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Studies on Aspartase

III. On the Specificity of Aspartase

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Aspartase has been shown to be strictly specific to fumaric and L-aspartic acid. No other amino-acid tested (glycine [1], alanine [2], leucine [3], phenylalanine [4], tyrosine [4], dioxyphenylalanine [4], histidine [4] and glutamic acid [1]) are deaminated by the enzyme. Nor was D-aspartic acid attacked by aspartase [5,6]. It has been shown that with maleic [1], glutaconic [1], crotonic [7], pyruvic [7], mesaconic [3], aconitic [3] and sorbic acid [3] no addition of ammonia occurred. A change in the carboxyl groups of fumaric acid seems to be decisive for the action of aspartase, *e. g.* the diamide [3] and mono- and diethyl ester [8] of this acid were not acted on by aspartase.

The present investigation has been undertaken to elucidate the structural requirements of the substrate and of the competitive inhibitors of aspartase. Aspartase was obtained from propionic acid bacteria, and its action was studied using compounds with different chemical structure competing with L-aspartic acid.

EXPERIMENTAL

Enzyme material: A finely ground dry preparation of *Propionibacterium peterssonii* (strain of this laboratory) was used as the enzyme preparation. The culture procedure was the same as that used earlier [9].

Activity measurements: The activity of the preparations was determined by the rate at which aspartic acid was deaminated. The experiments were performed in exactly the same way as before [10].

Competition experiments: Before adding the competitors to the enzyme, their pH was adjusted to 7.0—7.5 with sodium hydroxide. The dry bacterial mass and 5 ml of 0.1 *M* solution of competitor were incubated at 37° C for 30 minutes, after which the reaction was started by adding aspartic acid, phosphate buffer and distilled water.

When testing the ability of the enzyme to deaminate D-aspartic, L-cysteic and α,β-diamino-succinic acid, concentrations similar to that used in the activity determinations of L-aspartic acid, *i. e.* 0.02 *M* solutions, were used.

L-α-hydroxy-β-sulphopropionic acid was prepared by deamination of L-cysteic acid, which was treated with nitrous fumes. The fumes were prepared from sodium nitrite and diluted hydrochloric acid [11]. After deamination, the solution was boiled in a water bath with a small amount of concentrated hydrochloric acid (0.3 ml) till the nitrous gases were removed, after which the solution was concentrated to a small volume, neutralized with sodium hydroxide to pH 7 (glass electrode) and made up to a 0.1 *M* solution of L-α-hydroxy-β-sulphopropionate.

RESULTS

Preliminary experiments were performed to test the ability of this enzyme preparation to attack D-aspartic acid, L-cysteic acid and α,β-diamino-succinic acid (Fig. 1). Practically no deamination was observed with cysteic acid and α,β-diamino-succinic acid, whereas a very weak deamination of D-aspartic acid was noticed (*cf.* later).

Changes in the carboxyl groups of fumaric acid have been found to determine the synthetic action of aspartase. In order to find out how the carboxyl groups and their interatomic distance in compounds may determine their affinity for aspartase, experiments were made with carboxylic acids competing with L-aspartic acid (Fig. 2). The strongest competitive effect was noticed with oxalic acid. The difference in competing ability between citric acid and tricarballylic acid is of interest, and emphasizes the importance of the hydroxyl group (tricarballylic acid differs from citric acid in having a hydrogen atom in the place of the hydroxyl group). The presence of the hydroxyl group markedly enhances the competing effect of citric acid. Succinic acid exhibited a weak competition whereas malonic acid displayed no competitive ability at all. Nor was any competition observed with glutaric and adipic acids. In view of the weak effect produced by succinic acid it was decided to investigate in detail the four-carbon dicarboxylic acids. The competing effect of the *trans*- and *cis*-isomeric forms were investigated (Fig. 3). Maleic acid showed no affinity for aspartase, whereas mesaconic acid had a noticeable, and fumaric acid a strong affinity.

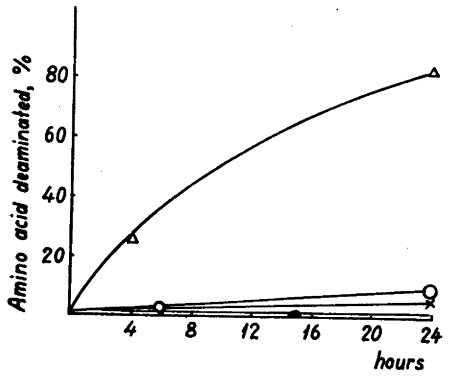

Fig. 1. Ability of the aspartase preparations to deaminate:

△ L-*aspartic acid*
○ D-*aspartic acid*
× L-*cysteic acid*
● α,β-*diamino-succinic acid*

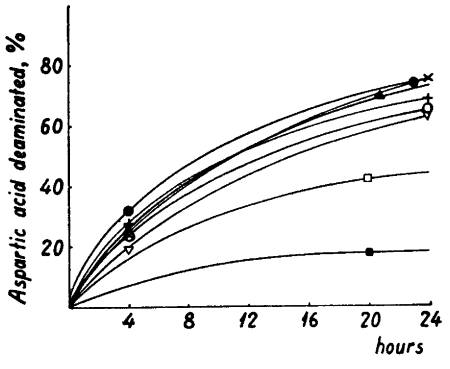

Fig. 2. Inhibiting effect of different carboxylic acids on the action of aspartase:

● *0.1* M *malonic acid*
○ *0.1* M *succinic acid*
\+ *0.1* M *glutaric acid*
× *0.1* M *adipic acid*
■ *0.1* M *oxalic acid*
▽ *0.1* M *tricarballylic acid*
□ *0.1* M *citric acid*
▲ *control without addition of inhibitors*

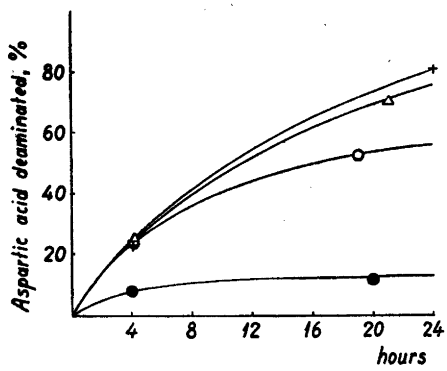

Fig. 3. Inhibiting effect of isomeric four carbon dicarboxylic acids on the action of aspartase:

○ *0.1* M *mesaconic acid*
\+ *0.1* M *maleic acid*
● *0.1* M *fumaric acid*
△ *control without addition of inhibitors*

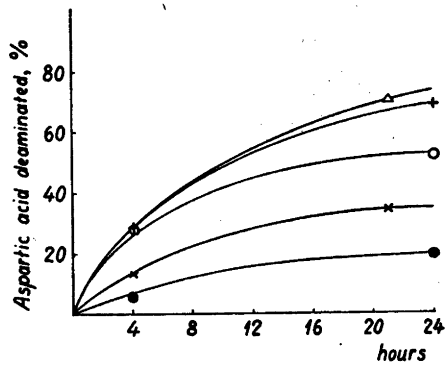

Fig. 4. Inhibiting effect of different sulphonic acids on the action of aspartase:

● *0.1* M *1,2-ethane disulphonic acid*
○ *0.1* M *sulphoacetic acid*
\+ *0.1* M p-*sulphobenzoic acid*
× *0.1* M L-*α-hydroxy-β-propionic acid*
△ *control without addition of inhibitor*

To show to what extent the carboxyl groups can be replaced by sulphonic acid groups *etc.*, compounds were tested in which one or both carboxyl groups are replaced by sulphonic acid groups (Fig. 4). The effect of 1,2-ethane disulphonic acid is almost as great as that of oxalic acid and much stronger than the effect of the corresponding dicarboxylic acid *i. e.* succinic acid. The affinity of L- *α*-hydroxy-*β*-sulphopropionic acid is strong compared with the affinity of succinic acid. L-*α*-hydroxy-*β*-sulphopropionic acid is identical with

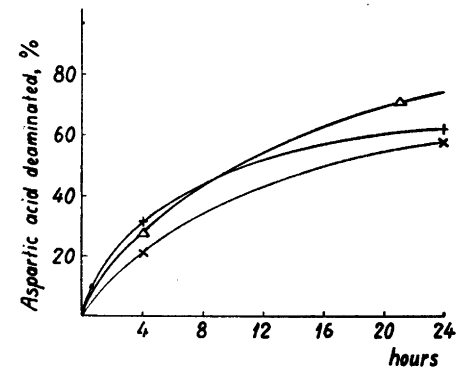

Fig. 5. Inhibiting effect of gluconic and salicylic acid on the action of aspartase,

\+ *0.1* M *gluconic acid*
× *0.1* M *salicylic acid*
△ *control without addition of inhibitor*

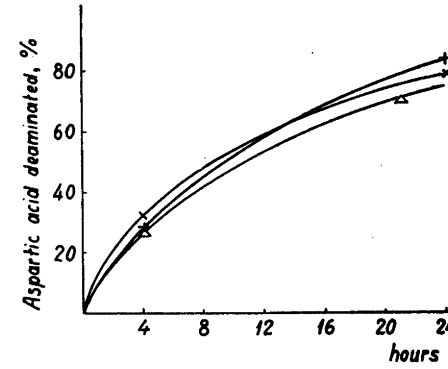

Fig. 6. Inhibiting effect of L-*cysteic and* D-*aspartic acid on the action of aspartase,*

× *0.1* M L-*cysteic acid*
\+ *0.1* M D-*aspartic acid*
△ *control without addition of inhibitor*

L-malic acid except that the carboxyl group further away from the hydroxyl group is replaced by a sulphonic acid group. If one of the two carboxyl groups in malonic acid is replaced by a sulphonic acid group, its affinity for aspartase increases compared with the original affinity of malonic acid.

Tests were made on the degree to which one of the strongly acid groups is replaceable by a phenolic group or by several alcoholic groups (Fig. 5). Salicylic acid and gluconic acid show a rather weak competing effect. Of these two acids, salicylic acid seems to possess the strongest affinity for aspartase. To ascertain whether L-cysteic acid and D-aspartic acid have any affinity for aspartase, although they do not play the role of substrates, their competing ability with L-aspartic acid was investigated (Fig. 6). In both cases no competition was noticed. Hence the weak evolution of ammonia observed earlier in the deamination experiments with cysteic acid and D-aspartic acid can hardly be due to a deamination of these acids, but ammonia is evidently liberated from other sources.

DISCUSSION

The results taken as a whole indicate that the combination of enzyme and substrate or enzyme and competing inhibitor is largely ionic in nature. Thus it appears that a two-point attraction of the substrate or competing inhibitor to the active centre is necessary for the occurrence of a combination.

The importance of the ionic attraction of the enzyme and substrate in the formation of a complex is shown by the strong inhibition produced by 1,2-ethane disulphonic acid. This molecule is identical with succinic acid except that the carboxyl groups are replaced by sulphonic acid groups. The fact that L-cysteic acid does not function as a substrate of aspartase is evidence of the great specificity of this enzyme, and is indicative of the ionic balance that must be required for the catalytic action of the enzyme.

The difference in affinity observed between citric acid and tricarballylic acid may also be explained on the basis of a difference in ionic attraction. All polar substituents almost invariably increase the acidity of an ionizing group.[12] The complexing properties of citric acid for Ca and Sr have been shown to be several times more powerful than those of tricarballylic acid, showing the importance of a hydroxyl group in this case, too [13].

The residual negative charges in a molecule should play an important role in this respect. These charges may be derived from the amount of residual charge on certain groups in a molecule due to the attractive forces of unequal kernel charges. An approximate calculation according to Latimer and Porter [14] shows that the charge on an oxygen atom in a hydroxyl group, for instance, actually possesses a residual negative charge equal to —0.20 [15]. The uncharged amino-group of amino-acids has a negative charge equal to —0.11 [15], when the charged group has a positive charge equal to +0.89 [15].

The most obvious ionic attraction would be between the ionized carboxyl groups of the substrate and the positively charged center in the enzyme. An explanation of the nature of this center may be that aspartase is a metal-protein [10] containing one of the alkaline-earth metals as activator. These

metals are known to form essentially ionic bonds with the more non-metallic elements [16].

The alkaline-earth metals tend to form rings, oxalic acid forming a five-membered, malonic acid a six-membered and succinic acid a seven-membered ring. Of these compounds, the five- and six-membered rings exhibit the greatest stability. The stability of these ring-compounds decreases steadily with increasing members in the ring [17].

Compounds unable to form rings probably have a low affinity for the enzyme. Hence the monocarboxylic amino-acids should have a low affinity for aspartase, although it may be assumed that the carboxyl group is combined with the metal as in the usual substrate, but since no ring formation is possible the affinity is low. The amino-dicarboxylic acids, aspartic and glutamic acid, may be expected to have an affinity of the same order as that of succinic and glutaric acid. However, the positively charged amino-group (positive residual charge = +0.89) can be expected to repel a positively charged ion from the adjacent carboxyl group. This should have an inhibiting effect on the combination with the enzyme. Convincing evidence to justify this suggestion is given in the competition experiments with L-cysteic acid and its deamination product L-α-hydroxy-β-propionic acid. In the case of cysteic acid no affinity to aspartase could be demonstrated but when deaminated the compound showed a strong affinity for the enzyme. The fact that no association has been found to take place between aspartic acid and calcium [18] and magnesium [19] in neutral solution confirmed these observations. Only in alkaline solution, where the amino-group possesses a negative residual charge ($pK = 9.60$ [12]) has a strong association between magnesium and aspartic acid been observed [19]. Accordingly, it is quite evident that a combination between an amino-acid and the enzyme is possible only if the charged amino-group is neutralized or blocked.

On the basis of all this the existence of a third point of attraction in the aspartase should be possible. Combining with the positively charged amino-group of the substrate the repulsion effect of the uncombined charged group may be prevented. In D-aspartic acid no combination takes place and the repulsion between the positive charges prevents the complexformation between the amino-acid and the enzyme. This should explaine why no competition between L-aspartic acid and D-aspartic acid was observed.

No reliable information on the nature of the third point of attraction can be given. There is, however, reason to assume that this point consist of a thiol group, since it has earlier been shown that thiol groups are necessary for the activity of aspartase [10].

To explain the stereochemical specificity, it is suggested that there should be at least three points of specific interaction between enzyme and substrate. In the case of aspartase, these three points seem to be made up of the two valencies of the metal assumed to bind the two essential carboxyl groups, and a third, possibly a thiol group, which combines with the positively charged amino-group, neutralizing it and so making possible the formation of the complex with the enzyme, which is necessary for the catalytic action.

SUMMARY

Aspartase has been shown to have no action on α,β-diamino-succinic acid, L-cysteic acid and D-aspartic acid. Nor did L-cysteic acid and D-aspartic acid show any affinity for aspartase. Strong affinity for the enzyme has been observed in the sodium salts of oxalic, citric, 1,2-ethane disulphonic and L-α-hydroxy-β-sulphopropionic acid. Succinic, mesaconic, sulphoacetic and salicylic acid show a weak affinity for this enzyme, whereas no affinity could be observed in the case of malonic, maleic, glutaric, adipic, *p*-sulphobenzoic and gluconic acid.

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REFERENCES

1. Quastel, J. H., and Woolf, B. *Biochem. J. (London)* **20** (1926) 545.
2. Cook, R. P., and Woolf, B. *Biochem. J. (London)* **22** (1928) 474.
3. Virtanen, A. I., and Tarnanen, J. *Biochem. Z.* **250** (1932) 193.
4. Jacobsohn, K. P., and Soares, M. *Compt. rend. soc. biol.* **125** (1937) 554.
5. Jacobsohn, K. P., and Pereira, F. B. *Compt. rend. soc. biol.* **120** (1936) 551.
6. Virtanen, A. I., and Laine, T. *Suomen Kemistilehti* **B 9** (1936) 28.
7. Ichihara, T. *Fukuoka-Ikwadaigku-Zasshu* **24** (1931) 1231; *Chem. Abstracts* **26** (1932) 3539.
8. Jacobsohn, K. P., and Soares, M. *Enzymologia* **1** (1936) 183.
9. Ellfolk, N. *Acta Chem. Scand.* **7** (1953) 824.
10. Ellfolk, N. *Acta Chem. Scand.* **7** (1953) 1155.
11. Warburg, O. *Biochem. Z.* **189** (1927) 354.
12. Cohn, E. J., and Edsall, J. T. *Proteins, Amino acids, and Peptides*, Reinhold, New York, 1943.
13. Schubert, J. *Nature* **166** (1950) 913.
14. Latimer, W. M., and Porter, C. W. *J. Am. Chem. Soc.* **52** (1930) 206.
15. Schmidt, C. L. A. *Chemistry of the Amino Acids and Proteins, 2nd ed.*, Thomas, Springfield, 1945.
16. Pauling, L. *The Nature of the Chemical Bond.* Ithaca, New York, 1939.
17. Cannan, K., and Kibrick, A. *J. Am. Chem. Soc.* **60** (1938) 2314.
18. Heinz, E. *Biochem. Z.* **321** (1951) 314.
19. Greenwald, J. *J. Phys. Chem.* **43** (1939) 379.

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