Enzymic reactions in mammalian metabolism of γ-hydroxyglutamic acid

Glyoxylic acid has recently been shown to be formed from γ-hydroxyglutamate by an enzyme system present in rat-liver extracts[1]. Paper-chromatographic identification of alanine as one other product of this process suggested the possibilities of either a direct cleavage of the substrate or a series of reactions with formation of alanine as a terminal product. The present experiments show that the latter alternative is correct.

Using glyoxylate formation to measure enzymic activity[1], we have purified the system approx. 40-fold from dialyzed KCl–ethanol extracts of rat liver. The procedures include acetone precipitation, heat treatment, and the adsorption of inert proteins first by bentonite and then by carboxymethylcellulose. A serious loss (80–90 %) of activity is observed when the bentonite-treated fraction is dialyzed exhaustively or passed through columns of Sephadex G-25. After either treatment, full activity is restored by adding heat-deproteinized rat-liver extract. The active component present in this boiled extract has been isolated and shown to be replaceable by and identical with L-glutamine. Other findings substantiate the participation of L-glutamine in the enzymic system. For example, the stimulatory factor present in boiled extract of rat liver is destroyed by glutaminase purified from *Escherichia coli* extracts. Also, the observed stimulation of glyoxylate formation by added boiled extract of rat liver or L-glutamine is strongly inhibited by the known glutamine antagonists, 6-diazo-5-oxo-norleucine and γ-glutamyl hydrazide. The inhibition shown by either of these two compounds is prevented by the presence of excess L-glutamine.

As shown in Table I, L-glutamine greatly accelerates the reaction and only L-isoglutamine and L-glutamate, when present at a level of 1 μmole in 3 ml of incubation mixture, partially stimulate glyoxylate formation above the degree of activity

TABLE I

SPECIFICITY OF L-GLUTAMINE FOR STIMULATION OF GLYOXYLATE FORMATION FROM γ-HYDROXYGLUTAMATE

The reaction mixture (3.0 ml) contained 300 μmoles Tris-HCl buffer (pH 8.1), 5 μmoles EDTA (pH 7.4), 10 μmoles GSH, 5 μmoles γ-hydroxyglutamate, 0.73 mg protein (bentonite fraction exhaustively dialyzed), plus 1 μmole of each of the compounds listed. Gas phase, N_2. Incubated for 60 min at 37°. Protein was precipitated with HPO_3 and 1-ml aliquots of the supernatant removed for assay of glyoxylate. D-Glutamine, L-homoglutamine, D-homoglutamine, *N*-acetylglutamine, β-methylglutamine, *N*-carbobenzoxyglutamine, L-asparagine, and L-aspartic acid failed to stimulate above base-level activity.

Compound tested	*Glyoxylate formed* (% maximal activity)*
None	15
L-Glutamine	100
L-Isoglutamine	58
L-Glutamic acid	46
NH_4^+	12
L-Glutamic acid + NH_4^+	32

* Estimated by an adaptation of the colorimetric procedure of D. N. KRAMER *et al.*[9].

observed when no glutamine is added. At higher levels (5 μmoles/3 ml), L-glutamate markedly inhibits the system whereas at much lower levels (0.1–0.2 μmole/3 ml) this amino acid is as effective as L-glutamine. L-Glutamine, in contrast, is just as effective at high as at low levels. Both L-glutamine and L-glutamate serve in a catalytic role in stimulating the formation of glyoxylate as well as of alanine, the latter determined as acetaldehyde after reaction with ninhydrin[2]. As much as 1.8 μmoles of glyoxylate and alanine have been found to accumulate in the presence of only 0.2 μmole of L-glutamine or L-glutamate when a typical reaction mixture is incubated for 2 h.

Glyoxylate and alanine formation are also markedly accelerated when either α-ketoglutaramate, α-ketoglutarate or oxaloacetate is added to the enzymic system, but only α-ketoglutaramate and α-ketoglutarate function catalytically. This catalytic involvement of L-glutamine, L-glutamate, and their corresponding α-keto acids, and of pyridoxal 5-phosphate[1], suggested the presence of one or more transamination steps as part of a sequence of reactions.

Enzymic activities were separated using DEAE-cellulose. When a 10-fold purified preparation of the enzymic system is applied to a DEAE-cellulose column and subsequently eluted batchwise with increasing concentrations of Tris·HCl buffer (pH 7.4), three individual protein fractions (A, B, and C) are obtained. Protein fraction B is devoid of any activity alone or in combination with the other two. In contrast, protein fractions A and C in the presence of catalytic levels of either L-glutamine or L-glutamate form essentially no glyoxylate from γ-hydroxyglutamate when tested alone, but when tested in combination show a higher specific activity than the original preparation. The incubation of γ-hydroxyglutamate and α-ketoglutarate with fraction A followed by heat denaturation of the protein, and subsequent addition of fraction C to the same tube results in glyoxylate formation. No product is formed when the protein fractions are added serially in the reverse order showing that the reaction catalyzed by C follows that catalyzed by A.

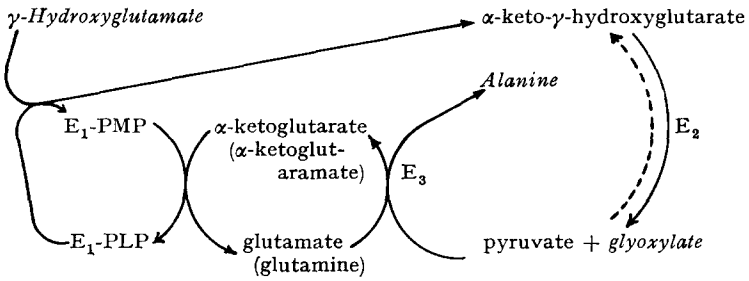
The assay of protein fractions A and C for specific enzymes has demonstrated the following. Fraction A catalyzes a transamination involving γ-hydroxyglutamate, which is stimulated by added α-ketoglutarate or oxaloacetate. This fraction can be replaced by a 500-fold purified pig-heart preparation of glutamate–aspartate transaminase*, an enzyme shown to require pyridoxal 5-phosphate as coenzyme[3,4]. The protein fractions obtained in the various stages of purifying the pig-heart enzyme as well as in preparing fraction A from rat liver have been compared for transaminase activity using γ-hydroxyglutamate and glutamate as substrates with oxaloacetate as the amino group acceptor. A constant ratio of activity toward the two substrates is observed with all the enzyme fractions obtained from either source. These results we interpret as evidence that γ-hydroxyglutamate participates in the glutamate–aspartate transamination reaction, a conclusion contrasting to that of GOLDSTONE AND ADAMS[5].

α-Keto-γ-hydroxyglutarate, the expected product of transamination between γ-hydroxyglutamate and α-ketoglutarate, has been prepared by non-enzymic transamination with pyridoxal and also by the enzymic reaction described above. The isolated α-keto-γ-hydroxyglutarate serves as immediate substrate for glyoxylate formation with protein fraction C. This fraction, therefore, probably contains the

* Kindly furnished by Dr. W. G. ROBINSON, Ann Arbor, Mich.

enzyme[6] that cleaves α-keto-γ-hydroxyglutarate to form glyoxylate plus pyruvate. In addition, protein fraction C has been shown to contain glutamine transaminase, free of α-ketoglutaramate deamidase, which catalyzes the formation of alanine from pyruvate. In accord with the observation of MEISTER *et al.*[7], we have also found that L-glutamate functions more effectively than L-glutamine in this conversion of pyruvate.

These findings indicate that the formation of glyoxylate and alanine from γ-hydroxyglutamate results from a sequential loss of the amino group of γ-hydroxyglutamate, followed by a cleavage and a reamination of one of the fragments, as shown in the accompanying scheme.



E_1 = enzyme present in protein fraction A, replaceable by highly purified pig-heart glutamate–aspartate transaminase;
E_2 = cleavage enzyme present in protein fraction C;
E_3 = ketoamidase-free glutamine transaminase of fraction C;
PMP = pyridoxamine monophosphate;
PLP = pyridoxal monophosphate.

This sequence requires that α-ketoglutaramate transaminates with γ-hydroxyglutamate. In direct tests, α-ketoglutaramate prepared enzymically by the procedure of MEISTER[8] has been found capable of replacing α-ketoglutarate in the first transaminase. Glutamine as well as glutamate, therefore, serves as the amino group donor in the second transaminase yielding α-ketoglutaramate and α-ketoglutarate, respectively, either of which, in turn, can again transaminate with γ-hydroxyglutamate. Details of the individual reactions proposed in this scheme are under investigation.

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