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O-Diphenol:Oxygen-Oxidoreductase from *Musa cavendishii* *

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O-diphenol:O_2-oxidoreductase from banana *(Musa cavendishii)* presents a certain degree of multiplicity. The enzyme is easily extractable with low ionic strength buffers (*p*H 6-6.5) and is not assumed to be associated with membranes. K_I values are higher in the order: cyanide, phenylhydrazine, dieca, azide (non-competitive inhibitor) and 8-hydroxyquinolin (competitive inhibitor). There is not a defined relationship between fruit ripening and enzymatic activity.

The enzyme O-diphenol:oxygen oxidoreductase (14) (E.C. 1.10.3.1), or, as it has recently been denominated monophenol monooxigenase (4), (E.C. 1.14.18.1), is also known under other names more or less related to the primary action of the enzyme, such as: catecholase, cresolase, phenolase, polyphenolase, laccase, tyrosinase, etc. Concretely, we will refer to the transformation of an o-diphenol, such as catechol, into the corresponding o-quinone when catalyzed by the enzyme, the result being expressed as: o-diphenol + $\frac{1}{2}$ O_2 $\rightleftharpoons$ o-quinone + H_2O.

The physiological role of this enzyme in many and diverse living systems has been the subject of numerous investigations, specially in terms of the enzyme's participation in flavonoid, lignin and tropolone biogenesis; in animal processes of melanization; in functional brownings; and finally in its action as a terminal oxidase in the respiratory chain.

PALMER (12), in his report on some properties of the enzyme from banana *(Musa acuminata)*, stated that the enzyme seemed to be associated to cell walls, while our results, working with the enzyme extracted from banana pulp *(Musa cavendishii)*, have been different. A more

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detailed study of this enzyme seemed justified. We shall report on the extraction and characterization of the O-diphenol: oxygen oxidoreductase, and on the inhibitory action of several substances.

Materials and Methods

The fruits used were those from banana plants *(Musa cavendishii)* of Tenerife (Canary Islands), in a similar grade of ripeness and with a good external aspect. In the studies on maturation, three predetermined zones of harvesting were chosen, distinguished by their height above sea level (50, 100 and 150 meters). Samples were always taken from the same plants in each collection area, choosing fruits from the bottom, top and middle of each spike of bananas. Enzymatic and specific activities and also proteins were determined every week during the maturation process.

The crude extracts of the enzyme were prepared from pulp slices weighting about 7 g, ground with 5 g of purified sea sand and 50 ml of buffer which if not otherwise specified, was 0.025 M citric acid-potassium phosphate *p*H 6.0. After grinding at 0° C for five minutes the mixture was centrifuged at 10,000 × g for fifteen minutes using the supernatant as the enzyme source.

Necessary dilutions were made in order to have a maximum activity of 2 units/ml. The enzyme showed good activity after several hours at 0° C and after several days at —20° C.

The procedure for measuring the banana catecholase was essentially that described by SOLER, SABATER and LOZANO (18). The substrate, 12.5 mM catechol, was placed in a 0.05 M potassium phosphate *p*H 6.0 buffer in the presence of 7.5 mM ascorbic acid. The reaction time was 180 seconds, with good oxygen uptake maintained by means of magnetic stirring. At the end o the reaction the remaining non-oxidized ascorbic acid was titrated with a 5 mM iodine solution. The difference between ascorbic acid at the beginning and end of the reaction period was proportional to the enzyme present in the medium. Blank controls were performed (a) with a previously boiled enzyme, (b) by substituting buffer for the substrate, (c) by the addition of the enzyme after adding the 10 % phosphoric acid solution which ended the reaction. The results were always coincidents. One unit of enzyme is the quantity which transforms 1 micromol of substrate in 1 minute at 20° C. Specific activity is the number of units per milligram of proteins.

For measuring cresolase activity, the procedure was similar to the above one, with the new substrate, p-cresol, substituted for catechol. In order to avoid a possible lag period (11), catechol was added to the medium until a concentration of 0.5 mM was reached.

Ascorbic acid oxidase was determined in a similar way: in the presence of 7.5 mM ascorbic acid and in the absence of mono- and diphenols.

Proteins were measured by the LOWRY method (10). Disc electrophoresis operations with polyacrylamide gels were carried out in an analytical Shandon instrument, in accordance with manufacturer's instructions. Tris-hydrochloric acid buffer (*p*H 8.0) and 7.5 % polyacrylamide were normally used.

The applied potential was 200 volts and the running time about 30 minutes. Gels were developed by inmersion in a 0.1 M solution of catechol in 0.05 M potassium phosphate buffer *p*H 7.0, during 30 minutes.

When preincubations of the enzyme with inhibitors were effectuated, they remained together about 120 minutes at 0° C with the indicated concentrations in the corresponding assays.

Results

Extraction. Although the enzyme is considered as soluble in water, other fac-

tors can affect the extracts, such as the enzyme's relationship with cell membranes, subcellular organelles, lipids, etc. Investigations were therefore undertaken on the use of different buffers, *pH*, ionic strenghts; on the addition of detergent; lisozyme; and on freezing and thawings processes. In addition to bidistilled water, the following buffers were employed in extractions: glycine-hydrochloric acid from *p*H 2 to *p*H 4, citrate-potassium phosphate (*p*H 6-8), tris-hydrochloric acid (*p*H 8-9), sodium barbital-hydrochloric acid (*p*H 8-9) and glycine-sodium hydroxide (*p*H 9-10). The following concentrations were used: 25, 50, 100 and 200 mM.

Results can be seen in figure 1. Good specific activities were achieved with sodium barbital-hydrochloric acid and glycine-sodium hydroxide buffers, but these buffers were not normally used since at higher *p*H oxidation of phenolic substances is favoured. Highly satisfactory extractions were obtained using phosphate buffers of low ionic strenghts and *p*H between 6.0 and 6.5.

In other experiments, fifteen sucessive freezings and thawings of the enzyme were performed. In each operation about 3 per cent of the enzymatic activity was lost, passing from 17 units/ml at the beginning to 11 units/ml at the end.

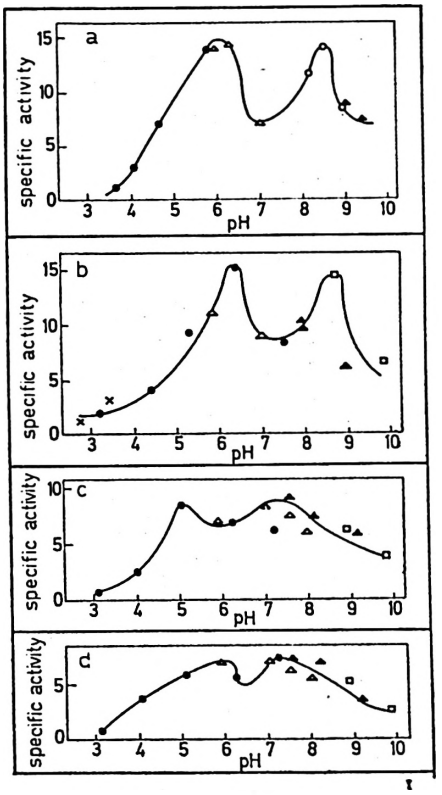

Fig. 1. *Influence of the pH and the molarity of buffers on the specific activity of the extract.*
1 a: 0.025 M; 1 b: 0.05 M. 1 c: 0.1 M; 1 d: 0.2 M. (●) citrate-phosphate buffer; (×) glycine-HCl buffer; (△) phosphate-phosphate buffer; (□) glycine-HONa buffer; (▲) tris-HCl buffer; (○) veronal-HCl buffer.

Table I. *Extraction of the enzyme.* Extractions were made as indicated in Material and Methods, but adding to the buffer the substances indicated below. Each value is the average of at least four determinations.

Extraction Medium	Concentration % (W/v)	Specific activity (units/mg protein)
0.025 M phosphate citrate *p*H 6.0	—	9.9
+ Sodium lauril sulphate	0.2	7.1
	0.1	9.4
	0.05	9.8
+ Lysozyme	0.01	8.2
+ Dowex 1×8 200-400 m	5.0	11.1
+ Dowex 1×8 20-50 m	5.0	9.5
0.025 M potassium phosphate *p*H 8.0	—	6.9
+ Sodium lauril sulphate	0.2	4.2
	0.1	4.3
	0.05	5.4
+ Lysozyme	0.01	6.6
+ Dowex 1×8 200-400 m	5.0	7.6
+ Dowex 1×8 20-50 m	5.0	8.2

The possible relationship between the enzyme and membranes was studied using different concentrations of the lauryl sulphate detergent. In other experiments lisozyme was added to the extraction medium. In none of these cases was a higher specific activity obtained (Table 1), and given the fact that water extracts a large percentage of the enzyme, it can be postulated that the enzyme is soluble and easily extractable. If there were any insoluble forms of the enzyme, they would not be affected by any of the above treatments.

LAM and SHAW (8) have shown the good properties of the anionic resin Dowex 1 × 8 in the extraction of some enzymes, but with banana enzyme (Table I) only a 20 % improvement was reached using this resin, due to the low concentration of phenols present in the extract. This scarcity of phenols is observed even at room temperature with a good oxygen supply, since no appreciable oxidation can take place in the medium without the addition of substrate.

Enzymatic preparations had no ascorbic acid oxidase nor any cresolase activity.

Activity measurements. The possibility existed that ascorbic acid or its oxidation product, dehydroascorbic acid, could alter measurements because of their activator or inhibitory effect on the enzyme, but by varying ascorbic acid concentrations in the medium from 0.3 to 12 mM, coincident results were obtained. On the other hand, enzymatic activity was not changed by the addition on the medium of a mixture of ascorbic acid-dehydroascorbic acid, ratio 1:9, as calculated with iodine titration.

Enzymatic or non-enzymatic products of the oxidation of catechol had no effect on the activity of the enzyme. Such products were prepared beforehand by oxygen oxidation of catechol at room temperature during a period of 24 hours in the presence or absence of the enzyme. At that time, the brown oxidation products were not reduced by the addition of ascorbic acid, and after their concentration, any proteins present in the solution were denatured by heat.

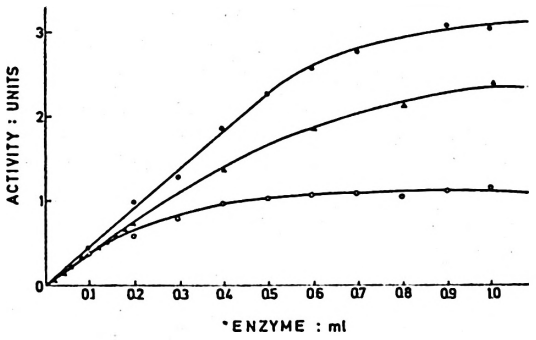

Fig. 2. *Influence of the oxygen uptake on the activity of the enzyme.*
(○) no stirring; (▲) low stirring; (●) high stirring.

The enzyme catalyzes a bisubstrate reaction in which oxygen is the second substrate, and if enzyme concentrations are increased in the assays, the oxygen uptake must be higher. But oxygen has a relatively low solubility in aqueous solutions, and therefore the velocity of the catalytic reaction would be limited by the interchange speed of the gas between the gaseous and liquid phases. Figure 2 shows the results of three different conditions of oxygen supply. The existence of proportionality between activities and enzyme quantities could be observed under normal conditions of stirring if the activities were lower than 2 units. Therefore, if necessary, the enzyme was always diluted. At low concentration of the enzyme, three different graphs corresponding to the three diverse stirring speeds were obtained. This behaviour was similar to that described in potato (20), and can be contrasted with that of apricot (11), this last showing only one line at low values of the catalyst.

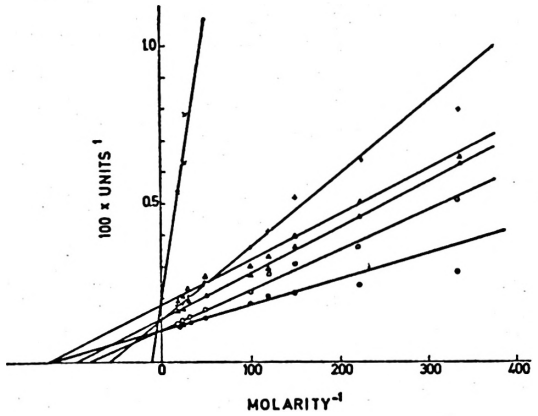

Fig. 3. *Lineweaver-Burk graphs for inhibition.* Inhibitor concentration: 10^{-3} M. (●) no inhibitor; (○) 8-hydroxiquinolin; (▲) dieca; (△) azide; (+) phenylhydrazine; (×) cyanide.

Inhibitors. The enzyme is a copper metalloenzyme and therefore could be inhibited by substances that complex the metal: sodium dienthyldithiocarbamate (Dieca), 8-hydroxyquinolin, sodium azide and potassium cyanide. On the other hand, the inhibitory effect of phenylhydrazine on similar systems has been reported (9) and was, therefore, included in the study.

Plots of $1/v_i$ against $1/(s)$ resulted in representations of the Lineweaver-Burk type (Figure 3). The competitive nature of the inhibition produced by 8-hydroxyquinolin and the non-competitive action of the sodium azide can be easily deduced. In the other cases, intermediate graphs were obtained. All these inhibitors were studied in greater detail, using the treatment of HUNTER and DOWNS (6), which results from the equation:

$$\frac{(I)\ (1-i)}{i} = \frac{\alpha \times K_i}{\alpha + \frac{(s)}{K_s}} \times \left(1 + \frac{(s)}{K_s}\right)$$

An inhibitor can alter both the enzyme affinity towards the substrate and the velocity of the transformation of the enzyme-substrate complex into products. α represents the change of the affinity and β the change in the velocity. i is the fractional inhibition and K_i the dissociation constant of the enzyme-inhibitor complex, while (I), (s) and K_s have the classical meaning of enzyme kinetics.

It can be calculated from the above expression that dieca is a mixed inhibitor ($K_i = 0.3$ mM; $\alpha = 8$; $K_s = 1.4$ mM), that 8-hydroxyquinolin is competitive ($K_i = 1.77$ mM; $\alpha = \infty$; $K_s = 0.5$ mM), that sodium azide is non-competitive ($K_i = 1.24$ mM; $\alpha = 1$), that potassium cyanide is an excellent mixed inhibitor ($K_i = 0.01$ mM; $\alpha = 28$; $K_s = 0.5$ mM) and finally that phenylhydrazine is also mixed ($K_i = 0.2$ mM; $\alpha = 10$; $K_s = 1.2$ mM). The changes in free energy of inhibitor binding at 20° C, as calculated from $\Delta G = RT \ln K_i$, were: —6.7 (potassium cyanide), —4.9 (phenylhydrazine), —4.7 (dieca), —3.9 (sodium azide) and —3.7 kcal × mol^{-1} (8-hydroxyquinolin). Inhibitory mechanisms varied from purely non-competitive ($\alpha = 1$) to absolutely competitive ($\alpha = \infty$), in the following order: azide, dieca, phenylhydrazine, cyanide, and 8-hydroxyquinolin.

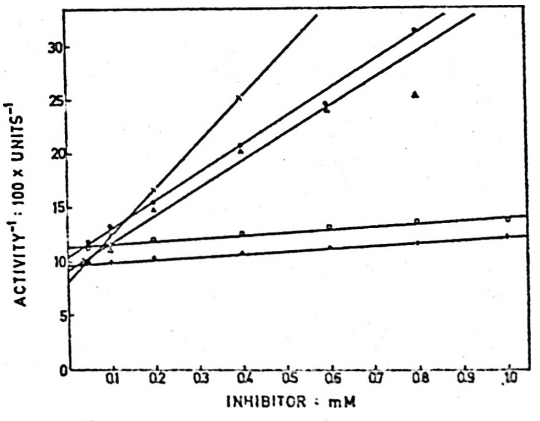

Fig. 4. *Dixon's graphs for inhibition.* (×) dieca; (●) cyanide; (△) phenylhydrazine; (○) 8-hydroxyquinolin; (+) azide.

The equation developed by DIXON (3) can be used at standard concentration of the substrate and at variable inhibitor concentration:

$$\frac{1}{v_i} = \frac{1}{V_M}\left[1 + \frac{K_M}{K_s}\right] + \frac{K_M}{V_M(s) \times K_i} \text{ (I)},$$

$$\text{plotting } \frac{1}{v_i} \text{ against I}$$

Figure 4 shows that dieca, cyanide and phenylhydrazine — with higher slopes — produced inhibitions more sensitive to the increments of the inhibitor.

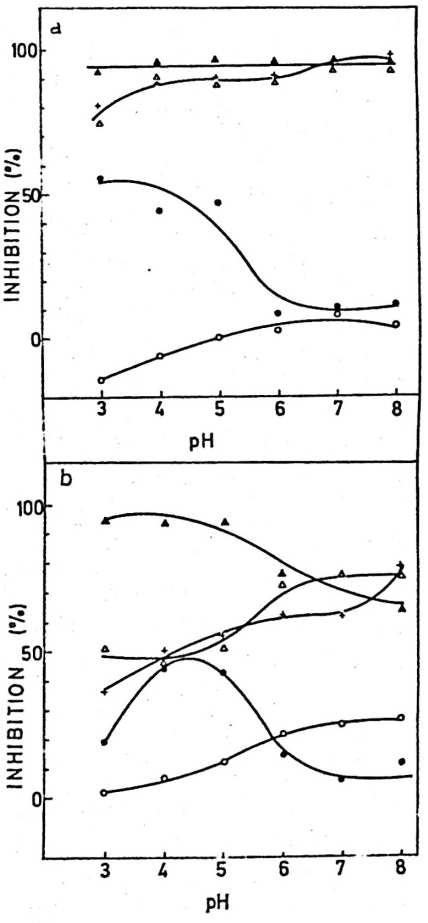

Fig. 5. *pH effect on the inhibition.* a) Inhibitors nor preincubated with the enzyme. b) Inhibitors preincubated with the enzyme. (▲) dieca; (△) phenylhydrazine; (+) cyanide. (●) azide; (○) 8-hydroxiquinolin.

Another noteworthy point about inhibition is its dependence on *p*H. Figure 5 shows two different results, since in the first case the enzyme was not preincubated with each inhibitor, and in the second one preincubation was carried out at 0° C for two hours. The main characteristics of the inhibitors related to the *p*H were similar to those described in the previous work of LOZANO (11) with apricot phenolase. 8-hydroxyquinolin, when preincubated in this way, presented at low *p*H a clear activator effect. Dialysis assays indicated the existence of an easily dialyzable and thermostable substance which inhibits at low *p*H and could be eliminated by preincubation with 8-hydroxyquinolin. The excellent inhibitory action of dieca at high *p*H was made patent only if there was previous preincubation. In relation to sodium azide, the inhibitory form was N_3H and not N_3^-, having in mind that $K_a = 1.8 \times 10^{-5}$ and calculating et each *p*H the real concentration of N_3H, the fractional inhibition was the same as that deduced fron figure 4. As regard phenylhidrazine and potassium cyanide at low *p*H, their inhibitions were potentiated by preincubation, with quite similar characteristics between them. This similarity also exists in relation to their inhibition type and kinetic pattern, but phenylhydrazine possibly acts upon a carbonyl group in the enzyme molecule while cyanide forms complexes of copper ion.

Table II shows that some ions could also inhibit the enzyme. Fluoride and chloride ions were better inhibitors than acetate ions.

Multiplicity. Disc electrophoresis with polyacrylamide gels was performed under different conditions of *p*H and pore sizes. The results in regard to electrophoretic mobility and the number of bands were not always reproducible, but at least two well-defined bands could be seen after the

Table II. *Inhibition produced by some ions.* Activities measurements were carried out as indicated in Material and Methods, but in each case, the corresponding quantity of salt was added to the 0.05 M potassium phosphate *p*H 6.0 buffer.

Inhibitor [M]	Fractional Inhibition			
	NaCl	KCl	NaF	CH_3-COONa
2.5×10	—	—	—	0.59
1×10	1.00	1.00	1.00	0.52
5×10^{-1}	0.95	0.94	1.00	0.30
1×10^{-1}	0.68	0.56	0.85	0.05
5×10^{-2}	0.52	0.40	0.76	—
1×10^{-2}	0.21	0.10	0.59	—
5×10^{-3}	0.09	0.04	0.44	—
1×10^{-3}	0.07	0.01	0.37	—
5×10^{-4}	0.06	0.00	0.35	—

development of gel with catechol solutions. The possible presence of at least two different forms of the enzyme could be in accord with additional studies actually in course on other properties of the enzyme, such as the number of peaks obtained with molecular filtration on Sephadex G-100, multiplicity of properties during thermal denaturation, different behaviour in respect to the *p*H of extraction, etc.

Ripening. During the process of maturation of fruits a number of assays of enzymatic and specific activities were carried out weekly on different samples obtained from the 3 zones of harvesting.

However, it was impossible to deduce any relation between specific activities and degree of maturation, due to the fluctuations of the figures during the physiological process. No defined tendency was patent.

Discussion

Phenolase enzymes from vegetable sources are very often associated with membranes or have insoluble forms which are extractable only with the aid of detergents as some other treatment. Detergents are essential for the solubilization of the enzyme from grapes (5), latex of *Papaver somniferum* (15), *Musa acuminata* fruits (12) and melanoma (2).

The results reported here show that *Musa cavendishii* does not follow this pattern.

The different properties of the enzymes from *Musa cavendishii* and *Musa acuminata* are also shown in the results obtained from freezing and thawing. This treatment did not affect the phenolase studied by PALMER (12) in *Musa acuminata*. PALMER claimed that, due to the association of the enzyme with the cell wall, 80-100 % of the polyphenolase activity was sedimented at 500 × g 10 min. Our enzyme, on the other hand, remained practically in the supernatant at 10,000 × g 10 min.

Absence of ascorbic acid oxidase and cresolase activities in both fruits confirm the fact that vegetable materials with abundant polyphenolase activities very often lack ascorbic acid oxidase.

The possible role of ascorbic acid as an activator or inhibitor on enzymes of this type had been discussed some time ago (7), but contradictory conclusions could have resulted from the use of inadequate activity measurement procedures. The presence of ascorbic acid is very useful since the substrate then remains at the same concentration during the course of the reaction. Our earlier results (11) and those reported by other workers using spectrophotometric techniques (1, 16, 19) are similar, and it is possible to assert that ascorbic acid does not directly affect the activity of the enzyme.

Some enzymes like phenolase have a low affinity for oxygen, and this fact has, unfortunately, not always been considered, and working conditions under which oxygen access is a limitating factor in the reaction are all too likely. This problem is even more acute when such techniques as spectrophotometric methods are used, since in these cases stirring is not usual.

In our banana enzyme, oxidation products of the substrate did not inhibit, in contrast with other systems, as demonstrated in melanization studies (17) carried out with epidermis enzyme. The latter is inactivated by quinoid groups of melanin. There are similar examples of inhibition in vegetables.

Inhibitors such as those used in this work differ in their effects depending upon the enzyme source, and at times some striking results are produced. For instance, cyanide is not a good inhibitor on latex phenolase from *Papaver somniferum* (15) nor on *Mycobacterium leprae* phenolase (13). In the latter, azide was not an inhibitor. Phenylhydrazine in some plants (9) presented a non-competitive kinetic instead of the kinetic observed by us.

Dieca with good specificity towards copper ions, forms a very stable enzyme-dieca compound at all *p*H ranges and is the best inhibitor. 8-hydroxyquinolin, which also has good specificity towards copper ions, has however a very high dissociation constant of the enzyme-inhibitor complex and is, therefore, not as efective as dieca.

The possible existence of phenolase multiplicity is significant. Some forms have been demonstrated in other biological materials, their number being 8 in grape (5). Additional studies will be required in order to assess the significance of the existence of multiple phenolase forms in banana, but in a preliminary way we can hypothesize their relation to other observed and still unpublished properties of the enzyme, such as the different behaviour of the enzyme depending on the *p*H of the extraction; the double kinetics of thermical inactivations; the results of urea treatment, etc.

Resumen

El enzima catechol:O_2 oxidorreductasa de banana, que parece presentar cierta multiplicidad, es fácilmente extraíble con sistemas tampones de *p*H 6-6,5 y bajas fuerzas iónicas y no parece estar asociado a membranas, siendo baja su afinidad por el oxígeno. El valor de la K_I respecto a ciertos inhibidores aumenta en el orden siguiente: cianuro, fenilhidrazina, dieca, azida (no competitivo) y 8-hidroxiquinoleína (competitivo). No parece existir una relación directa definida entre el proceso de maduración y la actividad enzimática.

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