyme occurs in the cytosol [8].

Evidence has also been provided that at least three transporting systems are involved in the passage of glutamate across the (inner) mitochondrial membrane of various types of mitochondria. These systems are the glutamate transporter (system I, which is probably a glutamate- OH^- antiporter) [9,10], the glutamate-aspartate antiporter (system II) and the glutamate-glutamine antiporter (system III) [11]. In the present context only systems I and II are important. Reference to the following situations enables the functions of system I and II to be assessed:

- 1) When isolated mitochondria oxidize glutamate to oxoglutarate using glutamate dehydrogenase system I is involved; this is necessarily so since no aspartate, which is required for the action of system II, is available.

- 2) When mitochondria oxidize glutamate to aspartate, a pathway which commonly occurs with isolated mitochondria and involves their aspartate aminotransferase and the enzymes for the oxidation of oxoglutarate to oxaloacetate, then carrier II is involved, since the only way in which the end-product aspartate can leave the mitochondria is by this route. System I cannot be involved to any extent since, if it were, aspartate would accumulate in the mitochondria and osmotic lysis would occur.

System I is inhibited by low concentrations of some sulphhydryl combining reagents, e.g. *N*-ethylmaleimide, has an apparent K_m for the inward transport of glutamate of 4 mM and permits a maximum rate of glutamate entry of 20 nmol/mg of protein/min at 37°C [12,13]. At this time there are no kinetic data for system II available to us, but this carrier is known to be inhibited by the anti-diabetic drug 1-(hexahydro-1-H-azepin-1-yl)-3-*p*-2-(5-methyl-isoxazol-3-carboxy-amido)-ethyl phenyl-sulphonyl-urea (Glisoxepide, Prodiaben: Bayer Company) [14] and is most probably electrophoretic in action [15].

The properties of the oxoglutarate-dicarboxylate antiporter are relevant in the present context; the apparent K_m for inward transport of oxoglutarate into malate-loaded mitochondria is 46 μM , with a maximum velocity of 43 nmol/mg of protein/min at 9°C [16]. Note that this velocity is twice that of system I for

glutamate, even when no correction for temperature is applied.

3. The effects of leucine

3.1. *On soluble glutamate dehydrogenase*

L-Leucine is a potent activator of the glutamate dehydrogenase activity of sonicated mitochondria [17]. The V_{\max} in the direction of glutamate synthesis was increased ten-fold; that in the direction of glutamate dehydrogenation two- to three-fold. The apparent K_m values for substrates, and nicotinamide nucleotides were increased in the presence of leucine. The Briggs-Haldane relationship may be shown to apply both in the presence and absence of leucine. The extent of activation of the glutamate dehydrogenase of sonicated liver mitochondria is higher than that reported for the purified bovine liver enzyme, and is observed at lower leucine concentrations (half-maximal effect at 1 mM).

3.2. *On the glutamate dehydrogenase activity of isolated mitochondria*

It has been shown that leucine readily penetrates to the matrix space of isolated liver mitochondria, the location of the glutamate dehydrogenase [17]. Further it has been shown that added leucine markedly stimulates glutamate synthesis from added 2-oxoglutarate (in the presence of L-malate to activate its entry) and NH_3 ; both the rate of oxidation of intra-mitochondrial NAD(P)H, measured spectrophotometrically, and the steady-state rate of glutamate synthesis (when reducing equivalents were provided continuously by the concomitant oxidation of pyruvate) were increased. By these criteria leucine activates the glutamate dehydrogenase of isolated mitochondria.

In contrast, no effect of leucine could be demonstrated on the rate of deamination of glutamate by intact mitochondria; this was the case when the rate of reduction of intra-mitochondrial NAD(P) was followed subsequent to the addition of glutamate or when the steady-state rate of NH_3 production was followed.

The simplest explanation of these findings is that the rate of inward transport of glutamate is limiting. This contention is supported by the following observations: 1) The observed rate of glutamate deamination is ten-fold higher with sonicated mitochondria, than with untreated mitochondria, i.e.

the enzyme is 'latent'. 2) The rate at which isolated mitochondria produce NH_3 from added glutamate is close to the rate of glutamate entry; this is the case with mitochondria derived from rats on a normal diet and rats on a high protein diet. In the latter both the rates of glutamate entry and of NH_3 production were two-fold higher [18]. 3) The NH_3 produced by glutamate deamination may in turn be used for citrulline synthesis, when ornithine and CO_2 are provided and ATP is used as an energy-source [18]. Under these conditions leucine had no effect on the rate of citrulline synthesis, either with mitochondria derived from rats fed a normal diet or those fed on a high protein diet. In the latter case both the rates of glutamate entry and of citrulline synthesis were increased two-fold in mitochondria from protein fed animals. Under the conditions of these experiments the rate of citrulline synthesis is limited by the availability of NH_3 . The rate is much higher when exogenous NH_3 is provided, but importantly, under these circumstances leucine had no effect on the rate of citrulline synthesis [18].

3.3. *On the metabolism of alanine by isolated liver cells* [19]

Isolated liver cells suspended in a suitable saline medium convert exogenously added alanine to glucose, glutamate and glutamine, lactate and ketone bodies and urea. Without changing the overall rate of utilization of alanine, the addition of 5 mM leucine markedly affects the distribution of the C and N atoms of alanine amongst the different metabolic products (table 1). Urea synthesis was decreased from a rate of 366 nmol of N atoms/mg of liver cell protein/hr to a rate of 100 in the presence of leucine; there was a corresponding increase in the rate of synthesis of glutamate and glutamine. Gluconeogenesis is also inhibited in the presence of leucine.

The following additional points should be noted. Leucine itself was not metabolized to any detectable extent; the recovery of added radio-actively labelled leucine was the same after incubation as it was initially. The effects observed were not due to a change in the pattern of metabolism of endogenous substrate; the pattern of metabolite production was virtually the same as judged by assays of the quantities of metabolites as it was when the fate of ^{14}C -labelled alanine was determined (table 1).

Table 1
Pattern of distribution of metabolic products resulting from the incubation of liver cells with alanine in the presence and absence of leucine

<i>Metabolic assays:</i>				
Conditions	<i>Contribution of metabolic assayed (%)</i>			
	Glucose	Glutamate	Glutamine	Lactate
No leucine	51	16	25	9
With leucine	30	27	37	5
<i>Distribution of ^{14}C</i>				
No leucine	56	18	26	—
With leucine	26	31	43	—

Note that there was no significant change in the total amount of alanine metabolized in the presence and absence of leucine. Data from [19].

3.4. Possible interpretations

The effect of leucine in decreasing the amount of urea synthesized by isolated rat liver cells may be accounted for in at least two ways. One way, based on traditional views, would be to postulate that leucine has another effect than its action on glutamate dehydrogenase, this other effect might be for example on some part of the urea cycle itself; glutamate dehydrogenase may well be activated by leucine but this is not the major effect. An alternative postulate, which we favour, is that glutamate dehydrogenase is not particularly important in hepatic NH_3 production; if it were, increasing its activity should either have no effect at all if it were not a rate-limiting enzyme or should stimulate if it were rate-limiting and was involved in ammoniogenesis for urea synthesis.

To take the first alternative. No evidence for inhibition (or activation for that matter) of any of the reactions of the urea cycle itself has been obtained. Thus, as was stated previously, leucine does not affect the synthesis of citrulline from ornithine, CO_2 and exogenous NH_3 , with ATP as an energy-source [17]. Leucine does not affect that part of the urea cycle which occurs in the mitochondrion. Using the cytosol fraction of rat liver it has also been shown that leucine does not affect the conversion of citrulline to ornithine and urea, in the presence of ATP and aspartate (Scheme 1) [19]. There is no evidence that leucine acts directly on the urea cycle.

To take the second alternative; glutamate dehydrogenase has less importance in ammoniogenesis than is usually thought to be the case. This hypothesis readily

accounts for the increased rate of synthesis of glutamate and glutamine which is observed in the presence of leucine, but carries with it two immediate implications; firstly, at least in the absence of leucine, the glutamate dehydrogenase is rate-limiting, and secondly, the NH_3 for urea synthesis comes from elsewhere.

In our opinion these findings are best explained in the following way: NH_3 is derived from some, as yet unspecified, source at a rate such that there is competition between the glutamate dehydrogenase on the one hand and the carbamoyl phosphate synthetase on the other; activation of the glutamate dehydrogenase leads to increased glutamate synthesis and consequently to decreased carbamoyl phosphate production; thus activation of the glutamate dehydrogenase leads to inhibition of urea synthesis. The feasibility of this interpretation is supported by the experiment described in the next section.

3.5. A mitochondrial model system [17]

The system was set up by providing conditions such that mitochondria were able to synthesize at the same time both glutamate (from added oxoglutarate, with pyruvate providing the necessary reducing equivalents) and citrulline (from added ornithine and CO_2 , with ATP as energy source). NH_3 was generated at a constant steady rate by the addition of adenosine and limiting, but varied, amounts of adenosine deaminase. In the presence of leucine, glutamate synthesis was markedly stimulated and that of citrulline inhibited [17].

There would seem to be therefore no necessity to

invoke further sites of action of leucine other than on the glutamate dehydrogenase. Other things being equal and making the not too-extravagant assumption that mitochondria in the cell work along similar lines to those in vitro, the expected outcome of increased mitochondrial glutamate synthesis would be an increase in glutamine also, and the expected result of decreased citrulline synthesis would be a decrease in urea production.

One important feature of this model is that the effects observed were obtained when NH_3 was generated exogenously to the mitochondrion. The possible nature of such a generating system is discussed in the next section.

4. The importance of glutamate dehydrogenase

We are now in a position to re-examine the four postulates on which the accepted importance of glutamate dehydrogenase in hepatic NH_3 production were said to rest. 1) Will be left to last, for its fall will depend on the demolition of 2) to 4). To take 2); the

contention that glutamate dehydrogenase activity is high in relation to the rate of urea synthesis (see table 2). In fact this is just not the case; the rate of deamination of added glutamate by intact mitochondria prepared from the livers of rats fed on a standard laboratory diet was found to be 4.3 ± 0.7 (S.E.M.) nmol of NH_3 /mg of mitochondrial protein/min at 30°C [18]. Previously, values for glutamate dehydrogenase activity, corresponding to more than ten times this value have been used in calculations; this totally ignores the contribution of the rate-limiting glutamate transporter (I). Indeed often values for the rate at which glutamate synthesis occurs have been employed; the V_{\max} for glutamate dehydrogenase in this direction is approximately ten times higher than in the direction of deamination, in the case of rat liver [18]. In these cases, that is where the enzyme activity has been assayed in the wrong direction and the permeability barrier ignored, the estimate is two orders of magnitude too high.

The situation appears to be this: working on the basis of 1 g wet weight of liver and with alanine as substrate, urea synthesis occurs at a rate of 350 nmol/

Table 2
A comparison of the rate of urea synthesis with rates of NH_3 production from glutamate by sonicated and intact rat liver mitochondria

Reaction	System	Conditions	Observed rate (nmol/min/mg of mitochondrial protein)	Equivalent or actual rate of urea synthesis* (nmol/min/gm wet wt at 37°C)	Ref.
Urea synthesis	Perfused liver	37°C ; alanine	—	350	[20,21]
Urea synthesis	Isolated cells	37°C ; alanine	—	540	[19,22]
Glutamate dehydrogenase (deaminating)	Sonicated mitochondria	30°C ; V_{\max}	55 ± 10	1260**	[18]
Glutamate dehydrogenase (aminating)	Sonicated mitochondria	30°C ; V_{\max}	406 ± 116	16 200	[18]
Glutamate dehydrogenase (deaminating)	Intact mitochondria	30°C ; 20 mM-glutamate	4.3 ± 0.7	80***	[18]

* The equivalent rates were derived on the assumption that there are 40 mg of mitochondrial protein/g wet wt of liver.

** This value has been derived on the assumption that the intracellular concn. of glutamate is 2 mM and application of the Michaelis-Menten relationship taking a K_m value for glutamate of 4 mM [17], and that the Q_{10} for glutamate dehydrogenase is 2.

*** This value has been derived on the assumption that the intracellular concn. of glutamate is 2 mM and that the K_m value for inward transport of glutamate is 4 mM [12]. The correction for temperature was calculated from ref. [12] and was 1.5.

min with perfused livers and 540 with isolated liver cells at 37°C [19–22]. The maximum rate at which glutamate dehydrogenase would appear to be able to produce NH_3 , taking into account the permeability barrier to glutamate, is 160 nmol/min at 30°C and 20 mM glutamate; correction to 37°C and a more physiological concentration of glutamate (2 to 3 $\mu\text{mol/g}$) gives a value of 80 nmol/min, which is clearly not enough.

Postulate 3), which is entirely negative, states that glutamate dehydrogenase is important in ammonio-genesis because no other enzyme or enzyme system is known to be sufficiently active in liver. Lowenstein [see 6] has shown that in skeletal muscle there is an active 'AMP cycle', involving the deamination of AMP to NH_3 and IMP, and the conversion of the latter to AMP with aspartate acting as amino donor (see Scheme 1). Lowenstein has also considered this system in liver, but in this case he concluded that the AMP cycle was not likely to be so active and was probably of lesser significance than glutamate dehydrogenase. However, it has been shown in this Laboratory [J. D. McGivan and K. Moss-unpublished observations] that the AMP deaminase activity of rat liver cytosol fractions is relatively low in the presence of AMP alone; the addition of ATP or of analogues of ATP (adenylyl (β,γ -methylene)diphosphate or adenylyl-imidodiphosphate) caused a marked increase in activity. Under the conditions of these experiments and in the presence of 2 mM ATP the rates of AMP deamination observed are approximately 20 nmol of NH_3 /mg of cytosol protein/min at 37°C; this corresponds to a rate of 2000 nmol NH_3 /g wet weight of liver/min, a rate four times greater than that needed to support urea synthesis from alanine. Of course, other factors must be taken into account; particularly the prevailing concentrations of AMP and ATP and the rates at which IMP can be aminated are obviously important. With regard to this latter process less definite results have been obtained; rates equivalent to a rate of urea synthesis of 350 nmol/min/g wet wt have been routinely observed. Improvement in assay conditions may well lead to an inflation of this value.

Postulate 4) is that the level of glutamate dehydrogenase activity in liver cells is so high that at all times the level of substrates, products and oxidized and reduced nicotinamide nucleotides in the mitochondrial matrix space is held at 'thermodynamic equilibrium'.

Thus by measuring the amounts of substrates and products of the glutamate dehydrogenase, and making the further assumption that there are no significant permeability barriers or substrate-anion gradients across the mitochondrial membrane, calculations of the redox state of intramitochondrial nicotinamide nucleotides have been made (see [7]). However the fact that leucine is able to perturb the substrate flow through glutamate dehydrogenase does not appear to us to be compatible with the assumption that the reactants and products of this enzyme are already at 'equilibrium'. Either postulate 4) is incorrect or leucine does not affect the glutamate dehydrogenase of intact cells. If the latter is the case, then how does leucine exert its effects on liver cells?

Postulate 1) states that it is plausible that glutamate dehydrogenase is involved in NH_3 production for urea synthesis. This would seem not to be the case since the addition of leucine, which by other criteria may be seen to affect glutamate dehydrogenase activity (glutamate and glutamine synthesis in whole cells, glutamate dehydrogenase activity in the direction of amination in intact isolated mitochondria), did not promote an increase in urea synthesis by isolated cells; on the contrary increasing the activity of glutamate dehydrogenase inhibited urea synthesis. We can conceive of no set of circumstances under which the activation of an enzyme can lead to an inhibition of the pathway in which it is involved.

5. Illations

If the views expressed in this article are ultimately found to have any semblance to reality, then the following generalizations may be made. Aspartate, not glutamate, is the amino acid which occupies the central position in nitrogen excretion processes. Aspartate would provide *both* of the N atoms of urea, one through the AMP cycle and one at the level of arginosuccinate synthesis; aspartate acts as N donor in purine and pyrimidine synthesis (when this is not so glutamine, not glutamate is donor); in bacteria aspartase cleaves aspartate to form NH_3 and fumarate.

Glutamate dehydrogenase is involved mainly in glutamate synthesis; thermodynamically this is very much favoured; it is the first reaction on the pathway to glutamine synthesis; it is an enzyme involved in N storage.

Historically, much useful knowledge has been obtained from a study of the effects of metabolic inhibitors, e.g. the effects of malonate on succinate dehydrogenase and the elucidation of the nature of the tri-carboxylate cycle. In theory at least, and with certain reservations with regard to rate-limitation, activators of enzyme, when they can be found, should be equally useful. It is suggested that the effect of leucine on glutamate dehydrogenase is a case in point.

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