BBA 65676

STUDIES ON TWO GLUTAMINASE SYSTEMS FROM RAT KIDNEY

CARLOS LAMAR, JR.

The Department of Medicine, University of Wisconsin, School of Medicine and the Veterans Administration Hospital, Madison, Wisc. (U.S.A.)

(Received June 26th, 1967)
(Revised manuscript received September 4th, 1967)

SUMMARY

An enzyme, glutamohydroxamase, that accomplishes the hydrolysis of γ-glutamohydroxamate and the deamidation of glutamine at similar rates has been described. This enzyme is predominantly present in rat kidney, is absent from liver, and is bound to the microsomal fraction of the cell. It is activated by SO_4^{2-} and inhibited by $HgCl_2$ and reduced glutathione in a similar manner as is glutaminase, which it resembles in several other ways as well. However, the failure of glutaminase to hydrolyze γ-glutamohydroxamate serves to distinguish the two enzymes. The products of the glutamohydroxamase reaction were shown to be glutamic acid and hydroxylamine and the reaction was found not to be reversible.

INTRODUCTION

Early work by ERRERA[1] and ERRERA AND GREENSTEIN[2] demonstrated the presence of two enzymatic activities resulting in the deamidation of glutamine. One was particulate and was present in liver, kidney, brain and spleen, and was activated by the presence of phosphate, sulfate or arsenate ions. It was designated as glutaminase I. A second activity present in the supernatant fraction of liver and activated by pyruvate was named glutaminase II. Subsequent work disclosed that the latter enzyme activity was associated with a transamination reaction which was followed by the hydrolysis of α-ketoglutaramate to yield ammonia and α-ketoglutarate[3,4]. More recently, work involving the purification[5] and studies on the characteristics[6] have been devoted largely to true glutaminase activity (glutaminase I).

KLINGMAN AND HANDLER[5] noted that pig renal glutaminase activity was located exclusively in mitochondria. They were able to show that phosphate ions gave an apparent enzyme activation by exerting a protective effect against enzyme inactivation, and to use this phenomenon in accomplishing a 25-fold purification of the enzyme.

The essence of this report is to suggest that there exists in rat kidney two en-

Abbreviations: CETAB, hexadecyltrimethylammonium bromide; BAL, 2,3-dimercaptopropanol.

zymes capable of hydrolyzing glutamine. One (glutaminase I) is found predominantly in mitochondrial preparations and has a high degree of specificity for glutamine. The other (here designated glutamohydroxamase) is associated with the microsomal pellet and is able to hydrolyze both glutamine and glutamohydroxamate. Other differences between the two enzyme activities are noted.

MATERIAL AND METHODS

L-Glutamine and 2,3-dimercaptopropanol (BAL) were products of the Mann Research Laboratories, New York, N.Y. γ-Glutamohydroxamate and sodium deoxycholate were purchased from General Biochemicals, Chagrin Falls, Ohio. Hexadecyltrimethylammonium bromide (CETAB) was a product of the Matheson, Coleman and Bell Co., Cincinnati, Ohio. 3-Acetylpyridine–adenine dinucleotide was obtained from P-L Biochemicals, Inc., Milwaukee, Wisc. The L-glutamate dehydrogenase (bovine liver), oxidized and reduced glutathione, cysteine, and mercaptoethanol were products of Calbiochem, Los Angeles, Calif. All other reagents were of the best commercial grade available.

Male Holtzman rats (150–250 g) were used in all experiments, unless otherwise stated. After decapitation, the kidneys and liver were removed within 40 sec and the brain within 60 sec. The organs were weighed, minced, and homogenized in 9 vol. of either 0.01 M sodium barbital buffer (pH 8.0) with 0.1% CETAB, or 0.25 M sucrose when the differential centrifugation studies were done. The tissue, kept at 0°, was transferred to a homogenizer tube fitted with a teflon pestle (clearance 0.005 inch) and homogenization was accomplished mechanically with 8 strokes. Centrifugation was carried out in a Sorvall refrigerated centrifuge Model RC 2B. Nuclei were removed at 700 $\times$ *g* for 10 min, mitochondria at 5000 $\times$ *g* for 10 min and the lysosomal fraction at 12 500 $\times$ *g* for 10 min. The various cellular fractions were washed twice in 0.25 M sucrose and were homogenized manually in a homogenizer tube fitted with a teflon pestle in 4 times the original kidney weight of 0.01 M barbital buffer (pH 8) containing 0.1% CETAB. The post-lysosomal supernatant was spun at 145 000 $\times$ *g* for 2 h in a Spinco Model L ultracentrifuge and the microsomal pellet was homogenized in barbital–CETAB buffer. Unless otherwise stated, the mitochondrial fraction or the microsomal pellet were used as the enzyme source in all assays. All experiments were performed on the same day that tissues were obtained from fasted rats.

Enzyme assays

Glutaminase was assayed in Conway diffusion flasks. The outer well contained 1 ml and in final concentrations, L-glutamine 22 mM, Na_2SO_4 100 mM, sodium barbital buffer (pH 8.0) 30 mM, and 0.2 ml of the enzyme solution. After a 10-min incubation at 37°, the enzymatic reaction was terminated by the injection through the rubber stopper of 1 ml of borate buffer (pH 11.5) 0.15 M. The liberated ammonia was captured in 2 ml of 2 M H_2SO_4 in the center well of the Conway diffusion flask during a 2-h incubation at 37°. A flask which was to serve as a blank was treated identically except that water replaced the enzyme solution. Ammonia concentration was determined in two 0.5-ml aliquots of H_2SO_4 by the Nessler reaction. NH_4Cl was used as a standard. One unit of enzyme activity was defined as the amount of enzyme necessary to release 1 μmole of ammonia per h.

Glutamohydroxamase

The basic incubation mixture of 1 ml contained γ-glutamohydroxamic acid 3 mM, Na_2SO_4 100 mM, and sodium barbital buffer (pH 8.0) 24 mM. Additionally, when kidney and brain were assayed, 21 and 3 mM, respectively, of L-glutamine were added to the incubation mixture. Glutamine was not added when the activity of this enzyme in other tissue was assayed. The assay was terminated after a 1-h incubation at 37° by the addition of 2 ml of $FeCl_3$ solution (8% $FeCl_3$ and 10% trichloroacetic acid (w/v) in 0.5 M HCl). The solution was filtered and the concentration of unreacted glutamohydroxamate was determined at 500 mμ in a Gilford Model 2000 spectrophotometer. The absorbance of the reaction mixture was subtracted from the absorbance of an identical mixture to which $FeCl_3$ solution had been added prior to the addition of the enzyme. The resulting absorbance change was compared to a standard glutamohydroxamate curve and the μmoles of reacted glutamohydroxamate determined. The final result was multiplied by either 7 or 2 in accordance with the ratio of glutamine/glutamohydroxamate present. One unit was defined as the amount of enzyme necessary to hydrolyze 1 μmole of glutamohydroxamate per h.

Proteins were measured by the method of LOWRY *et al.*[7] with bovine serum albumin as standard.

Evidence that the products of the glutamohydroxamase assay were glutamic acid and hydroxylamine was accumulated in this manner: The glutamohydroxamase reaction was allowed to proceed in the absence of glutamine until nearly complete disappearance of the glutamohydroxamate. An aliquot of the protein-free supernatant was used in two reactions: (1) one portion was added to the ingredients necessary for glutamine synthesis as described by SELLINGER AND DEBALBIAN VERSTER[9]. The reaction mixture thus contained only the supernatant described above, ATP, Mg^{2+}, BAL, Tris buffer, and a purified preparation of brain glutamine synthetase. Following a 1-h incubation, the quantity of glutamohydroxamate formed was determined. Calculations revealed that in excess of 50% of the previously hydrolyzed glutamohydroxamate had been reformed by the glutamine synthetase reaction. (2) A second portion of the protein-free-supernatant from the glutamohydroxamase reaction as described above, calculated to contain the products of 0.25 μmole of reacted glutamohydroxamate was compared with a standard known to contain 0.25 μmole of glutamic acid. The blank contained the same quantity of unreacted glutamohydroxamate. The samples were incubated in the presence of 0.5 μmole of *N*-acetylpyridine-DPN and 0.2 mg of glutamate dehydrogenase. The reaction rate was followed in a Gilford Model 2000 spectrophotometer equipped with a constant temperature heating unit. Using the absorption coefficient for *N*-acetyl-DPN of $8 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$ per mole, total conversion of 0.25 μmole of glutamic acid would result in an absorbance reading of approx. 2. At the end of a 1-h incubation the absorbance recorded for the cuvette having the unknown was 1.25, for the one containing the known amount of glutamic acid was 1.0 and for the one containing the unreacted glutamohydroxamate the absorbance was 0.17. No further change in rate was noted after 4 h and the final absorbance was 2.13 in the unknown cuvette, 2.1 for the cuvette containing standard glutamic acid and 0.25 in the cuvette containing the unreacted glutamohydroxamate.

RESULTS

Initial difficulties in following the enzymatic hydrolysis of γ-glutamohydroxamate were due to the occurrence of non-enzymatic hydrolysis of the compound in the presence of phosphate ions in an alkaline solution. Glutamine has been reported to behave similarly and to yield ammonia and pyrrolidone carboxylic acid[6,10]. These problems were largely overcome by using SO_4^{2-} in the place of phosphate in accordance with the report by GILBERT, PRICE AND GREENSTEIN[6]. These workers showed that SO_4^{2-}, when compared to phosphate, effected a 94% activation of glutaminase activity and caused virtually no non-enzymatic deamidation of glutamine. Initial studies showed that the enzymatic hydrolysis of γ-glutamohydroxamate by crude kidney homogenates was directly proportional to tissue concentration up to 40 mg of wet weight and was virtually absent when homogenates heated to 90° for 5 min were used. A time-course reaction showed linearity with time of incubation up to 120 min.

That the glutamohydroxamase assay system could not be used to determine glutaminase activity became apparent when both glutaminase and glutamohydroxamase enzyme levels were determined in various tissues. Results showed that although the activity of glutaminase is higher in rat brain than in kidney, the activity of glutamohydroxamase in brain is approx. one-tenth of that in kidney. The presence of considerable glutaminase activity in rat liver and the virtual absence of glutamohydroxamase indicated that the two enzyme systems were not identical. Table I shows the distribution of both enzyme activities in various tissues of the rat. It can be noted further that the activity of glutamohydroxamase in pancreas is about the same as its activity in brain, with lesser amounts being present in lung, heart and spleen.

The possibility that two different enzymes were being assayed was strengthened when the study of the subcellular distribution of the two activities was investigated. Table II shows that while glutaminase activity predominates in mitochondria, glutamohydroxamase is present primarily in the microsomal pellet, a fact that was continually borne out in many additional experiments not included in the table. It is quite evident that mitochondrial preparations hydrolyzed almost exclusively gluta-

TABLE I

THE DISTRIBUTION OF GLUTAMINASE AND GLUTAMOHYDROXAMASE IN VARIOUS TISSUES OF THE RAT

Assays on kidney, brain and liver are average of at least twelve experiments. Assays on spleen, pancreas, heart, lung and hepatoma are average of two experiments.

Rat	*Glutaminase**	*Glutamohydroxamase**
Kidney	1150	360
Brain	1580	33
Liver	240	3
Spleen	195	5
Pancreas	170	32
Lung	100	8.4
Heart	84	6.8
7800 Hepatoma	13	5

* Units per gram of tissue.

TABLE II

THE DISTRIBUTION OF GLUTAMINASE AND GLUTAMOHYDROXAMASE IN THE SUBCELLULAR FRACTIONS OF RAT KIDNEY

Average of at least four experiments.

Fraction	*Glutaminase*		*Glutamohydroxamase*	
	*Units**	*%*	*Units**	*%*
Homogenate	1525	100	636	100
Nuclear	145	9.5	35	6
Mitochondrial	1020	67	60	10
Lysosomal	16	1	10	4
Microsomal	260	17	284	45
Cytosol	40	2.6	97	15
Recovery		97		80

* Total units in fraction.

mine but not glutamohydroxamate, while the enzyme activity found in the microsomal pellet was able to hydrolyze both glutamohydroxamate and glutamine at nearly equal rates. The recovery of glutamohydroxamase was poor, a fact that is related to the instability of the enzyme; enzyme activity dropped approx. 40% when samples were frozen overnight.

Attempts at purification of glutamohydroxamase disclosed that the enzyme could be solubilized in 1% deoxycholate. Since this detergent resulted in a spurious increase in the color development of the assay, it was removed by passage of the supernatant through a Sephadex G-50 column. The enzyme was eluted in the first 20 ml following the passage of the bed volume. Treatment of mitochondrial preparations in an identical manner with 1% deoxycholate completely inactivated glutaminase activity. A more extensive purification of glutamohydroxamase is now in progress and will be the subject of further reports.

It is of interest to note that in Table II the microsomal preparation contains apparent glutaminase activity; however, since the major part of the activity can be ascribed to the glutamohydroxamase system, the real distribution of glutaminase is appreciably changed.

Fig. 1 shows the activity of both enzyme systems at various pH values. Both enzymes have a pH optimum of 8, but it can be seen that the fall in activity at the more acid pH value is greater for glutaminase than it is for glutamohydroxamase.

The results of kinetic studies are shown in a composite graph in Fig. 2 and are represented by double-reciprocal plots[11]. The K_m value calculated for glutaminase (Fig. 2, A) was $5.5 \cdot 10^{-3}$ M when either crude kidney homogenate or mitochondrial preparations were used. The results of kinetic studies with glutamohydroxamase activity using the microsomal pellet as the enzyme source and glutamohydroxamate as the substrate are seen in Fig. 2, B. The K_m value in this case was $4 \cdot 10^{-3}$ M. When glutamine and glutamohydroxamate in a ratio of 7:1 were present as substrate (Fig. 2, C) the calculated K_m was $6 \cdot 10^{-3}$ M. The K_m was approximately the same when either crude homogenates or resuspended microsomal pellet was used. Significantly, when microsomal membranes were used as the enzyme source (presumably measuring glutamohydroxamase activity) in conditions satisfactory for glutaminase assay

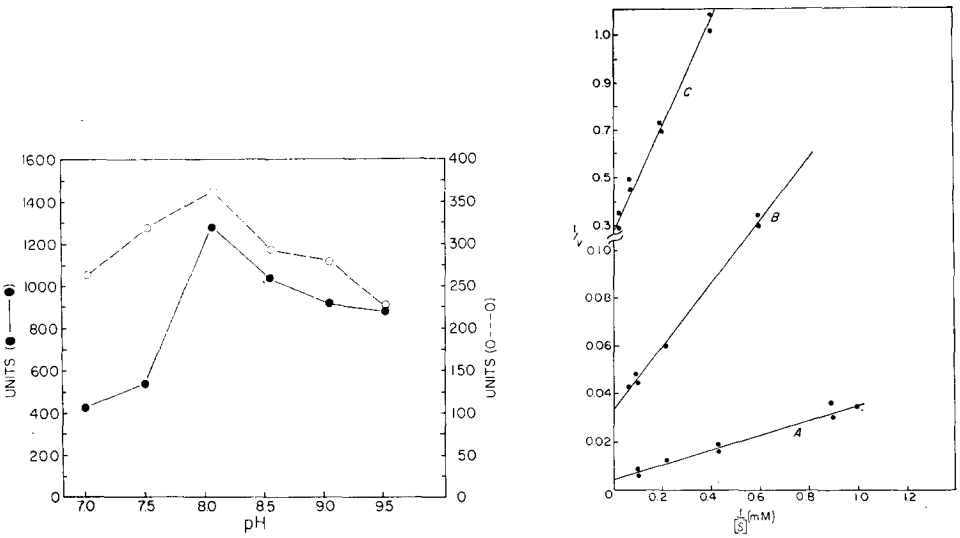

Fig. 1. The effect of pH on glutaminase (●—●) and glutamohydroxamase (○- - - -○) activity. Enzyme source was rat-kidney mitochondria for glutaminase and the microsomal pellet for glutamohydroxamase. Units are referred to per g of original tissue.

Fig. 2. A composite representation of the enzyme activity from several subcellular fractions plotted against certain substrates according to LINEWEAVER AND BURK[11]. A. Glutaminase I from rat-kidney mitochondria *vs.* glutamine concentrations. K_m, $5.5 \cdot 10^{-3}$ M. B. Glutamohydroxamase activity from rat-kidney microsomal pellet *vs.* glutamohydroxamate as substrate. K_m, $6 \cdot 10^{-3}$ M. (See text.)

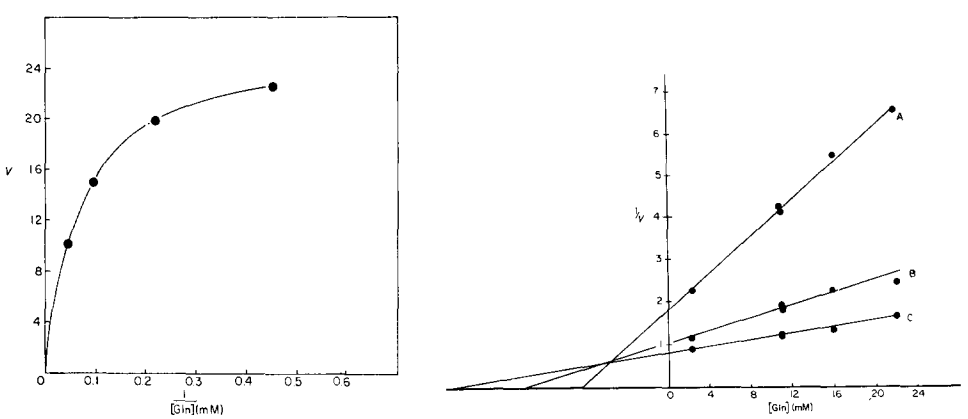

Fig. 3. The effect of various concentrations of glutamine (Gln) on the rate of the hydrolysis of constant glutamohydroxamate concentration (3 mM) by rat-kidney preparations. *v*, μmoles of glutamohydroxamate degraded per h.

Fig. 4. The effect of increasing concentrations of glutamine (Gln) on glutamohydroxamase activity at three glutamohydroxamate concentrations (mM): A, 6; B, 3; C, 1.5. The enzyme source was rat-kidney microsomal membranes. The data are plotted according to DIXON[12]. The calculated K_i for glutamine as an inhibitor to glutamohydroxamate in the reaction was $5.5 \cdot 10^{-3}$ M. *v*, μmoles of glutamohydroxamate hydrolyzed per h.

(Conway diffusion flask, only glutamine as substrate) the resulting K_m value was $6 \cdot 10^{-3}$ M. These values were considered to be within the limits of experimental error. The rate of hydrolysis of a constant concentration of glutamohydroxamate (3 mμ) by glutamohydroxamase in the face of increasing glutamine concentrations is demonstrated in Fig. 3. It is quite evident that as glutamine concentrations increase, the rate of the reaction is reduced. Fig. 4 demonstrates the results of kinetic studies when both the glutamine concentrations and the γ-glutamohydroxamate concentrations are varied. The data represent the reciprocal of the velocity plotted against the inhibitor concentrations (glutamine at three different glutamohydroxamate concentrations). Using the method of DIXON[12] the K_i for glutamine was calculated to be $5.5 \cdot 10^{-3}$ M. The results as represented in Fig. 4 are characteristic of a competitive inhibition. Since glutamine competes with glutamohydroxamate in the glutamohydroxamase reaction it effectively acts as an inhibitor with a K_i of $5.5 \cdot 10^{-3}$ M. When glutamine

TABLE III

THE EFFECT OF VARIOUS INHIBITORS AND OF SO_4^{2-} ON GLUTAMINASE AND GLUTAMOHYDROXAMASE ACTIVITY

Addition (M)	*Inhibition (%)*	
	Glutaminase	*Glutamo-hydroxamase*
$HgCl_2$		
10^{-2}	97	80
10^{-3}	90	12
10^{-4}	81	10
p-Chloromercuribenzoate		
10^{-3}	98	16
10^{-4}	86	3
10^{-5}	18	0
Evans blue		
$5 \cdot 10^{-3}$	55	0
Bromocresol purple		
$5 \cdot 10^{-3}$	20	0
Glutamate		
$2 \cdot 10^{-2}$	—	32
$5 \cdot 10^{-3}$	60	16
Hydroxylamine		
$2 \cdot 10^{-2}$	0	23
NH_4Cl		
$5 \cdot 10^{-3}$	55	13
$CaCl_2$		
10^{-2}	—	30
$MgCl_2$		
10^{-2}	5	—
Glutathione, reduced		
10^{-3}	68	87
$5 \cdot 10^{-4}$	—	25
Glutathione, oxidized		
10^{-3}	6	25
2,3-Dimercaptopropanol	0	0
Mercaptoethanol	0	0
Na_2SO_4	78*	63*

* Activation (%).

is studied as substrate in the glutamohydroxamase reaction the resulting K_m value is the same. This suggests that glutamohydroxamase hydrolyzes both substrates at very similar rates. Consequently, in order to perform the assay with an excess of substrate and yet conserve γ-glutamohydroxamate, the reaction was routinely run with a set ratio of substrates (7:1, see METHODS). This modification proved quite practical except in situations when tissues having low glutamohydroxamase activity were assayed. In such cases the glutamine:γ-glutamohydroxamate ratios were lowered to 1 in the case of brain and in other cases glutamine was omitted from the incubation mixture completely.

The effect of various inhibitors on the enzyme activity of both glutaminase and glutamohydroxamase can be seen in Table III. Some of the data is similar to that reported by ROBERTS[4], and SAYRE AND ROBERTS[13]. $HgCl_2$ and *p*-chloromercuribenzoate have considerable inhibitory effect on glutaminase activity at the concentration of $1 \cdot 10^{-4}$ M. Evans blue and bromocresol purple at concentrations of $5 \cdot 10^{-3}$ M cause a 55 and 20% inhibition of glutaminase, respectively. In contrast, glutamohydroxamase activity is inhibited 80% at $1 \cdot 10^{-2}$ M concentrations of $HgCl_2$ and only 10% at $1 \cdot 10^{-3}$ M concentrations. The marked discrepancy in the sensitivity of the two enzyme systems to these inhibitors suggests that the two enzyme systems are of different molecular composition. It should be noted that Na_2SO_4 activates both enzyme systems to a considerable degree and $CaCl_2$ inhibits glutamohydroxamase at $1 \cdot 10^{-2}$ M concentrations by 30%. $MgCl_2$ has virtually no effect on glutamohydroxamase activity in either the presence or the absence of Na_2SO_4. Of considerable interest was the marked inhibitory effect of reduced glutathione on both glutamohydroxamase and glutaminase. This finding has been described[8] but was shown to disappear when purified glutaminase preparations were used. Kinetic data subjected to reciprocal plots in the manner of LINEWEAVER AND BURK[11] indicates that in both instances the inhibition is of a non-competitive nature. This information, along with a substantially smaller inhibition when oxidized glutathione was used, implies that glutathione is not serving as substrate in the glutamohydroxamase reaction.

DISCUSSION

The above data demonstrate that while the microsomal membrane has enzymatic capacity to hydrolyze glutamine and glutamohydroxamate at nearly equal rates, mitochondrial preparations are active only against glutamine and not against glutamohydroxamate. Alternative explanations for the data presented are difficult to exhaust but several of the more possible ones should be considered: The glutamohydroxamase system might be due to: (1) glutamine transferase activity; (2) transpeptidase activity; and (3) transglutaminase activity.

The absence of glutamohydroxamase activity in liver together with the presence of glutamine synthetase–glutamine transferase activity there precludes the possibility that the enzymatic hydrolysis of glutamohydroxamate is being accomplished by glutamine synthetase. It is possible that the glutamohydroxamase system described is due to a transpeptidase activity. This latter enzyme, initially described by HANES, HIRD AND ISHERWOOD[14], and investigated further by KINOSHITA AND BALL[15], is involved in the transfer of glutamic acid bound to a protein or a small polypeptide (glutathione) to an acceptor amino acid (arginine). REVEL AND BALL[16] have shown that the rate of

the transpeptidase reaction with glutathione as substrate increases with increasing glutamine (acceptor) concentrations up to 13 μmoles. These workers were unable to ascribe deamidation of glutamine to the transpeptidase reaction. ORLOWSKI AND MEISTER[17] using glycyl-*p*-nitroanilide as substrate, accomplished a 1000-fold purification of γ-glutamyl transpeptidase from hog kidney. They reported a 30% activation by Mg^{2+}. Using glutamine as substrate, incubation in the presence of purified enzymes resulted in the formation of some glutamic acid and a series of γ-glutamylglutamine compounds. CLIFFE AND WALEY[18] used γ-glutamylglycine as substrate and reported that transpeptidase activity was predominantly present in the nuclear fraction of rat liver. SZCZEKLIK, ORLOWSKI AND SZEWCZUK[19], were able to demonstrate transpeptidase activity in the serum of some patients with liver disease. The substrate used was glutamyl amino propionitrile. A transfer of γ-bound glutamine of glutathione to water by bacterial preparations has been reported by MILBAUER[20]. The variability in the reported results may be explained by the variety of substrates used.

Several aspects of the studies presented largely eliminate the possibility that the glutamohydroxamase reaction is the result of transglutaminase activity. The quantity of glutamic acid produced was equivalent to the amount of glutamohydroxamate degraded. Also, the addition of glutamine to the glutamohydroxamase reaction mixture decreases apparent glutamohydroxamase activity. The activation of glutamohydroxamase conferred by SO_4^{2-} has not been reported for the transglutaminase reaction, and the activation of Mg^{2+}, which has been reported[16] for the transglutaminase reaction is lacking. Glutathione non-competitively inhibits both glutaminase and glutamohydroxamase activity. It is concluded that glutathione is not serving as a substrate in the glutamohydroxamase reaction.

EHRENFELD, MARBLE AND MEISTER[21] isolated and partially purified from *Azobacter agilis* an enzyme activity that formed γ-glutamohydroxamate from L-glutamine or L-glutamic acid and hydroxylamine in the absence of nucleotides and metal ions. The enzyme also catalyzed the hydrolysis of γ-glutamohydroxamate. It is possible that a similar enzyme is being described here. A definitive decision concerning these alternatives awaits the purification of the microsomal glutamohydroxamase activity, which is now in progress. However, it should be emphasized that mitochondrial preparation purified to the best extent possible failed to hydrolyze γ-glutamohydroxamate while glutamine was actively deamidated.

Entirely compatible with the results is the hypothesis that glutamohydroxamase is a unique enzyme, mechanistically similar to glutaminase, but differing in its localization and specificity.

ACKNOWLEDGEMENTS

The tireless and competent technical assistance of Mrs. B. IMM is gratefully acknowledged.

This investigation was supported in part by a grant from the Epilepsy Foundation.

REFERENCES

1 M. ERRERA, *J. Biol. Chem.*, 178 (1949) 483.
2 M. ERRERA AND J. P. GREENSTEIN, *J. Biol. Chem.*, 178 (1949) 495.

3 A. MEISTER AND S. V. TICE, *J. Biol. Chem.*, 187 (1950) 173.
4 E. ROBERTS, in P. D. BOYER, H. LARDY AND K. MYRBÄCK, *The Enzymes*, Vol. IV, Academic Press, New York, 1960, p. 285.
5 J. D. KLINGMAN AND P. HANDLER, *J. Biol. Chem.*, 232 (1958) 369.
6 J. B. GILBERT, V. E. PRICE AND J. P. GREENSTEIN, *J. Biol. Chem.*, 180 (1949) 209.
7 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
8 J. D. KLINGMAN AND P. HANDLER, *J. Biol. Chem.*, 232 (1958) 369.
9 O. Z. SELLINGER AND F. DEBALBIAN VERSTER, *J. Biol. Chem.*, 237 (1962) 2836.
10 A. C. CHIBNALL AND R. G. ULESTALL, *Biochem. J.*, 26 (1932) 122.
11 H. LINEWEAVER AND D. BURK, *J. Am. Chem. Soc.*, 55 (1934) 658.
12 M. DIXON, *Biochem. J.*, 55 (1953) 170.
13 F. W. SAYRE AND E. ROBERTS, *J. Biol. Chem.*, 233 (1958) 1128.
14 C. S. HANES, F. J. R. HIRD AND F. A. ISHERWOOD, *Nature*, 166 (1950) 288.
15 J. H. KINOSHITA AND E. G. BALL, *J. Biol. Chem.*, 200 (1953) 609.
16 J. P. REVEL AND E. G. BALL, *J. Biol. Chem.*, 234 (1959) 577.
17 M. ORLOWSKI AND A. MEISTER, *J. Biol. Chem.*, 240 (1965) 1.
18 E. E. CLIFFE AND S. G. WALEY, *Biochem. J.*, 79 (1961) 118.
19 E. SZCZEKLIK, M. ORLOWSKI AND A. SZEWCZUK, *Gastroenterology*, 41 (1961) 353.
20 R. MILBAUER, *J. Gen. Microbiol.*, 41 (1965) 185.
21 E. EHRENFELD, S. J. MARBLE AND A. MEISTER, *J. Biol. Chem.*, 238 (1963) 3711.