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INHIBITION OF ENZYMIC TRANSAMINATION OF ASPARTIC ACID BY HYDROXYASPARTATE, 2,3-DIAMINOSUCCINATE AND 2,3-DIAMINOPROPIONATE*

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It was reported from this laboratory (KUN AND GARCIA-HERNANDEZ[1], KUN[2]) that oxaloglycolic and diketosuccinic acids were among the main products of the enzymic oxidation of tartaric acid. One of the enzymic pathways by which these keto acids may be further metabolized is transamination. So far we have been able to show only one transamination, *viz.* the reaction of oxaloglycolate with glutamate, to yield hydroxyaspartate and α-ketoglutarate. This reaction is catalyzed by an aqueous extract of acetone powder of isolated pig kidney mitochondria. In the course of an independent investigation, SALLACH[3] also described this reaction, brought about by an enzyme preparation obtained from sheep brain. Diaminosuccinic and diaminopropionic acids were obtained only by chemical synthesis. It has been previously known (HASKELL *et al.*[4]) that diaminopropionic acid is a constituent of a tuberculostatic antibiotic isolated from certain actinomycetes, while hydroxyaspartic and diaminosuccinic acids inhibited bacterial growth (SHIVE AND MACOW[5], SUZUKI *et al.*[6,7]). One of the many possible mechanisms by which these uncommon amino acids may exert their inhibitory action is an interference with certain enzymic transaminations. In order to test this hypothesis we determined their effect on the transamination of aspartate and α-ketoglutarate.

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EXPERIMENTAL

Preparation and properties of hydroxyaspartic, diaminosuccinic and diaminopropionic acids

Syntheses of these acids were carried out by a procedure similar to methods described by DAKIN[8] and more recently by HAUPTMANN AND BERL[9]. The precursor of hydroxyaspartic acid, chloromalic acid, was prepared by treating maleic anhydride with a solution of freshly made hypochlorite. This was done by dissolving 6.25 g $Ca(OCl)_2$ in 250 ml H_2O + 44 g K_2CO_3 + 12.5 g KOH in 200 ml H_2O followed by filtration. The hypochlorite solution is chilled to 10 to 15° C and acidified with 100 ml conc. HCl, whereupon 20 g of maleic anhydride is added with continuous stirring. The reaction mixture is set aside overnight. Extraction of carboxylic acids is carried out with ether (500 ml) in a Kutscher-Steudel extractor. The ether extract contains some unreacted maleic acid in addition to chloromalic and dichlorosuccinic acids. A mixture of these acids were obtained in crystalline form after evaporation of the solvent *in vacuo* (total weight 13–14 g). A portion of this crude mixture (5 g) was dissolved in 50 ml of concentrated ammonia and heated in a sealed vessel at 100° for 10 hours. The resulting hydroxyaspartic and diaminosuccinic acids were separated as follows: after cooling 2.5 g of NaOH (dissolved in 10 ml H_2O) were added and the solution concentrated *in vacuo* (at room temperature) to ¼ of its original volume; the pH is then adjusted to 7.0 with glacial acetic acid and the bulk of crystalline diaminosuccinate (1 g) which settles overnight at 4° C is filtered off. The yellow mother liquor is treated successively twice with 0.5 to 1 g portions of activated charcoal and filtered. Crystalline hydroxyaspartate (1 g) is obtained from the filtrate by the addition of 200 ml of absolute methanol. Hydroxyaspartate, which is very soluble in water, can be further purified by redissolution, charcoal treatment and reprecipitation, followed by column chromatography as described by MOORE AND STEIN[10]. The separation of hydroxyaspartic acid by paper chromatography is shown in Fig. 1. Hydroxyaspartic acid reacts readily with the cadmium-ninhydrin reagent (BARROLIER[11]) to give an orange-red spot on paper (absorption maximum at 500 mμ). A pure preparation of 2,3-diaminosuccinic acid was also synthesized as described by RHINESMITH[12]. Diaminosuccinic acid does not react readily with the ninhydrin reagent (only after severe heating), but yields a yellow-colored quinoxaline derivative with glyoxal (reaction is carried out by heating the diamino acid with glyoxal in glacial acetic acid according to DECHARY, KUN AND PITOT[13]). Diaminopropionic acid gives both ninhydrin and quinoxaline color tests. A preparation of L(+)2,3-diaminopropionic acid was obtained from Mann Research Laboratories Inc. (New York).

Pyridoxal phosphate was also purchased from this firm. Pure L(+)-aspartic and L(+)-glutamic acids were received from Merck and Co.

Oxaloacetic acid was prepared and purified according to HEIDELBERGER[14] while α-ketoglutaric acid was purchased from Nutritional Biochemicals Inc. Dihydroxyfumaric acid was supplied by Aldrich Chemicals (Milwaukee, Wis.).

The best preparation of ninhydrin was one obtained from Eastman Organic Chemicals, and was used without further purification.

Enzyme preparation. Acetone powder of pig kidney mitochondria (isolated by differential centrifugation in 8% sucrose) was prepared following the procedure of DRYSDALE AND LARDY[15]. This powder was stirred mechanically for 30 minutes with 4 times its weight of 0.01 *M* phosphate or 0.1 *M* tris(hydroxymethyl)aminomethane (Tris) buffer of pH 7.4 at 0° C. After the addition of 1 g of Norite the slurry was centrifuged down (at 5000 × *g* at 0°) for 30 minutes and a clear supernatant obtained. The protein content of this extract was determined according to WARBURG AND CHRISTIAN[16] and by the biuret test.

Transaminase assay. The formation of oxaloacetate from aspartate in the presence of α-ketoglutarate and enzyme was determined spectrophotometrically with the Beckman DU and the Cary recording spectrophotometer. The micromolar extinction coefficient of oxaloacetate at pH 7.3 is 0.550 at 280 mμ and 1.125 at 260 mμ.

Chromatographic analyses were carried out as previously described (KUN AND GARCIA-HERNANDEZ[17]). Amino acids were determined quantitatively after extraction of the red cadmium-ninhydrin complex from paper chromatograms with absolute methanol. Absorbances for most amino acids were determined at 509 mμ. The lowest limit for quantitative amino acid analysis is 1 microgram. Beer's law is followed up to 20 micrograms. The cadmium ninhydrin color of amino acids is developed by dipping the dry chromatograms into a solution of this reagent (75 mg of $CdCl_2$ + 6 ml of H_2O + 0.3 ml glacial acetic acid + 200 mg ninhydrin dissolved in 100 ml of acetone).

RESULTS

Transamination of oxaloglycolic acid

The time sequence of this reaction was measured by quantitative determination of

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glutamic acid which was formed enzymically from hydroxyaspartate and α-ketoglutarate. Such an experiment is shown in Table I. The rate of dihydroxyfumarate formation from hydroxyaspartate can be followed spectrophotometrically by measuring the appearance of the dienol at 290 mμ (Fig. 2). Magnesium ion, while not affecting the initial rate, sustains the rate of appearance of the dienol peak. The effect of Mg^{++} on the enol–keto conversion of dihydroxyfumaric acid has been discussed elsewhere[2]. Enzymic formation of hydroxyaspartate from dihydroxyfumarate and glutamate was also demonstrated; however, this reaction is even slower than its reverse.

TABLE I

GLUTAMIC ACID PRODUCED FROM α-KETOGLUTARATE AND HYDROXYASPARTATE

The test system contained in a volume of 1 ml, 5 μmoles of α-ketoglutarate, 5 μmoles of hydroxyaspartate, 0.01 *M* phosphate buffer pH 7.3, 0.25 ml and 0.50 ml of extract of pig kidney mitochondria. Temperature 30°.

Incubation time min	*Glutamic acid produced (in μmoles)*	
	2.5 mg enzyme	*5.0 mg enzyme*
0	0	0
30	0.38	0.48
60	0.41	0.60
120	0.65	0.85

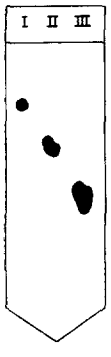

Fig. 1

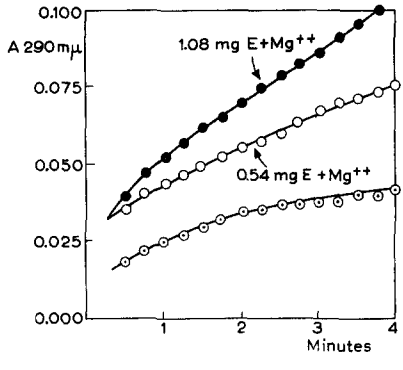

Fig. 2

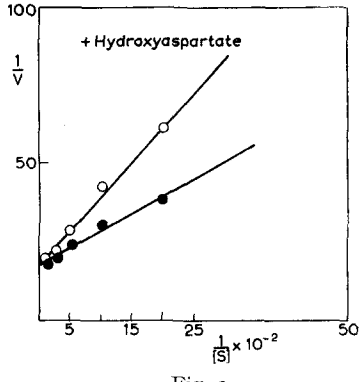

Fig. 3

Fig. 1. Paper chromatography of hydroxyaspartic (I), aspartic (II) and glutamic (III) acids. Descending chromatogram using Whatman No. 1 filter paper. Solvent system: *n*-butanol–acetic acid–water (4:1:5 v/v). Time of developing: three days at 25°.

Fig. 2. Rate of dihydroxyfumaric acid formation from hydroxyaspartic acid. Test system contained 10 μmoles of α-ketoglutarate, 10 μmoles of hydroxyaspartate, 10 μmoles of Mg^{++}, 20 μg of pyridoxal phosphate and 540 μg or 1.08 mg of protein in a total volume of 1 ml of 0.05 *M* phosphate buffer of pH 7.3. Readings were taken at 290 mμ. Lowest curve: no Mg^{++}.

Fig. 3. Competitive inhibition by hydroxyaspartate. Oxaloacetic acid formation was measured at a wavelength of 280 mμ. 10 μmoles of hydroxyaspartate. 20 μg of pyridoxal phosphate. 120 μg of protein. The velocity (initial velocity) is expressed in terms of change in absorbancy per 60 seconds. The substrate concentration is expressed in μmoles per ml. 0.1 *M* Tris buffer of pH 7.3.

Inhibition of transamination of aspartate and α-ketoglutarate

We may assume that if the same enzyme performs the transamination of both aspartic and hydroxyaspartic acids and if the second reaction is much slower than

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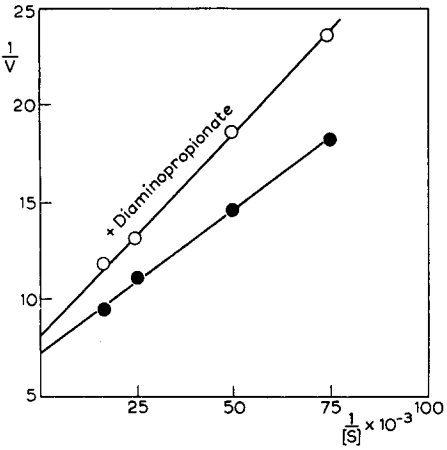

Fig. 4. Inhibition by 2,3-diaminopropionate. 10 μmoles of 2,3-diaminopropionate. 20 μg of pyridoxal phosphate. 150 μg of protein. The velocity (initial velocity) is expressed in terms of change in absorbancy per 60 seconds. The substrate concentration is expressed in μmoles per ml. 0.1 *M* Tris buffer of pH 7.3.

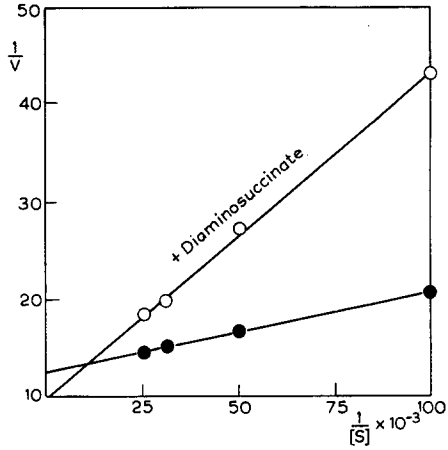

Fig. 5. Inhibition by 2,3-diaminosuccinate. 10 μmoles of 2,3-diaminosuccinate. 20 μg of pyridoxal phosphate. 142 μg of protein. The velocity (initial velocity) is expressed in terms of change in absorbancy per 60 seconds. The substrate concentration is expressed in μmoles per ml. 0.1 *M* Tris buffer of pH 9.0.

the first, hydroxyaspartate in the presence of aspartate should act as an inhibitor. Kinetic studies, dealing with the effect of hydroxyaspartic acid on the transamination of aspartate and α-ketoglutarate revealed a competitive relationship[18] between the two amino acids (see Fig. 3). This competitive relationship did not change when the concentration of the inhibitor (hydroxyaspartate) was varied.

On the other hand the two diamino acids tested as inhibitors did not show this "simple" competitive type of interaction[18]. As summarized in Figs. 4 and 5, a $1/v$: $1/S$ plot in the absence and presence of inhibitor results in straight lines which do not intersect at the ordinate. By varying the concentration of the inhibitor (all other conditions kept constant) the point of intersection may be moved. Presumably at one particular concentration of the inhibitor, the intersection may be just on the ordinate, thus imitating a "true" competitive inhibition. Similar observations were made by ALBERTY AND BOCK[19] during their study on the effect of phosphate and succinate on fumarase. The inhibition obtained by diaminopropionic acid is non-competitive while in the case of diaminosuccinic acid the type of inhibition is a combination of competitive and non-competitive one. An explanation might be visualized by assuming that the diamino acids react with one of the components of the transaminase system and in addition compete with aspartate for the enzyme site.

DISCUSSION

The results described above offer a partial answer to the question raised at the beginning of our work. A relatively slow rate of transamination of oxaloglycolate to hydroxyaspartate was readily demonstrated with an extract of mitochondria which also contains the dehydrogenase, oxidizing tartrate to oxaloglycolate. The coexistence of the two enzymes in mitochondria may suggest that under appropriate

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conditions this enzymic pathway is not improbable. In recent experiments, DAVIES AND KUN[20] isolated a malic dehydrogenase from mitochondria which among other α-hydroxydicarboxylic acids also attacks tartrate. It is a further question to be answered whether or not tartrate actually is formed in the course of intermediary metabolism.

Our earlier assumption, that certain metabolites of tartrate may act as enzyme inhibitors[1], gained support by the observed inhibition of transamination by hydroxyaspartate. Apart from the possibility—which is so far not decided by experimental findings—that these enzyme reactions actually occur in animal cells, the discovery of inhibitors of enzymic transamination is of interest by itself. As stated by BRANDENBERGER AND COHEN[21]: "Zur weiteren Erforschung der Transaminierung besteht ein grosses Bedürfniss nach einem spezifischen Inhibitor dieses Vorganges". A comparison of α,α'-acetone dicarboxylic acid—reported by these authors to be the "only highly active inhibitor of transamination"—with the effectiveness of either of the inhibitors described in this paper, is very favorable. BRANDENBERGER AND COHEN[21] needed 2.5 times higher concentration of α,α'-acetone dicarboxylate than substrate to achieve 50% inhibition. Hydroxyaspartate and diaminosuccinate in equimolar concentrations with aspartate inhibit transamination by 50%, while diaminopropionic acid is slightly less effective. Further work on the synthesis of more potent inhibitors of transamination is in progress.

SUMMARY

An extract of acetone powder of pig kidney mitochondria, which is rich in aspartic-α-ketoglutaric acid transaminase, also catalyzes the transamination of hydroxyaspartate. This second reaction is much slower than the first one. Hydroxyaspartic acid is a competitive inhibitor of the transamination of aspartate and α-ketoglutarate. Diaminopropionic and diaminosuccinic acids also inhibit transamination of aspartate. The inhibition by diaminopropionic acid is non-competitive. The inhibition by diaminosuccinic acid is a combination of competitive and non-competitive type.

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