Protective Actions of L-Aspartate from Acid or Proteolytic Inactivation

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- (1) L-Aspartate was found to replace L-asparagine in the protective action from acid inactivation of L-asparaginase (EC 3.5.1.1) produced by *Escherichia coli* A-1-3 and at the same time to inhibit the proteolytic inactivation by α -chymotrypsin.
- (2) L-Asparaginase changed in its chromatographic properties in the presence of L-aspartate and became to be absorbed on the CM Sephadex column.
- (3) The sedimentation patterns of L-asparaginase at pH 3.5 were identical either in the presence or absence of L-aspartate, showing partial dissociation. But the reversibility to the active state was observed only in the enzyme dissolved in the solution containing L-aspartate.
- (4) L-Aspartate did not prevent the enzyme either from the dissociation into subunits or from decrease in the activity by urea.
- (5) High concentration of L-aspartate was shown to inhibit the L-asparagine hydrolysis reaction.
- (6) L-Aspartate was suggested from ORD measurements to cause changes in the higher structure as well as the ionic properties or proteolytic inactivation.

Since the antineoplastic principle in guinea pig serum was identified with L-asparaginase,¹⁾ the enzymes from various origins were examined for the antitumor activities and many studies have been reported on the enzymological properties of the enzymes.

L-Asparaginase from *Escherichia coli* A-1-3, a mutant of *E. coli* HAP, was similar to EC 2 from *E. coli* B in the anti-leukemia activity,^{2, 3)} the physico-chemical properties^{4, 5)} and the substrate specificities.^{6, 7)} Some differences, however, were noticed between them, for example, the enzyme from *E. coli* A-1-3 did not contain cystine or cysteine residues.

Cooney and Davis⁸⁾ demonstrated that L-asparagine accelerated the thermal denaturation of the enzyme from *E. coli* B, while we observed with the enzyme from *E. coli* A-1-3 that L-asparagine did not accelerate the thermal inactivation, but showed a weak protection against it. Furthermore, L-asparagine remarkably protected the enzyme from

inactivation by acid or proteases. This protective action of L-asparagine could be reproduced by L-aspartate more effectively. In addition, L-aspartate changed the chromatographic behaviors of the enzyme, but did not interfere with the dissociation of the enzyme into subunits.

The present investigation was undertaken to characterize the effect of L-aspartate on the stability of the enzyme.

MATERIALS AND METHODS

L-Asparaginase was prepared from *Escherichia coli* A-1-3 as previously reported.⁵⁾ The enzyme activity was determined by measuring the amount of ammonia formed with nesslerization in the standard assay system,⁵⁾ unless otherwise specified.

Ultracentrifugal studies were carried out with a Hitachi U.C.A. 1A ultracentrifuge equipped with Schlieren optics. Sedimentation velocity was measured usually with the final speed of 60,000 rpm at 20°C. Under acidic conditions, it was measured at 51,200 rpm using a Kel-F cell. When multiple peaks appeared, the relative concentration of each component was

L-Asparaginase from *Escherichia coli*. Part III.

approximated by integrating the areas under each schlieren peak.

Optical rotatory dispersion measurements were performed with a Jasco ORD/UV-5. In every measurement, optical rotatory dispersion coefficient λ_e was calculated from the slope of Yang's plot, in which $[\alpha]_d$ was compensated for the effect of the solvent, and the helix content was deduced from λ_e , using Yang's empirical equation.⁹⁾

RESULTS

Protective effects of some amino acids on acidic inactivation of L-asparaginase

Since L-asparagine, the substrate, protects L-asparaginase from *E. coli* A-1-3 against acid inactivation,⁵⁾ some compounds related to L-asparagine were examined for their protective effect. As shown in Table I, only 26.4% of the original activity remained after the acidic incubation of the enzyme in the absence of amino acids. The enzyme solutions containing L-aspartate or L-asparagine retained about 90% of the original activity, and D-aspartate showed a weak protective effect. Other amino acids did not exhibit comparable positive effect.

Considering that the asparaginase is considerably active at pH 3.5 and D-aspartate showed a weak but more effective protection than D-asparagine did, the protective effect of L-asparagine might be ascribed to L-aspartate formed from L-asparagine during the acidic incubation.

The effects of L-aspartate and L-asparagine on acidic inactivation were compared at pH 2.8, at which the enzymatic hydrolysis of L-asparagine was expected to be negligible. The result (Table II) suggests that the protection against acidic inactivation was effected by L-aspartate but not by L-asparagine, the substrate of the enzyme.

The effects of L-aspartate on the stability of the enzyme at various pH are shown in Fig. 1. L-Aspartate showed a very weak protection at alkaline pH.

Effects of L-aspartate on chromatographic behaviors of L-asparaginase

TABLE I. EFFECTS OF AMINO ACIDS ON THE STABILITY AT pH 3.5

The incubation mixture consisting of the enzyme, 20 mM amino acid, and 50 mM buffer (acetate buffer pH 3.5 or phosphate buffer pH 8.0) was incubated at 47°C for 60 min and the remaining activity was assayed.

The remaining activity of the enzyme incubated at pH 8.0, which was nearly equal to the initial activity, was assumed to be 100%.

Addition	pH	Activity remained (%)
none	8.0	100.0
	3.5	26.4
L-Asparagine	3.5	91.2
L-Aspartate	3.5	87.3
D-Asparagine	3.5	29.8
D-Aspartate	3.5	53.5
L-Glutamine	3.5	25.7
L-Glutamate	3.5	26.3
L-Cysteine	3.5	29.3
L-Homoserine	3.5	22.7

TABLE II. COMPARISON OF PROTECTIVE ACTION AGAINST ACID INACTIVATION BETWEEN L-ASPARTATE AND L-ASPARAGINE

The incubation mixture was consisted of enzyme (50 units), buffer (100 mM) and L-aspartate or L-asparagine (0, 1, 5 and 20 mM). The remaining activity was assayed after the incubation at 47°C for 30 min.

pH	Amino acid concentration mM	Activity remained	
		L-Aspartate	L-Asparagine
8.0	0	100%	
2.8	0	35.8	
2.8	1	35.8	39.1
2.8	5	48.0	35.1
2.8	20	89.5	41.7

L-Aspartate was proved useful to stabilize the enzyme in CM Sephadex column chromatography. When L-asparaginase was applied on a CM Sephadex column equilibrated with acetate buffer of pH 5.0, and was eluted with the same buffer using a linear concentration gradient of sodium chloride, the enzyme activity was recovered in a yield of about 50% (Fig. 2A). On the other hand, when the enzyme was dialyzed against the acetate buffer containing 10 mM L-aspartate, and

FIG. 1. Effect of pH on the Stability of L-Asparaginase.

The incubation mixture of (a) 0.2 ml of the enzyme solution (50 u/ml), 0.7 ml of buffer (0.05 M) and 0.1 ml of water $\circ$ — $\circ$, or (b) 0.1 ml of L-aspartate (10 mM) in place of water $\bullet$ — $\bullet$, was incubated at 47°C for 60 min. The remaining activity was assayed at pH 8.0. pH 3~5: acetate, pH 6~8: phosphate, pH 9~11: borate buffer.

FIG. 2. CM Sephadex Column Chromatographies with and without L-Aspartate.

The columns and sample solutions were bufferized with 0.01 M acetate buffer, pH 5.0, (A) not containing or (B) containing 20 mM L-aspartate and the same buffers were used for the elution with linear gradient of NaCl concentration. O.D. 450 shows the color intensity of the nesslerization which stands for the enzyme activity of the fractions.

applied on a CM Sephadex column equilibrated with the same buffer, the enzyme activity was not absorbed on the column but was washed out with the buffer containing L-aspartate in a yield of 93% (Fig. 2B).

The differences between the elution profiles and the yields of the enzyme were obviously caused by the addition of L-aspartate to the buffer because these phenomena were observed solely with the asparaginase protein.

L-Glutamate did not show the similar effects.

Inhibition of proteolytic inactivation by L-aspartate.

L-Asparaginase from *E. coli* A-1-3 was inactivated by the action of proteolytic enzymes. The incubation of the enzyme with proteases, such as α -chymotrypsin, caused the decrease of the activity as shown in Fig. 3 curve A. L-Asparagine has been previously shown to protect the enzyme from proteolytic inactivation.¹⁰⁾ As shown in Fig. 3 curve B, L-aspartate protected the enzyme similarly against the inactivation by α -chymotrypsin. The protective effects of L-aspartate were observed against other proteases as well. Of several proteases examined, α -chymotrypsin inactivated the most effectively and trypsin, subtilisin and thermolysin in the succeeding order.

FIG. 3. Effect of L-Aspartate on the Proteolytic Inactivation.

The incubation mixture consisted of 1 ml of enzyme (1 mg/ml), 1 ml of α -chymotrypsin (5 γ /ml) and 1 ml of phosphate buffer (A) $\circ$ — $\circ$, or 1 ml of L-aspartate 30 mM in phosphate buffer (0.1 M, pH 7.4) (B) $\bullet$ — $\bullet$. At each time indicated, 0.1 ml of the mixture was introduced into an assay tube, containing L-asparagine 22 mM, pH 8. The reaction time for assay was 2 min.

FIG. 4. Effect of Temperature on L-Asparaginase Stability.

The incubation mixtures consisted of (a) enzyme and phosphate buffer pH 8.0 ○—○, or (b) enzyme, phosphate buffer and L-aspartate 10 mM ●—●, were incubated at temperature indicated for 30 min and the remaining activity was assayed.

The disc electrophoretic patterns of the asparaginases treated with α -chymotrypsin in the presence and in the absence of L-aspartate did not differ each other, though their enzymatic activities were entirely different.

Effects of L-aspartate on thermal treatment

L-Asparaginase was relatively stable against heat. As shown in Fig. 4, when the enzyme solution of pH 8.0 was incubated for 30 min at various temperatures, the activity began to decrease at 60°C and became to zero at 80°C. In this heat treatment, L-aspartate did not appreciably broaden the stable region. However, a weak but distinct suppression of heat inactivation was observed in the presence of L-aspartate from 60°C to 80°C, where the remaining activity was decreasing. For example, 52.3% of the initial value was retained in the presence of L-aspartate, while 40% remained in its absence at 66°C.

Effect of L-aspartate on inhibition by protein denaturants

L-Asparaginase was also relatively stable against alcohol and other solvents. In the purification procedure, the enzyme protein was

precipitated by adding *n*-propyl alcohol (50% v/v) at room temperature without loss of activity. Similar results were obtained when other solvents such as ethanol or acetone, were used. However, these solvents inhibited the enzyme activity. In 10 M methanol solution, the enzyme activity was reduced to 43% of the value measured in the absence of methanol, irrespective of the presence of L-aspartate, as shown in Table III. In both cases, the reduced activities were recovered up to the original levels by removing methanol.

Similar results were obtained with urea (Table III). The enzyme activity was completely recovered when urea was removed by dialysis.

TABLE III. EFFECT OF L-ASPARTATE ON THE ENZYME ACTIVITY IN THE PRESENCE OF METHANOL OR UREA

The assay mixtures in which methanol (10 mM) or urea (4 or 8 M) were added to the usual assay mixture (22 mM L-asparagine in 0.1 M phosphate buffer pH 8.0) were used in the assay. Other conditions were the same as in the usual enzyme assay.

	Denaturant M	L-Aspartate added mM	Activity remained %
Methanol	0	0	100.0
	10	0	42.8
	0	10	103.5
	10	10	43.2
Urea	0	0	100.0
	4	0	51.5
	8	0	2.9
	0	10	100.0
	4	10	53.8
	8	10	0

These results indicated that L-aspartate did not interfere with the inhibition of the enzyme by protein denaturants.

Effect of L-aspartate on subunit structure

It was already demonstrated in disc electrophoresis and sedimentation studies that L-asparaginase was composed of a few subunits and the treatment of the enzyme with urea or guanidine hydrochloride resulted in its dissociation into the subunits.¹⁰⁾

In order to determine whether L-aspartate interfered with the dissociation and the association of the enzyme, sedimentation measurements were carried out with the enzyme solutions both with and without L-aspartate, in the presence of 3 M urea. Figure 5 shows the photographs taken at 72 min after reaching 60,000 rpm at 20°C.

FIG. 5. Sedimentation Patterns of L-Asparaginase in the Presence of 3 M Urea.

The protein concentration was 1.5%, dissolved in 3 M urea of 0.05 M phosphate buffer pH 8.0 solution (A) containing 10 mM L-aspartate of (B) not containing L-aspartate. Picture was taken at 72 min after achieving maximum speed (60,000 rpm).

The sedimentation pattern of the enzyme in the absence of L-aspartate was very similar to the pattern obtained in the presence of L-aspartate. In both cases, two peaks were observed; the fast sedimenting peak obtained in the presence of L-aspartate was calculated to be 7.6s and that in the absence of L-aspartate 7.5s and the slowly sedimenting peaks were calculated to be 2.5s in both cases. The 7.5s peak appears to represent the native enzyme and the 2.5s peak its dissociated components, respectively. The relative concentrations of the two peaks were approximated by integrating the areas under the two schlieren peaks and the 7.6s components were estimated to constitute 58.4% of the total proteins in the presence of L-aspartate and 62.7% in its absence. The difference between these values appears negligible.

These findings show that L-aspartate did not affect the dissociation of the enzyme into the subunits by urea and its protective action against the inhibition of the enzyme might be

related to other mechanisms than the dissociation of the enzyme.

L-Asparaginase activity was lost at a low pH range and its loss was reduced in the presence of L-aspartate as shown in Fig. 1. On the other hand, L-asparaginase EC 2 from *E. coli* B was reported to be dissociated into the 3.9s components at pH 2.2.¹¹⁾ Thus, if this is true with L-asparaginase from *E. coli* A-1-3, there might be a possibility that the dissociation of the enzyme at acid condition was inhibited by aspartate, because it suppressed the acid inactivation of the enzyme. The effects of L-aspartate on the sedimentation patterns at pH 3.5 are shown in Fig. 6.

FIG. 6. Sedimentation Patterns of L-Asparaginase at pH 3.5.

The protein concentration was 1.5%, dissolved in acetate buffer (pH 3.5, 50 mM) containing 10 mM L-aspartate (A) or 0 mM (B). Pictures were taken at 72 min after achieving maximum speed (51,200 rpm).

The upper schlieren pattern (A) was obtained by sedimenting L-asparaginase in 50 mM acetate buffer of pH 3.5 containing L-aspartate; the lower pattern (B) was in the acetate buffer not containing the amino acid. Both were very similar. The sedimentation coefficients of the main peaks were 6.3s (A) and 6.2s (B) and those of the minor peaks were roughly

estimated to be 2.5s (A) and 2.6s (B), respectively. The rapid sedimenting components in both systems probably represented the native enzyme protein, though the question remained on the difference between *s* values of the native enzyme (about 7s) and of the acid treated enzyme (6.2s). The slow sedimenting components appeared to correspond to the dissociated form. There was no distinguishable difference in the ratios of the area of the main component to that of the minor component between the two systems.

Then, the reversibility of the dissociated form to the native one was examined. L-Asparaginase was incubated at pH 3.5 for 60 min at 47°C in the presence or absence of L-aspartate. Then, the enzyme solutions were dialyzed against 50 mM phosphate buffer pH 7.8 at 5°C over night. The enzyme treated without L-aspartate was irreversibly precipitated during dialysis. On the other hand, the enzyme solution treated in the presence of L-aspartate remained clear after the dialysis. The sedimentation pattern of the dialysate was obviously homogenous and its *s* value was calculated to be about 7.0s.

Therefore, the enzyme was dissociated partially into 2.5s components when dissolved in the pH 3.5 buffer, whether the buffer contained L-aspartate or not. However, heating of the enzyme solution at 47°C caused its irreversible denaturation in the absence of L-aspartate but not in its presence (Fig. 1). These findings mean that L-aspartate did not

protect the enzyme from the dissociation at an acidic pH but protected it from irreversible inactivation.

Effects of L-aspartate on activity and helix content

The effects of L-aspartate on a tertiary structure of the enzyme were examined with ORD measurements. The results are summarized in Table IV. The helix content (%) as a criterion of the state of the enzyme structure was estimated from the rotatory dispersion constant λ_e according to the Yang's equation.⁹⁾ The helix content of L-asparaginase was 31.4% at pH 7.9 and it decreased to 18.2% at pH 2.8, where the activity dropped to 45.9% of the activity at pH 7.9. However, if L-aspartate was added to the enzyme at pH 3.4, it showed 80% of the activity at pH 7.9. The addition of L-glutamate increased the activity only by 54%.

On the other hand, the addition of L-glutamate or L-aspartate resulted in the decrease of the helix content at pH 7.9 as compared with the enzyme solution without amino acid. The degree of the decrease in helix content did not differ each other. Moreover, in both enzyme solutions containing amino acids, lowering pH to 3.5 did not cause further decreases in the helix contents, which had already become about 20~24% at pH 7.9. Therefore, L-glutamate has the same effects on the changes in ORD spectrum as L-aspartate, while from the aspects of protective effect on

TABLE IV. EFFECTS OF L-ASPARTATE ON THE HELIX CONTENT AND ACTIVITY OF L-ASPARAGINASE

Enzyme solutions consisting of the L-asparaginase 2 mg/ml, buffer (phosphate, or glycine 33 mM) and the additives (L-aspartate or L-glutamate 10 mM) were used for ORD measurements.

pH	Additives	mm	$-\left[\alpha\right]_D$	λ_e m μ	Helix content %	Activity %
7.9	—		22.5	256.2	31.4	100.0
7.9	L-aspartate	10	35	239.4	19.5	98.8
7.9	L-glutamate	10	35	246.2	24.4	98.8
2.8	—		87	237.5	18.2	45.9
3.4	L-aspartate	10	42.5	236.7	17.7	80.3
3.5	L-glutamate	10	40	241.7	22.2	53.8
	0.1 N HCl		97.0	214	1.4	0

acid inactivation there was a remarkable difference between the two amino acids.

L-Aspartate as an inhibitor of L-asparagine hydrolyzing activity

L-Aspartate inhibited competitively the hydrolysis of L-glutamine and D-asparagine, and the K_i values were determined to be about 2.5×10^{-3} M against each substrate. The inhibition of L-asparagine hydrolysis by L-aspartate could be observed only when the concentration of L-asparagine was lowered to 2 mM which was still 100 times as high as its K_m value and the concentration of L-aspartate was increased between 25 and 100 times as high as the K_i values of L-aspartate against L-glutamine and D-asparagine hydrolyses (Table V).

TABLE V. INHIBITION OF L-ASPARAGINE HYDROLYSIS BY L-ASPARTATE

The reaction mixtures consist of L-asparagine 2 and 20 mM, L-aspartate 50~400 mM and the enzyme dissolved in phosphate buffer. Incubations were carried out at 37°C for 3 min.

Substrate mM	L-Aspartate added mM	Activity %
20	0	100.0
20	50	92.6
20	100	89.2
2	0	100
2	50	79.7
2	100	59.4
2	200	24.6
2	400	0

The type of inhibition by L-aspartate was not determined, but presumed to be competitive.

DISCUSSION

Protective action of L-aspartate on inactivation of L-asparaginase

The present studies have shown that L-aspartate protected L-asparaginase from acid as well as from proteolytic inactivation and weakly from heat inactivation. On the other hand, L-asparagine showed a similar but less

effective protection of the enzyme against acid inactivation. Consequently, the effects of L-asparagine⁵⁾ in the protection against inactivation and in the profiles of the DEAE cellulose chromatography could be attributed to L-aspartate which was formed from the added L-asparagine during the treatment. These protective actions of L-aspartate were also observed with the enzymes from *Serratia marcescens*¹²⁾ and *Proteus vulgaris*¹³⁾ and appeared to be common to microbial L-asparaginases (EC 2).

Ryoyama¹⁴⁾ reported that certain guinea pigs possessed a thermostable asparaginase in their sera, whereas others possessed a thermolabile form of the enzyme, and that both forms were stabilized against heat inactivation by Na^+ and K^+ . On the other hand, none of metal ions and nucleotides tested possessed the protective action with the L-asparaginase from *E. coli* A-1-3.

On the mode of interaction between L-asparaginase and L-aspartate

In the presence of L-aspartate L-asparaginase was not absorbed on CM Sephadex column, but it was absorbed more tightly on DEAE cellulose column. This means that in the presence of L-aspartate, L-asparaginase behaved in the chromatographic characteristics as if it has got negative charge. The interaction between L-aspartate and L-asparaginase seems to be highly specific and to relate closely to the protective action of L-aspartate. The binding force between the two was thought to be ionic from the above results. It appears, however, to be weak, because the protective action of L-aspartate was easily lost by a high concentration of sodium chloride (0.5 M).

Subunit nature and the effect of L-aspartate on it

A variety of evidences^{15, 16)} such as sedimentation studies, SDS gel electrophoresis, the stoichiometry of binding of DONV to the enzyme,¹⁷⁾ electron microscopy¹⁸⁾ and X-ray crystallography¹⁹⁾ have supported the tetrameric structure of *E. coli* L-asparaginase.

L-Asparaginase from *E. coli* A-1-3 was also proved to consist of four subunits by means of SDS disc electrophoresis,²⁰⁾ the molecular weight of each being about 36,000. There is indeed no direct evidence supporting that the four subunits are identical. However, only a single band was always observed for the subunits upon electrophoresis in 8 M urea or 0.1 % SDS, and in the sedimentation profile of the L-asparaginase dissolved in 8 M urea or 5 M guanidine hydrochloride. These results are consistent with an interpretation that the subunit may be identical.

Whether the separated subunits exhibit enzymatic activity or not has not been investigated. The remarkable ease with which the enzyme will reconstitute when agents such as urea are removed is one of the interesting properties of L-asparaginase. But, this property made it difficult to see whether the subunits have the enzymatic activity or not. However, as shown in the Results, the degree of the residual activity (about 60 %) in 3 M urea corresponds well to the percentage of the areas of the undissociated form (58.4~62.7 %) in 3 M urea, calculated from the sedimentation profiles. Thus, the subunits may not exhibit the enzymatic activity.

Kirschbaum *et al.*¹¹⁾ studied the effect of pH on the subunit structure of *E. coli* B asparaginase and observed two peaks at pH 3.3, the sedimentation coefficients of which corresponded to 4.0 and 8.6s, respectively. A possibility that L-aspartate might inhibit the dissociation of the enzyme into subunits and that was the mechanism of its protective action was examined by studying the behaviors of the enzyme in the ultracentrifuge. The results obtained, however, denied this possibility.

The effects of L-aspartate on the enzyme conformation

Proteases including α -chymotrypsin, trypsin, subtilisin and thermolysin were able to attack and inactivate more or less the native L-asparaginase, and their inactivating action was equally suppressed with L-aspartate. Therefore, the proteolytic inactivation does not

appear to depend on the cleavage of particular essential site(s) of the peptide chain, but on the degradation of the tertiary structure derived from the cleavage of many indefinite sites of the enzyme protein. If this is the case, the protective action of L-aspartate may be due to the induction of resistancy against proteases to the conformation of the active center and its surroundings, though many confirmative evidences will be required to establish this hypothesis.

In the case of acid inactivation, the situations appeared to differ from the proteolytic inactivation. The acid inactivation did not involve the cleavage of the peptide chain, but was accompanied by a partial dissociation. As mentioned above, L-aspartate exerted no influence on the dissociation and the association of the L-asparaginase. The protective action of L-aspartate on acid inactivation may have stemmed from its action to suppress the change, for example unfolding, of the tertiary structure of the enzyme by high concentration of hydrogen ions.

Thus, the influence of L-aspartate on the tertiary structure was examined with ORD measurement. Unexpectedly, results obtained did not explain the protective action of L-aspartate in terms of the preservation of the helical contents. Though L-glutamate caused a similar change in ORD spectrum, it did not exhibit a protective action on acid inactivation. Therefore, the remarkable decreases in the helical content by adding acidic amino acids seemed to have no relation to the induced resistancy against acid inactivation. It seems, therefore, better to think that the effective interaction between L-asparaginase and L-aspartate occurred on a limited part, for example, the active center on the enzyme.

L-Aspartate has been found to inhibit competitively the D-asparagine and L-glutamine hydrolysis reactions. It inhibited also to hydrolyze L-asparagine. These facts may suggest that L-aspartate occupy the active center and stabilize the enzyme against inactivation by acid, proteases or heat.

Jayaram demonstrated that L-aspartate acted

as an allosteric inhibitor of the pH 9.6 asparaginase from *Mycobacterium*.²¹⁾ Though similar phenomena have not been observed with *E. coli* L-asparaginase, a possibility²²⁾ can not be ruled out that there might be other effector sites for L-aspartate than the active site for stabilizing the enzyme against inactivation. This is one of the problems to be solved.

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