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Studies on Aspartase

II. On the Chemical Nature of Aspartase

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Our knowledge of the chemical nature of aspartase is negligible [1]. In 1938 Gale [2] notised that aspartase is composed of two enzymes, aspartase I and aspartase II, the latter requiring a coenzyme for its action. However, this observation has not been confirmed. Later Lichstein [3–6] and his collaborators have been able to show a biotin-linked deamination of aspartic acid with aged cells of different bacteria. The connection of this reaction with aspartase is unkown.

The present study has been undertaken in order to elucidate the chemical nature of aspartase. The enzyme was prepared from propionic acid bacteria. Different inhibitors were used, which in many cases have given results making possible a deeper understanding of the mode of action of the enzyme.

EXPERIMENTAL

Enzyme material: A finely ground dry preparation of *Propionibacterium peterssonii* (strain of this laboratory) was used as the enzyme preparation. The procedure was the same as was used earlier in cultivating bacteria mass [7].

Activity determinations: The activity of the preparations was determined by the rate at which aspartic acid was deaminated. The most convenient method for this purpose is to determine the liberated ammonia. The experiments were performed in 10 ml measuring cylinders. The following test solution was incubated in the cylinders at 37° C for 24 hours:

100	mg	dry bacteria mass
26.6	»	aspartic acid (in 2 ml, pH 7.2)
2	ml	phosphate buffer *M*/15 (pH 7.2)
6	»	distilled water
10	ml	total volume (0.3 ml toluene was added as antiseptic)

During the incubation, samples of 2 ml were taken for the determination of liberated ammonia, which was distilled after alkalization with a carbonate buffer [8] in a modified Pucher apparatus [9]. The receiver was charged with 5 ml 0.01 *N* sulfuric acid. Excess of acid was titrated iodometrically.

Inhibition experiments: Before adding the inhibitor solution to the enzyme the pH was adjusted to 7.0—7.5 with hydrochloric acid or potassium hydroxide. As phenylarsine oxide is insoluble in water a stock solution was prepared in *iso*-propanol. A stock solution of BAL (2,3-dimercaptopropanol) was prepared in ethyleneglycol monoethylether.

The dry bacteria mass and 5 ml inhibitor solution were incubated at 37° C for 30 minutes befor starting the reaction by adding aspartic acid (2 ml), phosphate buffer (2 ml) and distilled water (1 ml) to bring the total volume up to 10 ml.

In the experiments with acetonitrile, potassium cyanide and sodium sulfide the test cylinder was plugged with a rubber stopper.

RESULTS

Metal enzyme inhibitors: The effect of various metal inhibitors on aspartase is shown in Figures 1 to 3. Strong inhibition was produced by citrate, oxalate, versene (ethylenediamine tetraacetic acid) and pyrophosphate. The inhibition by cyanide, azide and acetonitrile is weak. No inhibition was seen with *ortho*phosphate and sodium sulfide. An apparent enzyme activation seems to take place in the presence of diethyldithiocarbamate. This reagent is not specific for copper only; silver mercury, nickel and manganese all give complex compounds. Hence the activation can be explained by removal of heavy metals from the enzyme surface.

These inhibitions point to the existence of an essential metal in the enzyme. The action of the four first-mentioned agents strongly suggest that one of the alkaline-earth metals is present, possibly magnesium.

The negligible inhibition produced by fluoride is surprising since fluoride has been regarded as a strong inhibitor of calcium and magnesium enzymes.

Thiol group inhibitors: Thiol groups essential to enzymes are usually detected through the inactivation of the enzyme by certain oxidizing and alkylating agents and mercaptide-forming compounds.

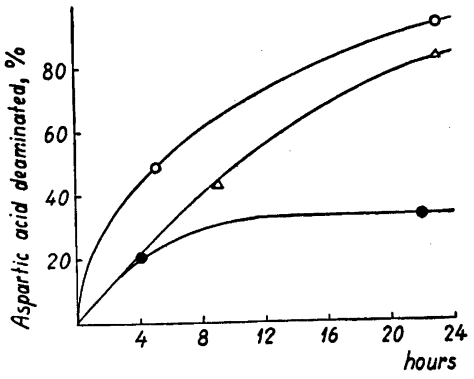


Fig. 1. Inhibitory effect of different anions on the deamination of aspartic acid:
○ *0.1* M *diethyldithiocarbamate*
● *0.1* M *ethylenediaminetetraacetic acid*
△ *control without addition of anions*

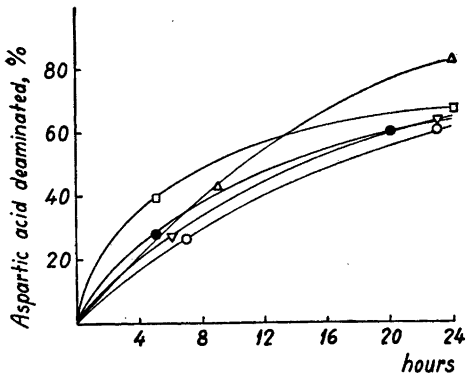


Fig. 2. Inhibitory effect of different metal reagents on the deamination of aspartic acid:
○ *0.1 M sodium azide*
□ *0.1 M sodium sulfide*
● *0.1 M potassium cyanide*
▽ *0.1 M acetonitrile*
△ *control without addition of reagent*

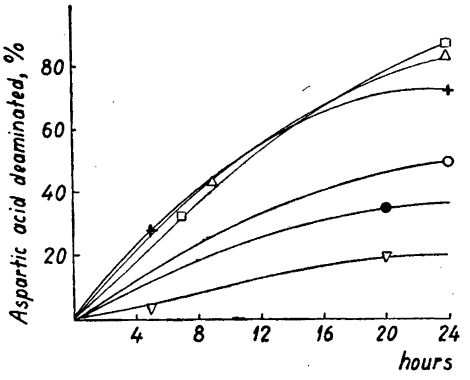


Fig. 3. Inhibitory effect of different anions on the deamination of aspartic acid:
○ *0.1 M citric acid*
● *0.1 M oxalic acid*
□ *0.1 M orthophosphate*
▽ *0.1 M pyrophosphate*
\+ *0.1 M potassium fluoride*
△ *control without addition of anions*

Barron and Singer [10] showed that *o*-iodosobenzoate was the most powerful of the oxidizing agents used in their studies. The effect of the reagent is based on the reaction:

$$\text{Protein}\begin{matrix}\diagup\text{SH}\\ \diagdown\text{SH}\end{matrix} + \text{IOC}_6\text{H}_4\text{COONa} = \text{Protein}\begin{matrix}\diagup\text{S}\\ \diagdown\text{S}\end{matrix} + \text{IC}_6\text{H}_4\text{COONa} + \text{H}_2\text{O}$$

Accordingly, the oxidizing agents act only on sulfhydryl groups close enough to allow disulfide formation. Fig. 4 shows the effect of different oxidizing agents. Iodine is the least specific and many groups in the protein molecule react with it either by being oxidized or by forming iodinated compounds. Ferricyanide has been extensively used for the determination of sulfhydryl groups in proteins. Only the sulfhydryl groups seem to reduce ferricyanide [11]. In metal-proteins, however, competition with the active metal may produce an inhibitory effect.

As detectors of sulfhydryl groups the alkylating agents are the least reactive of the commonly used agents. It is assumed that the agents react by replacing the hydrogen of the sulfhydryl group by the carboxymethyl group:

$$\text{Protein}-\text{SH} + \text{CH}_2\text{I}\cdot\text{COONa} = \text{Protein}-\text{S}-\text{CH}_2\cdot\text{COONa} + \text{HI}$$

The effect of iodoacetamide on aspartase is shown in Fig. 5. However, the action of iodoacetic acid or its amide is not confined to the sulfhydryl groups but may involve other groups in the protein molecule. Evidence of the

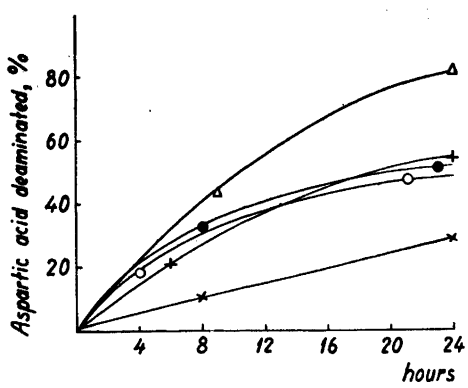


Fig. 4. Inhibitory effect of oxidizing thiol group reagents on the deamination of aspartic acid:
● *0.01* M *ferricyanide*
○ *0.01* M *o-iodosobenzoate*
\+ *0.0001* M *iodine*
× *0.001* M *iodine*
△ *control without addition of reagents*

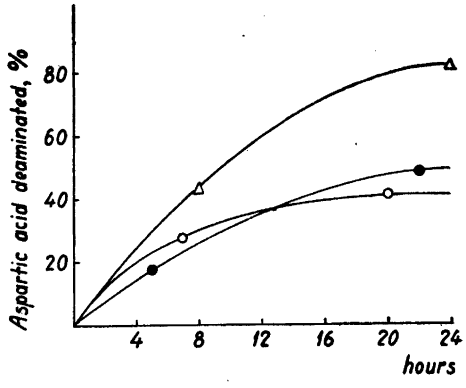


Fig. 5. Inhibitory effect of alkylating thiol group reagents on the deamination of aspartic acid:
○ *0.01* M *iodoacetamide*
● *0.05* M *iodoacetamide*
△ *control without reagent addition*

fact that iodoacetate inhibition, even in thiol enzymes, is produced by combination with groups other than sulfhydryl was given by Barron and Singer [10].

Among the mercaptide-forming agents *p*-chloromercuribenzoic acid has the advantage of combing with single sulfhydryl groups which makes possible the inhibition of sulfhydryl groups too far apart to form the disulfide linkage:

$$\text{Protein}-\text{SH} + \text{Cl}\cdot\text{Hg}\cdot\text{C}_6\text{H}_4\text{COONa} = \text{Protein}-\text{S}-\text{Hg}\cdot\text{C}_6\text{H}_4\cdot\text{COONa} + \text{HCl}$$

Thiol substances have a protecting effect against inhibition of this kind in the following way:

$$\text{Protein}-\text{S}-\text{Hg}\cdot\text{C}_6\text{H}_4\cdot\text{COONa} + \text{R}\cdot\text{SH} = \text{Protein}-\text{SH}+\text{R}-\text{S}-\text{Hg}\cdot\text{C}_6\text{H}_4\cdot\text{CCONa}$$

Fig. 6 shows the effect of *p*-chloromercuribenzoate and two arsenicals on aspartase. The antidotal effect of BAL (2,3-dimercaptopropanol) is shown in the same figure.

A possible protecting effect of aspartic acid, the natural substrate of the enzyme, against sulfhydryl reagents was tested with *p*-chloromercuribenzoate in the following way: Aspartic acid was added to the enzyme and incubated for 10 minutes before the mercurial was added to a concentration of $5 \cdot 10^{-3}$ *M* in 5 ml (the concentration was the same as in the experiment with BAL). No protection was noticed, at least not in this concentration, and the enzyme was completely inactivated.

Inhibitory effect of cations: Because an inactivation of aspartase by heavy metals can be assumed from the activation shown by diethyldithiocarbamate it was decided to investigate the influence of some metals on the activity of aspartase. Fig. 7 and 8 show the influence of different cations on the activity

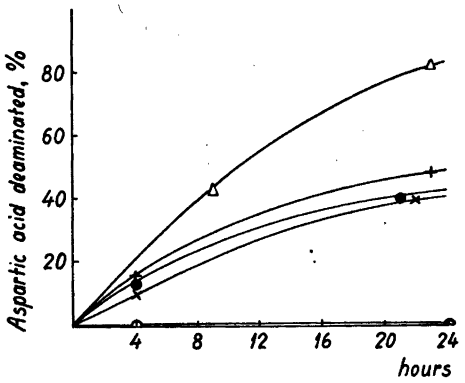


Fig. 6. Inhibitory effect of mercaptide-forming compounds on the deamination of aspartic acid:
- ○ *0.001* M p-*chloromercuribenzoate*
- ● *0.005* M p-*chloromercuribenzoate* + *0.01* M *BAL (2,3 dimercaptopropanol)*
- \+ *0.004* M *phenylarsine oxide*
- × *0.01* M *3-amino-4-hydroxydichlorarsine hydrochloride*
- △ *control without addition of mercaptide-forming compounds*

of the enzyme. Diethyldithiocarbamate gave the enzyme full protection against a mercurichloride concentration sufficient to inactivate it completely. The activation by the carbamate observed earlier may therefore be a protection against metals, as assumed above.

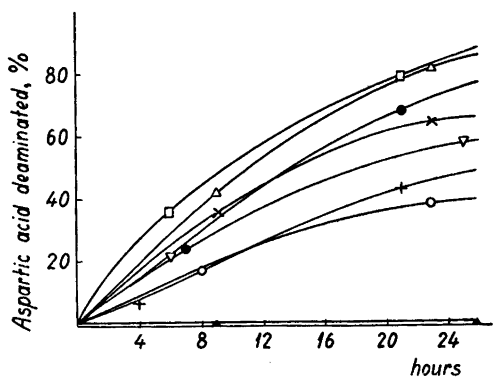


Fig. 7. Inhibitory effect of different metals on the deamination of aspartic acid:
- ● *0.001* M VCl_2
- ○ *0.001* M $AgNO_3$
- ▲ *0.001* M $HgCl_2$
- ■ *0.001* M SeO_2
- ▽ *0.001* M $CdCl_2$
- × *0.001* M $CuCl_2$
- \+ *0.001* M $ZnCl_2$
- △ *control without metal addition*

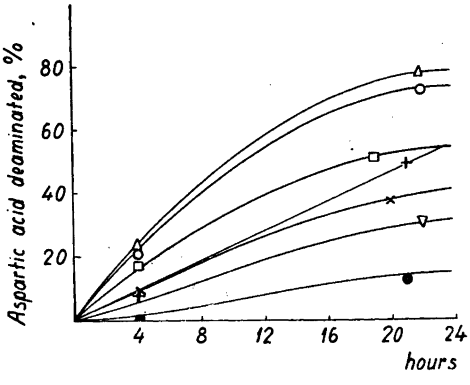


Fig. 8. Inhibitory effect of different metals on the deamination of aspartic acid:
- ● *0.01* M $CoCl_2$
- \+ *0.01* M $NiCl_2$
- ○ *0.01* M $MnCl_2$
- □ *0.01* M $FeCl_2$
- × *0.01* M $PbCl_2$
- ▽ *0.01* M $CdCl_2$
- △ *control without metal addition*

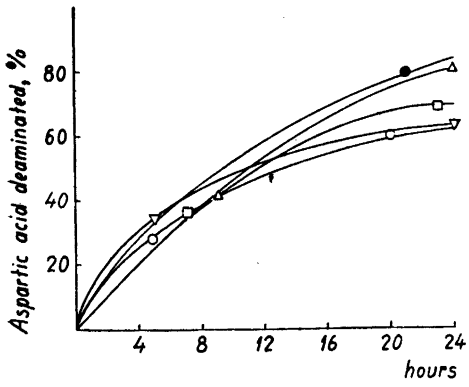


Fig. 9. The inhibitory effect of different carbonyl group reagents on the deamination of aspartic acid:
○ *0.01* M *potassium cyanide*
□ *0.01* M *semicarbazide*
▽ *0.01* M *hydrazine*
● *0.01* M *hydroxylamine*
△ *control without addition of reagents.*

Carbonyl group reagents: As cyanide is also known to react with carbonyl groups the effect of carbonyl group reagents on aspartase was examined. The results in Fig. 9 seem to indicate that the presence of an active carbonyl group in the enzyme can be excluded.

DISCUSSION

In the enzyme preparations used in this work fumarase was regularly present. Therefore the following reactions took place:

1) aspartic acid ⇌ fumaric acid + ammonia
2) fumaric acid + H_2O ⇌ malic acid

Accordingly, the velocity of the deamination of aspartic acid by the preparations used depends on the velocity of these two reactions. In the inhibition studies with aspartase the inhibitory effect observed may at least partially be explained as an inhibition of reaction 2, *i.e.* as an inhibition of fumarase. According to Massey [12], however, we know that crystalline fumarase is not inhibited by diethyldithiocarbamate, ethylenediamine tetraacetic acid, and citric acid, and hence fumarase cannot possibly be a metal protein nor responsible for the inhibitions with metal poisons used in this investigation. The inhibitions are limited, consequently, to the inhibition of aspartase, *i.e.* reaction 1.

On the basis of these studies it may be suggested that aspartase contains a metal ion essential for its activity. The metal in question seems to be one of the alkaline-earth metals, possibly magnesium. This being the case it is somewhat surprising to notice that aspartase was not inhibited by fluoride, although several experiments were performed with lengthening of the incubation time.

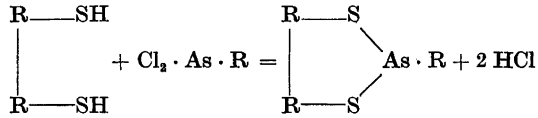
Warburg and Christian [13] have shown that enolase is a dissociating metal protein with magnesium as its activator. The protein component is inactive as such. The mechanism of the fluoride inhibition is not the binding of the magnesium of the enzyme but the displacement of the magnesium in the protein by a magnesium fluorophosphate molecule. It seems that the more readily the metal dissociates from the enzyme the stronger the effect of fluoride.

There are magnesium enzymes on which fluoride has no inhibitory effect at all *e.g.* carboxypeptidase [14] which is inhibited by cyanide. Alkaline phosphomonoesterase [15], a magnesium protein, is inhibited by fluoride in special experimental conditions only, whereas cyanide has a strong effect.

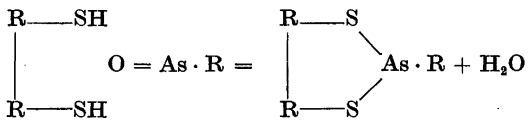
Aspartase apparently contains sulfhydryl groups. This is suggested by the complete inhibition achieved with some sulfhydryl reagents, which cannot be explained as an inhibition of fumarase.

Under the conditions of the experiments the inhibitory effect of the arsenicals and *o*-iodosobenzoic acid was weak compared with the effect of *p*-chloromercuribenzoic acid. The difference between the mercurial and the arsenicals and oxidizing agents may be explained in part from the manner in which they combine with sulfhydryl groups.

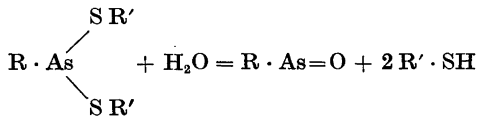
p-Chloromercuribenzoate reacts with one sulfhydryl group only. The arsenicals, on the contrary, react probably with two groups, in the following way:



or



Stocken and Thompson [16] have shown that a cyclic compound prepared by reacting arsenical with a dithiol is markedly more stable than the non-cyclic thioarsenites formed by interaction with monothiols, which undergo dissociation at physiological pH according to the reaction found by Cohen *et al.*[17]



Hence it is assumed that the high inhibitory effect produced with the arsenicals can be due to their ability to combine with two essential sulfhydryl groups forming a stable arsenical ring. A certain consonance between the effects of the arsenicals and *o*-iodosobenzoate can therefore be expected though it is known that arsenicals are able to react with sulfhydryl groups that are not attacked by oxidizing agents.

It is understandable that with only one sulfhydryl group and an inhibitor a reaction might occur more rapidly than with two groups. This, however, cannot provide a satisfactory explanation of all the facts, particularly as Barron and Singer [10] have shown with succinoxidase that the arsenicals and *p*-chloromercuribenzoate had about the same capacity to combine with the sulfhydryl groups in the protein *i.e.* 50 % inhibition was obtained with 3.2 $\times 10^{-5}$ *M* *p*-chloromercuribenzoate and 3.15 $\times 10^{-5}$ *M* 3-amino-4-hydroxy-

phenyldichlorarsine hydrochloride. However, in the experiments described above the effect of the arsenicals is only a fraction of that of the mercurial.

The evidence available is not sufficient to enable us to decide whether aspartase contains one or two sulfhydryl groups essential for its activity. If the enzyme contains only one essential group the weak inhibition obtained by the arsenicals and *o*-iodosobenzoate can be explained by assuming a reaction with two sulfhydryl groups from different molecules, an assumption that may explain the weak effect of the arsenicals. Hence it seems more likely that the activity of the enzyme depends on the presence of one sulfhydryl group or several sulfhydryl groups not situated close enough to permit formation of rings with arsenicals or disulfide linkages by oxidizing agents.

No protecting ability against *p*-chloromercuribenzoate could be proved with aspartic acid, which seems to indicate that no hindrance is produced by the substrate against attack of the inhibitor. Hopkins *et al.* discovered this protecting phenomenon in succinic dehydrogenase when the sulfhydryl reagent was added after malonate or succinate. The Hopkins phenomenon has since been shown to include several different enzymes *e.g.* carboxylase [19] and alcohol dehydrogenase [20].

Aspartase was strongly inactivated by metals. The toxicity of heavy metals seems largely to be due to their combining with sulfhydryl groups forming mercaptides or to their acting as oxidizing agents, as has been shown by Barron and Kalnitsky [21]. The inactivation of aspartase can be explained at least partially as an action on the sulfhydryl groups.

If, however, the action of different metals on aspartase is compared with the action of the same metals on urease [22], known as a sulfhydryl enzyme, a clear difference can be observed. Cobalt and nickel have a strong inhibitory effect on aspartase, on urease their inhibiting power is rather weak. The complete inhibition of urease with cobalt and nickel requires concentrations (molarities) thousands of times as great as with cadmium and zink [23]. Aspartase, on the contrary is inhibited more strongly with cobalt than with cadmium in the same concentration.

Accordingly, it is reasonable to assume still another inhibiting mechanism in the case of aspartase. The transition elements are known as strong formers of complex compounds. In their investigations of metal complex compounds Pfeiffer *et al.*[24] have shown that some metals are able to replace other metals in a metallo-organic complex compound. Pfeiffer obtained the following sequence, where the metal on the left was able to replace that on the right in a complex compound:

$$Cu \rightarrow Ni \rightarrow V, \quad Fe \rightarrow Zn \rightarrow Mg$$

In aspartase, cobalt and nickel replaced the alkaline-earth metal assumed to be responsible for the activity, and an inactivation was produced.

The negative results with the carbonyl group reagents show that these groups play no part in the activity of aspartase. Accordingly, the transfer of the amino group to an aldehyde group similar to that in the transaminase enzyme [25] can be excluded in the case of aspartase.

SUMMARY

1) The inhibition data obtained with the metal inhibitors show that in all probability aspartase is a metal-protein in which the metal is essential to its activity. The metal seems to be one of the alkaline-earth metals, possibly magnesium.

2) The activity of aspartase depends on thiol groups, which are sensitive to heavy metals and their compounds but less reactive to other thiol detectors like trivalent arsenicals, alkylating and oxidizing agents.

3) No evidence could be presented to justify the assumption of an active carbonyl group in aspartase.

4) In the discussion the assumption is made that aspartase contains only one active sulfhydryl group. To explain the strong inhibitory effect of cobalt and nickel, competition between these strong complex formers and the active alkaline-earth metal in the enzyme is assumed.

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