The Distinction Between γ -Glutamylhydroxamate Synthetase and L-Glutamine-Hydroxylamine Glutamyltransferase Activities in Rat Tissues

STUDIES IN VITRO

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Two common ways of measuring the potential for glutamine synthesis in a tissue are the rates of formation of γ -glutamylhydroxamate either by synthesis from glutamate (the glutamylhydroxamate synthetase reaction) or by transfer from glutamine (the glutamyltransferase reaction); it has not been established, however, that either reaction is a specific measure of glutamine synthetase. By differential extraction of glutamylhydroxamate synthetase and glutamyltransferase activities from water homogenates of several rat tissues I obtained an extract, rich in glutamylhydroxamate synthetase activity but nearly devoid of glutamyltransferase activity, and a fraction, solubilized by deoxycholate from the pellet, which contained virtually no glutamylhydroxamate synthetase activity but most of the original glutamyltransferase activity. Synthesis of glutamine, quantitatively similar to the γ -glutamylhydroxamate formed by glutamylhydroxamate synthetase, is catalysed in the water extract but not in the particulate fraction. γ -Glutamylhydroxamate formation by glutamylhydroxamate synthetase and glutamyltransferase shows discrepant substrate and metal specificities and can be differentially inhibited by L-methionine sulfoximine, phosphate and adenine nucleotides. The concordance between the formation of glutamine and γ -glutamylhydroxamate by glutamylhydroxamate synthetase but not by glutamyltransferase and the different solubilities of the glutamylhydroxamate synthetase and glutamyltransferase enzyme activities demonstrate that these two activities are not inextricably associated; they therefore cannot be catalysed exclusively by the same protein.

Glutamine synthetase [L-glutamate-ammonia ligase (ADP), EC 6.3.1.2] catalyses the formation of L-glutamine from L-glutamate by the following reaction:

Although a variety of procedures for specific measurements of glutamine have become available (Horowitz *et al.*, 1968; Kupchik & Knox, 1970; Prusiner & Milner, 1970; Buttery & Rowsell, 1971; Cooney *et al.*, 1971), glutamine synthetase is generally assayed colorimetrically by the formation of γ -glutamylhydroxamate from an activated glutamine residue. The reactive moiety is a postulated intermediate that reacts with hydroxylamine during the synthesis of glutamine (reaction 2) or one formed by arsenolysis of glutamine in the transfer reaction (reaction 3):

* Abbreviations: GS enzyme, γ -glutamylhydroxamate synthetase; GT enzyme, glutamyltransferase (L-glutamine-hydroxylamine glutamyltransferase).

The enzyme catalysing reaction (2) is here called GS enzyme; the catalyst of reaction (3) is called GT enzyme; the physiological reaction (1) is that of glutamine synthetase. The chemical basis for use of reactions (2) or (3) as measures of glutamine synthetase (reaction 1) rests on the studies of Meister (1962, 1969), who found that highly purified preparations from rat liver and from sheep brain catalysed all three reactions. A molecular mechanism involving an enzyme-bound glutamine residue was proposed, which could account for reaction (3) as part of the overall synthesis of glutamine or glutamylhydroxamate, depending on the acceptor used. It has not been established that in crude tissue extracts only glutamine synthetase catalyses the three reactions.

Since glutamine synthesis is a critical metabolic function and has been the subject of various crucial studies of enzyme induction and of biochemical differentiation *in vivo* as well as in tissue cultures (Fottrell & Paul, 1961; Moscona *et al.*, 1968; Reif-Lehrer & Amos, 1968; Chader, 1971), it is important

to clarify the question of the relationships of reactions (2) and (3) to actual glutamine synthesis. Many of the workers interested in the physiological role and regulation of glutamine synthetase have assumed that the reactions can be used interchangeably as presumptive measures of glutamine synthetase. One of the criteria for equating reactions (2) and (3) with measures of reaction (1) must be the constancy of their ratios at various stages of isolation or purification as well as among the several tissues. In fact, these ratios appear not to have remained constant even during the purification in the course of which both activities were measured (Lajtha *et al.*, 1953; Meister, 1962). There were also indications of inconstancy between the two reactions where comparisons among tissues have been recorded (Levintow, 1954; Raina & Ramakrishnan, 1964; Berl, 1966; Herzfeld & Estes, 1973).

A detailed comparison has now been made of their specificity with regard to substrates and inhibitors and of methods by which reactions (2) and (3) could be separated *in vitro*. The congruence between glutamine formation (reaction 1) and γ -glutamylhydroxamate formation by either of the other two reactions was also tested. It is apparent that crude tissue extracts contain GT enzyme activity not associated with GS enzyme or glutamine synthesis. The accompanying paper details further differences in the physiological behaviour of reactions (2) and (3) (Herzfeld & Estes, 1973).

Materials and Methods

Tissues were from inbred male Kx rats (formerly described as NEDH rats) between 60 and 90 days old. Freshly excised tissues were disrupted in glass-Teflon homogenizers or, in the case of muscle, in conical all-glass homogenizers, in 9 vol. of cold water. For the separation of GS enzyme and GT enzyme activities, the water homogenates were kept at 0–4°C for at least 60 min, then centrifuged at 105 000g for 30 min at 4°C and the supernatant fraction (water extract) was separated. The pellet was washed once in cold water, then resuspended to the original volume at a final deoxycholate concentration of 0.2%. After 30 min it was centrifuged as described above. The original water supernatant, the deoxycholate extract and the extracted pellet, resuspended in water, were all assayed for the various activities.

Enzyme assays

The assay for GT enzyme activity (reaction 3) was similar to that described by Thorndike & Reif-Lehrer (1971) except that each reaction mixture (total vol. 0.5 ml) contained 0.2 mM-ADP and the Mn^{2+} concentration was increased to 10 mM to obtain maximal activity. Samples (0.05–0.1 ml) of 0.5–1% (w/v) tissue preparations were used. Samples were

incubated for 10 min at 37°C, then deproteinized with 0.1 ml of 50% (w/v) trichloroacetic acid and the γ -glutamylhydroxamate was measured spectrophotometrically (at 500 nm) with 0.5 ml of the ferric chloride reagent described by Pamiljans *et al.* (1962). Non-incubated duplicate samples served as blanks, and the values for them were subtracted from those of the experimental samples.

GS enzyme activity (reaction 2) was assayed by the procedure of Wu (1963b), modified to contain an ATP-generating system (Knox *et al.*, 1971) and a lower concentration of ATP (6.4 mM) to give maximal GS enzyme activity. Duplicate reaction mixtures at pH 7.3 in total volumes of 0.5 ml contained 65 mM each of L-glutamate and hydroxylamine, 32 mM-Tris-HCl buffer, pH 7.3, 16 mM of both $MgCl_2$ and phosphoenolpyruvate, 6.4 mM-ATP, 5 units of pyruvate kinase and 0.05–0.1 ml of 5–10% (w/v) tissue preparations. After incubation at 37°C for 20 min, the reaction was stopped with 0.1 ml of 50% trichloroacetic acid, left for 10 min, mixed with 0.5 ml of ferric chloride reagent (Pamiljans *et al.*, 1962) and centrifuged. The E_{500} was measured in a Zeiss PMQ II spectrophotometer. γ -Glutamylhydroxamate (Calbiochem, La Jolla, Calif., U.S.A.) in the usual reaction mixture had a molar extinction coefficient (ϵ) of 0.7×10^3 litre $\cdot$ mol $^{-1}$ $\cdot$ cm $^{-1}$. Blanks, whose readings were subtracted from those of the experimental samples, were either complete mixtures that had not been incubated or incubated mixtures from which ATP and the generating system had been omitted. Since the values for both blanks were very similar, the latter ones were preferred.

For all measurements of glutamine synthesis by reaction (1) hydroxylamine was replaced by 60 mM- NH_4Cl in the standard GS enzyme incubation mixture. The amount of glutamine formed was determined after the reaction either by the glutamine aminotransferase (EC 2.6.1.15) reaction (Kupchik & Knox, 1970) or by the isolation of [^{14}C]glutamine formed from L-[U- ^{14}C]glutamate. In the first method, samples taken before and after 20 min incubation in the reaction mixture described above were added, without further manipulation, to an excess of kidney supernatant (105 000g for 30 min), as source of glutamine aminotransferase, and phenylpyruvate as the amino acceptor (Kupchik & Knox, 1970). The 300 mM-boric acid-NaOH buffer, pH 8.5, in the glutamine aminotransferase incubation mixture prevented further glutamine formation. The zero-time incubation mixtures served as blanks for the glutamine contents of the tissues. Because of the high tissue concentrations of glutamine (Kupchik & Knox, 1970), these samples always gave a significant blank value in the determinations of glutamine synthetase by glutamine aminotransferase. Glutamine formation was therefore measured alternatively by isolation of the L-[^{14}C]glutamine produced

from L-[U-¹⁴C]glutamate during the GS enzyme incubation. L-[U-¹⁴C]Glutamate (New England Nuclear, Boston, Mass., U.S.A.) (33 μ mol; 0.08 μ Ci/ μ mol) was incubated for 0 and 20 min with the usual reaction mixture containing NH_4^+ and 0.1 ml of 10% (w/v) tissue extract in a total volume of 0.5 ml. At the end of the incubation 0.12 ml of 25% sulphosalicylic acid was added. The deproteinized supernatants, neutralized with NaHCO_3 , were diluted to 1.0 ml with 5 mM-Tris-HCl buffer, pH 7.0. The extract was applied to columns (0.5 cm $\times$ 10 cm) of acid alumina (Bio-Rad, Richmond, Calif., U.S.A.) previously equilibrated with 5 mM-Tris-HCl buffer, pH 7.0, and the glutamine formed was washed out with the same buffer in three fractions in a total volume of 16.0 ml. Glutamate was retained on the column (Bessman, 1957) and eluted with 3 M- and 6 M-HCl. Then 0.01 ml of each fraction together with 0.05 μ mol of carrier L-glutamine and L-glutamate were chromatographed on silica-coated chromatogram sheets (Eastman Kodak, Rochester, N.Y., U.S.A.) in ethanol-aq. ammonia (7:3, v/v) (Brenner *et al.*, 1969) for 75–90 min. The R_f values for L-glutamine and L-glutamate were 0.65 and 0.45 respectively, when located with ninhydrin. Duplicate chromatograms were cut into 1 cm sections, eluted for 30 min in 1.0 ml of water and counted for radioactivity in 10.0 ml of Aquafluor in a Packard Tri-Carb liquid-scintillation counter at an efficiency of 90% for ¹⁴C. To determine non-enzymic as well as enzymic hydrolysis of glutamine during incubation and subsequent manipulations, mixtures containing non-radioactive L-glutamate but with L-[U-¹⁴C]glutamine (30 mM; specific radioactivity 7000 d.p.m./ μ mol) added at zero time were subjected to the same procedures, and the values found for glutamine formation were corrected for the amount of hydrolysis. This was relatively high in homogenates, especially of muscle, but lower in water extracts [cf. glutaminase location in mitochondria (Horowitz & Knox, 1968)].

All enzyme activities are expressed as units (μ mol of product/min at 37°C) per g of tissue.

Results

Assay conditions

To ascertain whether the conditions previously described for measuring GS enzyme activity (reaction 2) (Wu, 1963b) in rat liver and brain, and for GT enzyme activity (reaction 3) (Chader, 1971; Thorndike & Reif-Lehrer, 1971) in retina are optimum for other rat tissues, some of the requirements of the assays were determined in liver, brain, heart and muscle. The modified reaction conditions as described in the Materials and Methods section were optimum and kinetics were linear over a 25 min incubation time at 37°C for both reactions. The amounts of products formed were proportional to the amounts of enzyme

added, the only exception occurring with small amounts of muscle and heart, where less than 2.5 mg of tissue per GS enzyme assay gave disproportionately low γ -glutamylhydroxamate values. Doubling or decreasing the concentration of hydroxylamine in either GT enzyme or GS enzyme assays did not alter γ -glutamylhydroxamate formation significantly.

The alterations from assay conditions used previously consisted mainly of the use of an ATP-generating system in the GS enzyme reaction (Wu, 1963a) and an increase in the Mn^{2+} concentration from 2 to 10 mM in the GT enzyme reaction (Thorndike & Reif-Lehrer, 1971).

Table 1 demonstrates the more stringent requirement for an ATP-generating system for the GS enzyme reaction in muscle than in liver preparations. In muscle, perhaps because of its adenosine triphosphatase activity (Kielley & Meyerhof, 1948; Mommaerts & Green, 1954), only about 2% of its GS enzyme activity could be detected when phosphoenolpyruvate and pyruvate kinase were omitted from the incubation mixture, whereas their omission from incubations with liver homogenates yielded about one-half the usual GS enzyme activity. With the ATP-generating system, muscle was three times as active as liver (Table 1). Wu (1963b) found no GS enzyme activity in either skeletal muscle or heart when using 4 mM-ATP in the absence of a generating system. Iqbal & Ottaway (1970) found that muscle did catalyse γ -glutamylhydroxamate formation but at a rate much lower than liver.

Substrate, analogue and metal specificities for GS enzyme and GT enzyme

Table 2 shows that increases in the Mn^{2+} concentration from 2 to 10 mM enhanced the GT enzyme reaction by a factor of 1.6–2.0. This applied to all tissues except retina, for which the original assay

Table 1. *Dependence of γ -glutamylhydroxamate synthetase (GS enzyme) activities on ATP regeneration in muscle and liver homogenates*

Homogenates were assayed in duplicate in the complete standard incubation mixture (see the Materials and Methods section) containing 6.4 mM-ATP, 16 mM-phosphoenolpyruvate and 5 units of pyruvate kinase, or with omission of the last two components.

Incubation mixture	Enzyme activities (units/g)	
	Skeletal muscle	Liver
Complete	10.3	3.6
Minus phosphoenolpyruvate and pyruvate kinase	0.19	1.5

Table 2. Specificity for amino acids and metal ions in the γ -glutamylhydroxamate synthetase (GS enzyme) and L-glutamine-hydroxylamine glutamyltransferase (GT enzyme) reactions in liver homogenates

All assays were performed as described in the Materials and Methods section except for the alterations in amino acids and metal ions as noted. Enzyme activities are expressed relative to the standard assay value for adult male liver. The absolute GS enzyme and GT enzyme activities (units/g) are given in brackets in the first line of the Table.

GS enzyme					GT enzyme				
Final [L-glutamate] (mM)	Final [Mg ²⁺] (mM)	Additions (mM)		Relative enzyme activities	Final [L-glutamine] (mM)	Final [Mn ²⁺] (mM)	Additions (mM)		Relative enzyme activities
65	16	None	None	1.0 [4.2 ± 0.2]	256	2	None	None	1.0 [151 ± 18]
32	16	None	None	0.89	256	2	L-Glutamate	60	0.36*
125	16	None	None	0.66	256	2	L-Glutamate	100	0.25*
0	16	D-Glutamate	65	0.94	0	2	D-Glutamine	256	0.04
65	16	L-Glutamine	65	1.00	320	2	None	None	0.82
0	16	L-Aspartate	65	0.51	0	2	L-Asparagine	256	0.03
65	0	None	None	0.08	256	0	None	None	0.04
65	26	None	None	1.00	256	0	Mg ²⁺	10	0.08
65	16	Mn ²⁺	16	1.00	256	2	Mg ²⁺	10	0.98
65	0	Mn ²⁺	10	0.25	256	10	None	None	1.78
65	0	Mn ²⁺	20	0.08	256	20	None	None	0.95

* Studies in tissue extracts have not confirmed the inhibition of GT enzyme activity by L-glutamate found in liver homogenates (A. Herzfeld, unpublished work).

(Thorndike & Reif-Lehrer, 1971) was designed. Table 2 illustrates that the amino acid substrate concentrations used were optimum for the GS enzyme and GT enzyme activity determinations in liver. For the GS enzyme reaction 65mM-L-glutamate was saturating, whereas 256mM-L-glutamine saturated the GT enzyme reaction, and higher concentrations of the appropriate substrates inhibited both reactions somewhat. Table 2 also shows that the specificity for substrate isomers was not parallel in the two reactions. Although D- or L-glutamate served equally as substrates for the GS enzyme reaction, GT enzyme activity was virtually absent when the D-isomer replaced L-glutamine. These observations confirm the findings of Meister (1962). The addition of 65mM-L-glutamine did not decrease γ -glutamylhydroxamate formation in reaction (2) (GS enzyme); only 25–36% of the control amount of γ -glutamylhydroxamate was formed, however, when 60–100mM-L-glutamate was added to the GT enzyme reaction mixture (reaction 3). Recent studies (A. Herzfeld, unpublished work), however, have not confirmed this extensive inhibition of GT enzyme activity by L-glutamate. Some hydroxamate formation occurred when L-aspartate replaced glutamate in the GS enzyme reaction, but essentially no hydroxamate was formed when L-asparagine was substituted for L-glutamine in the GT enzyme mixture. The GS enzyme reaction was highly specific for Mg^{2+} and an increase in the Mg^{2+} concentration above that used as a routine yielded no further increments in GS enzyme activity. Replacement of Mg^{2+} by Mn^{2+} ions in a concentration range corresponding to the concentration of Mg^{2+} in the standard GS enzyme assay yielded only a small fraction of the GS enzyme activity found in controls. Tate & Meister (1971) found that the V_{max} of partially purified rat liver GS enzyme in the presence of Mn^{2+} was about one-quarter that when Mg^{2+} was present. Addition of 10mM- Mg^{2+} to the GT enzyme incubation mixture already containing 2mM- Mn^{2+} had no effect on the yield of product. When Mg^{2+} was omitted from the GS enzyme reaction mixture or Mn^{2+} from the GT enzyme reaction mixture, virtually no hydroxamates were formed. Most of the parameters shown in Table 2 were also tested on homogenates from brain or muscle, and the results confirmed those found with liver.

Distribution of GS enzyme and GT enzyme activities

By using the optimum assay conditions for GS enzyme and GT enzyme in muscle, liver and brain homogenates, one would expect to find the two activities proportional, though not equal, in the various tissues if both reactions were catalysed exclusively by a single enzyme. As shown by the absolute enzyme-activity values given in Table 3, they were neither equal nor proportional.

Brain was similar in enzyme content to liver. Muscle had more GS enzyme and less GT enzyme than either liver or brain and lung was relatively deficient in GT enzyme. (This is also shown by the ratios of the two activities in homogenates.) It should be noted that glutamine formation occurs at the rate of γ -glutamylhydroxamate formation by GS enzyme and not by GT enzyme.

Attempts were then made to separate the two activities by solubilization and to determine which of the two activities was associated with the formation of glutamine (reaction 1). Since Wu (1963a) has shown that GS enzyme in rat liver occurs bound to microsomal fractions, and Salganicoff & De Robertis (1965) found that 80% of the GS enzyme activity in rat brain was distributed among all intracellular organelles (44% of it in the microsomal fraction), we used several standard procedures in attempts to extract both GS enzyme and GT enzyme activities from rat tissue homogenates. Although water, 0.1–0.5% Triton X-100, sonication for 3min or three cycles of freezing and thawing released at least 70% of the GS enzyme activity into the 105000g supernatant, less than 15% of the GT enzyme activity could be recovered in the soluble fraction. None of the treatments inactivated the enzymes, and the unsolubilized activities were found in the resuspended pellets. The best results were obtained when tissue homogenates prepared in water were extracted for 1h at 4°C, separated by centrifugation at 105000g (30min) and the pellets were extracted with 0.2% deoxycholate or with 0.15M-KCl. Homogenates of liver, brain and lung required at least 1h at 4°C for extraction of the GS enzyme activity with water; less time was needed to solubilize the GS enzyme activity from muscle.

Table 3 demonstrates that the GS enzyme reaction (reaction 2) was catalysed primarily by the water extracts of all the tissues listed. The recovery of GS enzyme activity in the water fraction ranged from 70 to 98% over a series of experiments. Only a small portion of the GS enzyme activity remained associated with the pellet and was extracted into the deoxycholate supernatant. A large fraction of the GT enzyme activity resisted solubilization by water but was subsequently solubilized by deoxycholate or 0.15M-KCl. In liver, brain and lung, less than 15% of the GT enzyme activity was detectable in the water supernatants whereas deoxycholate treatment converted the major portion of it into soluble form. Water extracts from liver homogenates were subjected to DEAE-cellulose chromatography (Table 4). Although the GS enzyme activity was purified about 14-fold, it could not be dissociated from a residual fraction of GT enzyme activity. The GT enzyme/GS enzyme activity ratios remained constant at about 8.5 during this purification step of the water extract. Since, however, only about 15% of the total GT enzyme activity

Table 3. *Distribution and fractionation of glutamine synthetase, γ -glutamylhydroxamate synthetase (GS enzyme) and L-glutamine-hydroxylamine glutamyltransferase (GT enzyme) activities in some tissues*

The enzyme activities (means of determinations in three to six animals $\pm$ 1 s.d.) in units/g of tissue for each of the homogenates are given in brackets. Activities in the fractions are expressed relative to those in the appropriate homogenates. The ratios between GT enzyme and GS enzyme were calculated from the absolute enzyme activities (units/g) in each fraction. The preparation of the supernatants and the extraction of the remaining particulate fraction with 0.2% deoxycholate are described in the Materials and Methods section. All assays are described in the Materials and Methods section. In homogenates, glutamine formation (glutamine synthetase) was measured by the glutamine aminotransferase reaction; in the fractions, glutamine formed was determined by that assay or was isolated as [14 C]glutamine formed from L-[U- 14 C]glutamate (see the Materials and Methods section). Note that, in muscle, glutamine formation in the homogenate reflects only 20% of the γ -glutamylhydroxamate formed by the GS enzyme reaction.

Tissue	Fraction assayed	Activity (% of that of homogenate)			Ratio [calculated from enzyme activities (units/g)] of GT enzyme/GS enzyme
		GS enzyme	Glutamine synthetase	GT enzyme	
Liver	Water homogenate	100 [4.5 $\pm$ 0.5]	100 [4.3 $\pm$ 1.3]	100 [227 $\pm$ 30]	50
	Supernatant	76	69	11	6.7
	Deoxycholate extract of pellet	18	28	82	230
Brain	Water homogenate	100 [3.5 $\pm$ 0.5]	100 [3.4 $\pm$ 0.1]	100 [187 $\pm$ 22]	53
	Supernatant	80	97	13	5.1
	Deoxycholate extract of pellet	23	0	73	171
Skeletal muscle	Water homogenate	100 [10.5 $\pm$ 1.0]	100 [2.0 $\pm$ 0.8]	100 [15 $\pm$ 3]	1.4
	Supernatant	97	55	40	0.6
	Deoxycholate extract of pellet	13	0	80	8.6
Lung	Water homogenate	100 [1.65 $\pm$ 1.1]	—	100 [14 $\pm$ 4]	8.2
	Supernatant	97	—	7	0.56
	Deoxycholate extract of pellet	10	—	36	31

Table 4. *Partial purification of glutamylhydroxamate synthetase (GS enzyme) and L-glutamine-hydroxylamine glutamyltransferase (GT enzyme) activities from rat liver*

The water extract of liver (see the Materials and Methods section) containing 5 mm-2-mercaptoethanol and 1 mm-EDTA was applied to a DEAE-cellulose column (2.5 cm $\times$ 16 cm) (H. Reeve Angel and Co., Clifton, N.J., U.S.A.) The column was washed with 30.0 ml of 0.005 M-sodium phosphate buffer, pH 7.3, and eluted with 90 ml of 0.01 M-sodium phosphate buffer, pH 7.3, and 90.0 ml of 0.1 M-sodium phosphate buffer, pH 7.3. The GS enzyme and GT enzyme activities were recovered in about 10 fractions of 3.0 ml each after elution with 45 ml of 0.1 M buffer. Proteins were determined by the procedure of Lowry *et al.* (1951) with crystalline albumin as standard.

	Protein (mg/g of tissue)	Enzyme activities				Ratio of GT enzyme/ GS enzyme
		GS enzyme		GT enzyme		
		(units/g)	(units/mg of protein)	(units/g)	(units/mg of protein)	
Water homogenate	166	4.0	0.024	195	1.17	49
Water extract	61	3.4	0.055	29	0.48	8.5
DEAE-cellulose fraction	9		0.33		2.7	8.2
KCl extract		0.3		116		382

was released into the water extract, these results indicate that two forms of GT enzyme activity occur in rat liver: one firmly associated with the fraction that catalyses the GS enzyme reaction, the other independent of GS enzyme activity. A similar dichotomy of forms of GT enzyme activity was found in brain. Since muscle and lung contain little total GT enzyme activity, its measurements in the two fractions may be spurious. Preliminary evidence suggests that muscle contains no independent GT enzyme activity (A. Herzfeld, unpublished work).

Although the measurement of hydroxamate formation is useful operationally, in crude homogenates it is at best a measure of a partial reaction (activated intermediate) and at worst, a summation of many partial reactions, perhaps unrelated to glutamine synthesis. I therefore determined which of the solubilized enzyme activities contained the enzyme actually catalysing the synthesis of glutamine.

It is clear from Table 3 that in brain and muscle the water extract alone had the capacity to catalyse the formation of glutamine; in liver also most of reaction (1) occurred in the water extract. Both liver and brain homogenates formed an equimolar amount of glutamine and γ -glutamylhydroxamate from L-glutamate. Glutamine formation in muscle occurred to a significant extent in the same water extract as γ -glutamylhydroxamate formation, but it represents only a small fraction of the hydroxamate formed in reaction (2). However, water extracts of muscle hydrolysed as much as 30 μ mol of glutamine/min per g when incubated under conditions optimum for the glutamine synthetase reaction, in the presence of 0.050 M-L-[U- 14 C]glutamine. Under the same conditions, liver and brain extracts hydrolysed less than 0.6 and 0.1 μ mol of glutamine/min per g, respectively.

The ratio of the GS enzyme and GT enzyme activities was grossly altered by this fractionation without serious losses of either. The column in Table 3 showing these ratios of absolute units of enzyme activity emphasizes dramatically the disparity in the solubility of the two activities: the water extract, in all tissues, was enriched in GS enzyme six- to ten-fold over the homogenate whereas the deoxycholate extract (except for muscle) represented a three- to four-fold enrichment of the GT enzyme activity. Thus the differential solubilization of the two activities suggests that different proteins account for most of the activities of reactions (2) and (3). The stoichiometric relationship in liver and brain homogenates and in water extracts between glutamine formation (reaction 1) and γ -glutamylhydroxamate formation by reaction (2) also suggests that the GS enzyme measurements (reaction 2) are valid indicators for glutamine synthetase activity, whereas most of reaction (3) must measure an as yet unknown activity.

Inhibitors of glutamine synthetase and L-glutamyltransferase

To distinguish further the GS enzyme and L-glutamyltransferase (GT enzyme) activities, separable by their differential solubilities, some compounds were tested as inhibitors of one or the other reaction. L-Methionine sulfoximine has been used as a potent and specific inhibitor of glutamine synthetase (Meister, 1968). Table 5 shows that although 5 mM-L-methionine sulfoximine inhibited the GT enzyme reaction by 70%, it required 40 mM-L-methionine sulfoximine to attain a 50% inhibition of GS enzyme activity in liver homogenates. GT enzyme activity was also more sensitive to phosphate-containing compounds. As little as 6 mM- P_i inhibited the GT enzyme reaction by 50%; it was necessary to add 20 times as much P_i to obtain similar inhibition of the GS enzyme reaction. The GT enzyme reaction was even more sensitive to ATP than to P_i ; 1.0 mM-ATP virtually eliminated the reaction. Concentrations higher than 10 mM-ATP were needed to inhibit the GS enzyme reaction even slightly. Thus the optimum concentration of ATP for glutamine synthetase was completely inhibitory to GT enzyme activity. Five times the concentration of ADP used as a routine for the GT enzyme reaction had no effect on the GS enzyme activity. Further increases in the ADP concentration (10 mM) did inhibit the GS enzyme activity without corresponding inactivation of the GT enzyme activity. The sensitivity of the GT enzyme reaction to P_i and ATP, in addition to its inhibition by glutamate (Table 2) and its other properties, would substantially prevent its action in the usual GS enzyme assay. The P_i released during the conversion of ATP into ADP + P_i in the activation of glutamate alone sufficed to decrease the rate of the GT enzyme reaction by about 50%. These differential inhibitions, especially the relative sensitivity of GT enzyme reaction to L-methionine sulfoximine and to P_i , are further indications of a clear distinction between the two enzymes; such a distinction was also shown in their solubilization and their reactivity with substrates, analogues and metal cofactors.

Discussion

The partial purification of GS enzyme from various sources (Pamijans *et al.*, 1962; Meister, 1969; Ronzio *et al.*, 1969; Iqbal & Wu, 1971) has usually been monitored by measuring the formation of γ -glutamylhydroxamate with either glutamine (and ADP) or glutamate (and ATP) as substrates or cofactors. Observations that both GT enzyme and GS enzyme activities could be detected throughout purification were taken as evidence that both reactions were catalysed by the same protein. It was, however, mentioned by Levintow (1954) and also by Levintow *et al.* (1955) and Meister (1962) that the ratios between

Table 5. *Inhibitors of γ -glutamylhydroxamate synthetase (GS enzyme) and L-glutamine-hydroxylamine glutamyl-transferase (GT enzyme) activities in liver homogenates*

All activities are normalized to the activities in the standard assay (units/g = 1.0 reference unit). The absolute values per g of liver for control reactions with four animals are given in brackets; all the other values are averages from two closely agreeing determinations of water homogenates of two livers. L-Methionine sulfoximine was obtained from Calbiochem. The pH of the inhibitors was appropriate for the respective reactions (GS enzyme, pH 7.3; GT enzyme, pH 6.3).

Additions		Enzyme activities (reference units)	
	(mM)	GS enzyme	GT enzyme
None		1.0 $\pm$ 0.03 [4.75 $\pm$ 0.15]	1.0 $\pm$ 0.2 [195 $\pm$ 28]
L-Methionine sulfoximine	5	0.95 $\pm$ 0.08	0.26 $\pm$ 0.05
	10	0.72 $\pm$ 0.08	0.18 $\pm$ 0.02
	40	0.50 $\pm$ 0.17	0.13 $\pm$ 0.03
P _i	6	1.0	0.48
	30	1.0	0.15
	120	0.49	0.05
ATP	0.1	—	0.83
	1.0	—	0.27
	10.0	0.80	0.02
ADP	1	0.91	—
	10	0.63	1.0
	20	0.42	—

the two activities did not remain constant in the course of purification. This discrepancy was not investigated any further. Such inconstancy of ratios suggests that the activities may not reside exclusively in the same molecule. Assays under suboptimum conditions for either enzyme also would tend to obscure the magnitude of the real discrepancies.

By using optimum assay conditions in independent assays for the three reactions attributed to this single enzyme, our observations cannot be reconciled with the hypothesis that only a single protein catalyses all three reactions. A clear distinction between GS enzyme and GT enzyme in liver, muscle, lung and brain was established by the differential solubilization of the two activities. Most of the GS enzyme activity was extracted by simple water solubilization whereas the GT enzyme activity remained bound to particles unless extracted with deoxycholate or KCl. One was thus able to separate two fractions: one virtually free of GT enzyme, the other containing almost all of the GT enzyme and only a small amount of the GS enzyme activity. The constancy of the GT enzyme/GS enzyme activity ratios during partial purification of the water extract of liver, representing about 15% of the total GT enzyme activity (but 85% of the GS enzyme activity) suggests strongly that the total homogenate contains two separable forms of GT enzyme activity, catalysed by different enzyme molecules. It is very likely that the mechanism proposed by Meister (1962) to account for the multiplicity of reactions catalysed by his purified preparations is applicable to

that small portion of GT enzyme activity that is extracted conjointly with GS enzyme activity. A different mechanism of action and a new physiological function will have to be attributed to the newly discovered activity that is responsible for the formation of L-glutamylhydroxamate from L-glutamine and hydroxylamine in the presence of Mn²⁺ and ADP, and which appears to be independent of GS enzyme activity.

Little or no glutamine accumulated in the fractions that catalysed the GT enzyme reaction. That γ -glutamylhydroxamate formation from glutamate in the water extract may well be catalysed by glutamine synthetase itself was evident from measurements of the actual accumulation of glutamine synthesized in the same fraction under our assay conditions. Only muscle (and, to a lesser extent, heart), failed to show an equivalence between hydroxamate formation and glutamine formation. This discrepancy in the accumulation of products in muscle can probably be accounted for by the rapid hydrolysis of glutamine that occurs in water extracts of muscle homogenates under the conditions of our assay.

Muscle also hydrolyses ATP rapidly (Kielley & Meyerhof, 1948; Mommaerts & Green, 1954) and the low rate of γ -glutamylhydroxamate synthesis previously observed (Wu, 1963a,b; Iqbal & Ottaway, 1970) in the absence of an ATP-generating system may be partly attributable to a depletion of the concentration of ATP required, in stoichiometric amounts (Meister, 1962, 1969), for the GS enzyme

reaction. Muscle now emerges as a major site of glutamine formation and hydrolysis (Marliss *et al.*, 1971).

In liver and brain, glutamine synthesis occurred in the same fractions of tissue extracts as the GS enzyme reaction and was quantitatively similar to the γ -glutamylhydroxamate formed by the same preparations under the same assay conditions. In no instance could glutamine synthesis be associated quantitatively with γ -glutamylhydroxamate formation by the GT enzyme reaction.

The studies of the specificity of the GS enzyme and GT enzyme reactions, as well as the findings of their differential inhibition by several compounds, corroborated the evidence from the solubilization experiments. The GT enzyme reaction showed much greater specificity toward its substrate than did GS enzyme in reaction (2). The sensitivity of the GT enzyme reaction to a number of inhibitors also exceeded that of reaction (2). Further, the dissimilarity in metal ion requirements of the two activities is difficult to adapt to the hypothesis that the same enzyme site is responsible for both catalysed reactions. These differences plus the physical separation of the enzyme activities into differentially soluble fractions seem persuasive evidence that the GS enzyme and GT enzyme reactions in crude tissue extracts are catalysed largely by more than one enzyme. Glutamine synthesis cannot be measured by the GT enzyme reaction but can be measured by reaction (1) and probably by reaction (2). Although GS enzyme and GT enzyme can be clearly separated *in vitro*, it still remains probable that each enzyme may also catalyse the reaction of the other to a small extent.

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