FURTHER METABOLIC REACTIONS OF γ-HYDROXYGLUTAMATE: AMIDATION TO γ-HYDROXYGLUTAMINE; POSSIBLE REDUCTION TO HYDROXYPROLINE

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γ-Hydroxyglutamate (HGA), first discovered as a plant product (Virtanen and Hietala, 1955), is formed from hydroxy-L-proline by mammalian enzymes (Adams and Goldstone, 1960). Subsequent reactions (Kuratomi and Fukunaga, 1960; Dekker and Maitra, 1962; Goldstone and Adams, 1962) yield α-hydroxy-γ-ketoglutarate (HKG) and thence glyoxylate and pyruvate. Other metabolic reactions of these hydroxylated analogs of glutamate and α-ketoglutarate include α-decarboxylation of HGA (Bouthillier and Binette, 1961) and oxidation of HKG to malate (Payes and Laties, 1963).

We have investigated the possible reduction of HGA to hydroxyproline, a hypothetical pathway analogous to that postulated for the reduction of glutamate to proline. In initial experiments, 5-C^{14}-erythro-L-HGA, made enzymatically from 1-C^{14}-glyoxylate (Adams and Goldstone, 1963), was incubated in rat-liver homogenates with ATP, DPNH and unlabeled hydroxy-L-proline. Reisolation of the latter (by a sequence of ion-exchange and paper chromatography through several solvent systems) suggested extensive incorporation of label from HGA; a concurrently-observed formation of HGA-hydroxamate in liver extracts was at first thought to be related to the postulated reduction to hydroxyproline (Goldstone and Adams, 1963).

More searching repetition of these observations has revealed that most of the label present in hydroxyproline-containing fractions was associated with glycine, which could not be clearly separated from hydroxyproline in a number of paper-chromatographic solvents, but was separable

by high-voltage paper electrophoresis at pH 2. After electrophoretic and other purification methods, a small fraction of the counts (less than 1%) added as HGA remained with hydroxyproline. Although more conclusive identification of the label as hydroxyproline would be required to demonstrate a reductive pathway, it is of interest that Kuratomi et al (1963) observed the same order of labeling of hydroxyproline, relative to HGA, after incubation of rat-liver homogenate with 2-C^{14} pyruvate. In unpublished experiments with a child demonstrating hydroxyprolinemia (Efron et al, 1962), intravenously-administered 1-C^{14}-glyoxylate led to the significant labeling of free hydroxyproline in urine (private communication, Dr. Efron, 1964). A direct reductive pathway from HGA to hydroxyproline, while suggested by these observations, is not yet demonstrated.

Further investigation of the activation of HGA in rat-liver extracts led us to conclude that the product made when ammonia replaces hydroxylamine is the γ-amide, formed by an enzyme indistinguishable from glutamine synthetase. Identification of γ-hydroxyglutamine was based on the observations outlined below.

Purification of Liver Enzyme - HGA-activating enzyme in homogenates of fresh rat liver was purified using a hydroxamate assay (Speck, 1949), the substrates being erythro-L-HGA, ATP and NH_2OH. Purification steps included removal of material sedimenting at 100,000 x g, precipitation of enzyme at 0.4 saturation in ammonium sulfate, and an adsorption-elution step with $Ca_3(PO_4)_2$ gel. The final fraction, 200-fold purified, catalyzed hydroxamate formation with erythro-L-HGA, threo-L-HGA and both L- and D-glutamate. The ratio of hydroxamate-forming activity with L-glutamate and erythro-L-HGA as substrates was constant throughout purification.

Enzymatic Preparation of γ-Hydroxyglutamine - Erythro- or threo-L-HGA was incubated with 1 to 2 molar equivalents of ATP and NH_4Cl and purified liver enzyme for periods of 3 to 6 hours at 37° in dilute phosphate, pH 7.5. The reaction was stopped with 1N HCl and the centrifuged supernatant was chromatographed through Dowex-50 H^+. Two ninhydrin-positive peaks, sepa-

rated by several column volumes, were obtained by elution with 0.3 N HCl. The first peak contained HGA while the second contained γ-hydroxyglutamine in yields of 30 - 60% of the initial HGA. Eluates in the second peak were pooled and neutralized with AgO, and the supernatant was concentrated at reduced pressure. Further purification of γ-hydroxyglutamine was obtained by preparative paper chromatography.

Homogeneous sheep-brain glutamine synthetase, (Pamiljans et al, 1962) served as the enzyme source in similar preparative incubations using conditions described in the reference cited. Based on inorganic phosphate liberated from ATP, the reaction went to 70% or more of completion with erythro-L-HGA as substrate.

Enzymatic and Acid Hydrolysis of γ-Hydroxyglutamine - The product of HGA derived either with the rat-liver or sheep-brain enzyme yielded approximately stoichiometric ammonia on hydrolysis either with partly-purified glutaminase from Eschericia coli (Meister, 1955) or by acid (Table I). After either acid or enzymatic hydrolysis, HGA was detected by paper chromatography as the only ninhydrin-positive product.

Chromatographic Properties of γ-Hydroxyglutamine - The enzymatic product formed from HGA with the brain enzyme was chromatographically identical with that formed with the liver enzyme. γ-Hydroxyglutamine made with either enzyme yielded two spots in an ammoniacal propanol solvent. On repeated elution and chromatography, the slower-moving material continued to yield small amounts of the faster-moving material, indicating the latter as a probable chromatographic artifact rather than an additional enzymatic product; both bands yielded HGA and NH_3 on acid hydrolysis, but only the slower band was a substrate for glutaminase. The nature of the derivative of γ-hydroxyglutamine formed in propanol-NH_3 has not been established.

γ-Hydroxyglutamine as a Substrate for Glutamine Synthetase - The transfer of the amide group of glutamine for hydroxylamine is a well-established catalytic property of glutamine synthetase (Elliott, 1953).

Table I

Enzymatic and Acid Hydrolysis of γ-Hydroxyglutamine

Acid hydrolysis was carried out for 15 minutes at 100° in 1N HCl; glutaminase was incubated with substrates for 3 hours at 37° and pH 4.7. Substrates subjected to hydrolysis were assayed by a ninhydrin method with glutamate as standard. Ammonia was measured by Nessler's method and corrected by appropriate enzyme or zero-time blanks.

Compound Tested and Source	Micromoles Treated	Micromoles NH_3 Formed	
		Acid Hydrolysis	Glutaminase
Erythro-γ-hydroxy-L-glutamine			
Liver enzyme	1.0	0.9	0.8
	2.6	2.4	-
Sheep-brain enzyme	1.4	1.5	-
	1.0	-	0.9
Threo-γ-hydroxy-L-glutamine			
Sheep-brain enzyme	10.5	9.0	-
	1.0	-	0.6
L-Glutamine	1.0	-	1.1
L-Isoglutamine	10	9.4	-
	50	-	0.0

Table II

Chromatographic Behavior of γ-Hydroxyglutamine

Data shown are for erythro-γ-hydroxy-L-glutamine. Threo-γ-hydroxy-L-glutamine, made with the brain enzyme, was chromatographed in the first three solvents shown and migrated like erythro-HGA. Spots were detected by ninhydrin after ascending chromatography on Whatman No. 1 paper.

Solvent	Source of Compound	
	Liver Enzyme	Sheep-brain Enzyme
n-Propanol-0.2N ammonia (3:1)	0.15	0.15
	0.24	0.24
Ethanol-water (77:23)	0.28	0.28
t-Butanol-formic acid-water (70:15:15)	0.20	0.20
Phenol-water (4:1)	0.36	0.36
n-Butanol-pyridine-water (6:4:3)	0.10	0.10

When 5 μmoles of L-glutamine or of erythro-γ-hydroxy-L-glutamine (made either with the liver or brain enzyme) were incubated with 5 μmoles of ADP and 1 mmole of NH_2OH in the presence of sheep-brain enzyme, extensive formation of a hydroxamate was measured for each substrate; L-isoglutamine (10 μmoles) led to no measurable hydroxamate formation.

Discussion - The γ-amides of erythro - and threo-L-HGA have been identified as enzymatic products of the corresponding amino acids both with highly-purified sheep-brain glutamine synthetase and with a purified rat-liver enzyme. As measured both by hydroxamate and amide formation, both L-forms of HGA are active substrates for the liver enzyme. Because HGA has wider metabolic origins than free hydroxyproline, arising additionally from glyoxylate and pyruvate, it is worth considering the possible metabolic significance of the corresponding γ-amides, including their activity in or inhibition of the amino-donating functions of glutamine. While the present work was nearing completion, the first well-documented report appeared of the natural occurrence (in Phlox) of a γ-hydroxyglutamine (Brandner and Virtanen, 1963).

An incidental aspect of the present work has been the partial purification of glutamine synthetase from mammalian liver, a source which has received little attention. Recent studies by Wu (1963) have focussed on the solubilization and intracellular distribution of the enzyme in rat liver.

We thank Dr. A. Meister for providing samples of homogeneous sheep-brain glutamine synthetase. Meister (private communication, 1963) has independently observed a reaction of glutamine synthetase with erythro- or threo-L-HGA to yield presumptive γ-hydroxyglutamine.

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