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Development of Pyrimidine-metabolizing Enzymes in Cotyledons of Germinating Peas[1]

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ABSTRACT

Mechanisms controlling conversion of orotic acid-6-^{14}C to uridine-5′-phosphate in cotyledons of germinating Alaska peas (*Pisum sativum* L.) were investigated. The content of 5-phosphoribosyl-1-pyrophosphate was very low in dry seeds, increased to a maximum after about 12 hours of imbibition, and then rapidly declined. Orotidine-5′-phosphate pyrophosphorylase and orotidine-5′-phosphate decarboxylase activities more than doubled during the first 24 hours of germination and then also decreased. These results do not account for the continuous increases of orotate anabolism in such cotyledons as we observed previously. The initial increases in activities of these two enzymes were unaffected by cycloheximide, while the subsequent decreases were less rapid in the presence of this inhibitor. Activities of cotyledonary cytidine deaminase and uridine hydrolase also increased during imbibition, but the activity of only the latter showed a decrease after imbibition was completed. Cycloheximide inhibited the initial rapid increase in uridine hydrolase activity but had little effect on its subsequent decline. Cycloheximide had only slight inhibitory effects on the development of cytidine deaminase activity during the first 62 hours. The evidence suggests that uridine hydrolase might be synthesized *de novo* during the first few days of germination, but that the other three enzymes might not be.

We previously observed that ^{14}C-labeled orotic acid and uridine were converted into various pyrimidine nucleotides and RNA in cotyledons of germinating Alaska peas (15). This incorporation was more extensive in seeds germinated about 1 week than in those germinated for lesser times. It was suggested that the increasing capacity of the seeds to convert orotate into RNA might be due to an increase of OMP[2] pyrophosphorylase activity or of PRPP, a necessary substrate for this enzyme. Decarboxylation of OMP appeared to be nonlimiting at all germination stages, since no ^{14}C-labeled OMP was ever detected in the cotyledons. On the contrary, orotate-^{14}C accumulated in seeds which only weakly converted it into RNA. The present report describes results indicating that both OMP pyrophosphorylase and OMP decarboxylase become increasingly active in pea cotyledons during the first 24 to 36 hr of germination, but their activities then decrease. The decreases but not the increases in enzyme activities are inhibited by CH, a known inhibitor of protein synthesis. The effects of CH on protein synthesis and on the development of cytidine deaminase and uridine hydrolase, two enzymes suspected to be involved in the metabolism of RNA stored in the cotyledons, are also described.

MATERIALS AND METHODS

Germinaton. Pea (*Pisum sativum* L., cv. Alaska) seeds were germinated under conditions designed to minimize bacterial contamination. Seeds were shaken in 1.0% (w/v) NaOCl for 3 min and rinsed extensively in sterile water. Twenty seeds were then spread over the surface of three layers of Whatman No. 1 filter paper in 9-cm Petri dishes. The Petri dishes and filter paper were autoclaved prior to adding the seeds. Fifteen milliliters of CH (50 μg/ml) dissolved in sterile water were then added, resulting in a dose of 38 μg/seed. To the control group 15 ml of sterile distilled H_2O were provided. Germination occurred in the covered Petri dishes for periods up to 182 hr in darkness at about 27 C.

At various times seeds were removed, seed coats were discarded, and dry weights (85 C for 24 hr) of the combined root and shoot axes were determined. The excised cotyledons were separated and used for measurements of protein synthesis, PRPP content, respiration rates, or enzyme activity.

Protein Synthesis. Incorporation of L-phenylalanine-3-^{14}C into protein was used to estimate protein synthesis. Ten cotyledons were placed in each 2-ounce Skrip ink bottle containing 2.0 ml of 20 mM potassium phosphate buffer, pH 5.8, and 0.10 ml (2 μc) of L-phenylalanine-3-^{14}C (366 mc/mmole, New England Nuclear Corp.). The wells of the bottles contained 1.0 ml of 3% KOH to trap $^{14}CO_2$. After 6 hr of incubation at 27 C in normal laboratory light, radioactivity in CO_2 was analyzed after plating aliquots of the KOH on stainless steel planchets (13). The cotyledons were washed thoroughly with running water and each group of 10 was homogenized with 10 ml of 50 mM tris-HCl, pH 7.4, to which D-phenylalanine, 2 mg/ml, had been added. Homogenization occurred for 5 min at top speed in an Omni Mixer (Ivan Sorvall Co.). The homogenate was filtered through cheesecloth, the Omni Mixer cup and blade were rinsed with 5 ml of extracting buffer, and the combined filtrates were centrifuged at 27,000g for 15 min. Each supernatant solution was removed and subsequently combined with a solution obtained from washing the precipitate with extracting buffer. Protein was precipitated by adding cold trichloroacetic acid at a final concentration of 7% (w/v) to the supernatant solutions. After 30 min at 2 C, protein was centrifuged off and the pellet was washed with 6 ml of cold 5% trichloroacetic acid containing D-phenylalanine, 2 mg/ml. Analyses of ^{14}C in the trichloro-

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[2] Abbreviations: CH: cycloheximide; OMP: orotidine-5′-phosphate; PRPP: 5-phosphoribosyl-1-pyrophosphate.

acetic acid-soluble fraction were made. The protein pellet was further washed with 10 ml of ethanol by continuously stirring for 15 min. After centrifugation, the protein was dissolved in 10 ml of 2.5 N KOH and diluted to 50 ml with water, and the ^{14}C present was analyzed with a Nuclear-Chicago gas flow Geiger tube covered with a Micromil window. Self absorption corrections were made.

Enzyme Assays. Activities of OMP pyrophosphorylase and OMP decarboxylase were analyzed using carboxyl-labeled orotate and OMP (New England Nuclear Corp.), essentially as described before (18). At various germination times 20 cotyledons were homogenized in 10 ml of 50 mM tris-HCl, pH 7.4 (containing 0.5 mM dithiothreitol), for 3 min at full speed in an Omni Mixer cup surrounded by an ice bath. The blade and cup were rinsed with 5 ml of the grinding buffer and the homogenate was then strained through four layers of cheesecloth and centrifuged at 27,000*g* for 15 min at about 5 C. Supernatant solutions were used directly for enzyme assays and protein determinations (10) in the development studies. Enzyme extracts from dry seeds were prepared as above, except that they were initially ground to a fine powder in a mechanical mill and this powder was then further homogenized with the Omni Mixer and buffer as usual.

OMP pyrophosphorylase activity was measured by determining $^{14}CO_2$ release from labeled orotate. Bottles contained the following in a final volume of 2.6 ml: orotate-7-^{14}C, 0.1 μc, 1.3 μM; tris-HCl, pH 7.4, 56 mM; Mg_2PRPP (Sigma Chemical Co.), 0.19 mM; KF (to inhibit dephosphorylation of OMP and PRPP), 40 mM; $MgCl_2$, 3.8 mM; and 0.1 ml of enzyme (about 1.6 mg of protein). The reaction mixture was shaken gently at 29 C in a constant temperature water bath for 15 min. Reactions were stopped by injecting 0.5 ml of glacial acetic acid through the rubber gasket (16) into the mixture via a small hole previously cut in the metal part of the lid. After 15 min the lids were removed and the $^{14}CO_2$ trapped in 1.0 ml of 3% KOH held in the well was analyzed.

OMP decarboxylase activity was measured in a similar manner. Bottles contained the same amount of substances used in the OMP pyrophosphorylase assay, except that no Mg_2PRPP was added and 0.1 μc of OMP-7-^{14}C (final concentration 67 μM) was the substrate.

Uridine hydrolase activity was analyzed chromatographically. Small test tubes contained 0.5 ml of enzyme and 0.1 ml (5 nmoles, 2 μc) of uridine-2-^{14}C (International Chemical and Nuclear Corp.). After incubating for 30 min at 30 C, the reactions were terminated by adding 0.2 ml of glacial acetic acid and boiling for 1 min. The coagulated protein was centrifuged off and aliquots of the supernatant solutions were chromatographed with carrier uridine and uracil on Whatman 3MM paper strips in 1-butanol-HCOOH-H_2O (77:10:13, v/v). The ultraviolet-absorbing marker spots were cut out and the ^{14}C present in uridine (R_F 0.18) and uracil (R_F 0.37) was determined by liquid scintillation counting.

Cytidine deaminase (cytidine aminohydrolase) activity was also determined chromatographically. To tubes containing 0.1 ml (6 nmoles, 1.0 μc) of cytidine-5-^{3}H, 0.5 ml of enzyme solution was added. After incubating 30 min at 30 C the ^{3}H present in marker spots of chromatographically separated cytidine (R_F 0.08), uridine, and uracil was analyzed as for uridine hydrolase.

PRPP Analyses. Amounts of PRPP present in the cotyledons were estimated enzymatically by determining the amount of $^{14}CO_2$ released from carboxyl-labeled orotate in the presence of nonlimiting amounts of OMP pyrophosphorylase and OMP decarboxylase obtained from dry pea seeds. Dry whole seeds were ground with a mechanical mill and several 2.6-g samples of the resulting powders were frozen for use at various intervals. At the time of PRPP analysis these samples were further homogenized in the usual tris buffer, the homogenates were centrifuged, and 0.5-ml aliquots from the 12-ml resulting supernatant volumes were used as sources of the enzymes.

Table I. *Effects of Cycloheximide upon Metabolism of L-Phenylalanine-^{14}C and Embryo Growth*

Twenty seeds were aseptically germinated in 15 ml of water (controls) or 50 μg of CH per ml for 12 or 84 hr.

Treatment	^{14}C Recovered	^{14}C Distribution[1]			Embryo Dry Weight[2]
		$^{14}CO_2$	Trichloroacetic acid + ethanol soluble	Protein	
	cpm	%			*mg*
12 hr					
Controls	442,000	0.41	48.2	51.5	1.7
Cycloheximide	381,000	0.53	92.2	7.32	1.9
84 hr					
Controls	93,600	24.1	55.9	19.9	6.6
Cycloheximide	216,000	10.2	84.3	5.5	1.7

[1] Based on percentage of total cpm recovered. Values are means from two determinations in each of two experiments.

[2] Means of 20 seeds in each of 2 experiments.

Cotyledons were excised at various germination times and homogenized as described above for "Enzyme Assays," except that the homogenizing buffer contained KF at a final concentration of 0.1 M to minimize endogenous PRPP destruction by phosphatases. Two aliquots (50 and 100 μl) of the resulting PRPP-containing supernatant solutions were added to separate reaction bottles also containing 0.5 ml of dry seed enzyme preparation, 0.1 ml of 1 M $MgCl_2$, 0.1 ml of 1 M KF, 0.3 ml of orotate-7-^{14}C (1 μc/ml), 0.2 ml of 0.25 M tris at pH 7.4, and 1.0 ml of H_2O. Duplicate assays of $^{14}CO_2$ released were performed in the same way as for OMP pyrophosphorylase. The resulting $^{14}CO_2$ values were converted to nmoles of PRPP present from a standard curve. This curve was prepared with 0.5 ml of enzyme extract from dry seeds, PRPP final concentrations varying from zero to 9 μM, and the same concentrations of other components. The standard curve, not shown, was nearly linear over this PRPP concentration range.

Respiration Experiments. Oxygen uptake of cotyledons excised after various germination times was measured manometrically at 30 C. Ten cotyledons were placed in 2.0 ml of 20 mM potassium phosphate buffer, pH 5.8, held in 18-ml Warburg flasks. Evolved CO_2 was trapped in 10% KOH.

RESULTS

Respiration Studies. No significant influence of cycloheximide upon respiration of cotyledons was found in either of two experiments. Measurements at five periods between 48 and 157 hr of germination gave rates for both control seeds and those imbibed in CH of about 50 μl of O_2/2 cotyledons·hr.

Growth and Phenylalanine Metabolism. Table I lists the results showing the effects of CH on growth of embryonic axes, absorption of ^{14}C-phenylalanine, its incorporation into protein and trichloroacetic acid-soluble fractions, and its degradation to $^{14}CO_2$. Growth of nearly all of the embryonic axes between 12 and 84 hr was completely arrested by CH. Ab-

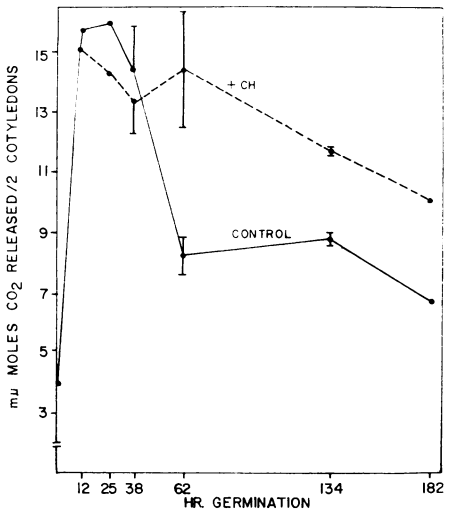

FIG. 1. Effects of cycloheximide on activity of OMP pyrophosphorylase in pea cotyledons during germination. Values are means from two experiments except at 182 hr. Bars indicate separate results for each experiment.

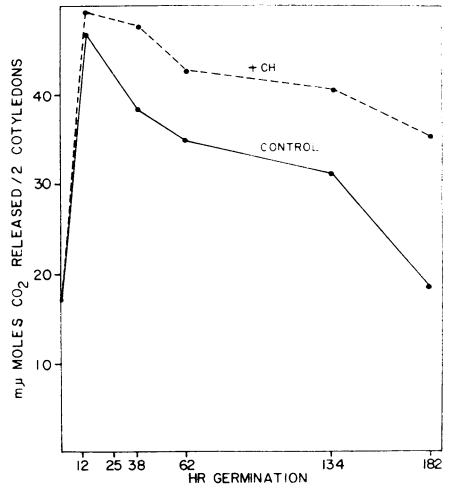

FIG. 2. Effects of cycloheximide on activity of OMP decarboxylase in cotyledons during germination. Results are means of duplicate determinations. Average percent deviations of individual values from the means were 5.8 for both control and cycloheximide treatments.

sorption of labeled phenylalanine was not significantly inhibited at 12 hr (as estimated by total ^{14}C recoveries), but protein synthesis was markedly reduced by CH. The antibiotic did not significantly affect $^{14}CO_2$ release at 12 hr.

After 84 hr of germination the cotyledons of control seeds absorbed only about one-fourth as much phenylalanine as at 12 hr. The apparent loss in ability to absorb this amino acid was partially prevented by CH. Cotyledons exposed to CH absorbed about twice as much phenylalanine as control cotyledons. Furthermore, the percentage of ^{14}C absorbed by the controls which was converted into protein decreased from about 50% at 12 hr to 20% at 84 hr. A possible explanation for this difference is that at 84 hr the control tissues may have contained more unlabeled phenylalanine arising from protein degradation than at 12 hr (2), thus reducing the specific radioactivity of internal phenylalanine-^{14}C. Because of greater phenylalanine absorption by cotyledons exposed to CH than by controls, the inhibition of incorporation of phenylalanine into protein was apparent at 84 hr only when the data were tabulated as percentages of ^{14}C absorbed.

It is interesting that control tissues converted about 60 times as much of the ^{14}C that they absorbed from phenylalanine into $^{14}CO_2$ at 84 hr than at 12 hr. This increased capacity to degrade phenylalanine, observed in both experiments, was inhibited by CH.

Effects of CH on Enzyme Development. Changes in activities of extractable OMP pyrophosphorylase and OMP decarboxylase and the influences of CH during the first 182 hr of germination are shown in Figures 1 and 2. Activities of both enzymes increased during imbibition and then declined. CH had no appreciable effect on the rise in activity of either enzyme; yet in both cases it significantly inhibited the subsequent decrease.

Influences of CH on development of cytidine deaminase and uridine hydrolase activities are shown in Figures 3 and 4.

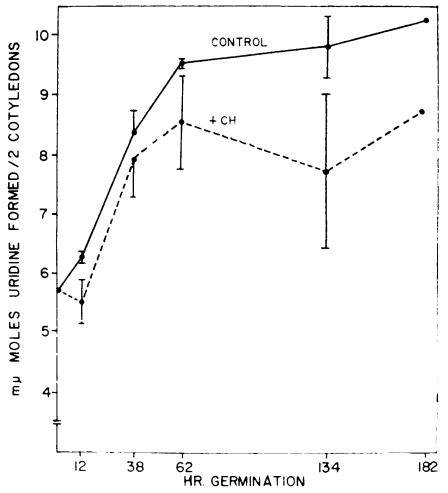

FIG. 3. Effects of cycloheximide on activity of cytidine deaminase in cotyledons during germination. Values are means from two experiments except at 182 hr. Bars indicate separate results for each experiment.

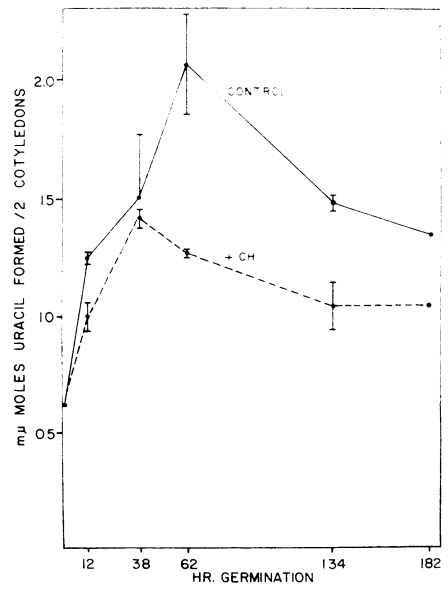

FIG. 4. Effects of cycloheximide on activity of uridine hydrolase in cotyledons during germination. Values are means from two experiments except at 182 hr. Bars indicate separate results for each experiment.

Results are means of two experiments; vertical bars indicate values for each experiment. Results from the two experiments were very similar in most cases, especially for uridine hydrolase. Cytidine deaminase was always more active than uridine hydrolase. Activities of both enzymes increased at least up to 62 hr in control cotyledons. CH inhibited the increase of uridine hydrolase with only a small inhibitory influence on increase of cytidine deaminase. As contrasted to results with OMP pyrophosphorylase and OMP decarboxylase, both cytidine deaminase and uridine hydrolase activities subsequently remained higher in the control seeds than in CH-treated seeds.

That the enzyme identified as uridine hydrolase is not uridine phosphorylase (which also produces uracil from uridine) was shown by experiments in which the crude enzyme extract was run through a Sephadex G-150 column to remove endogenous phosphate. The resulting activity elution profile was identical when assays were performed in the presence or absence of 30 mM phosphate. The presence of a uridine hydrolase and absence of uridine phosphorylase were previously reported for mung bean seedlings (1).

PRPP Contents. Figure 5 indicates changes in extractable PRPP in cotyledons of control seeds germinated for various times. Amounts of PRPP in dry whole seeds were almost undetectable, but these values rose rapidly during imbibition and a maximum of about 23 nmoles per pair of cotyledons was obtained in each of two experiments at about 12 hr. We are not aware of other analyses of PRPP contents in higher plants with which our values can be compared.

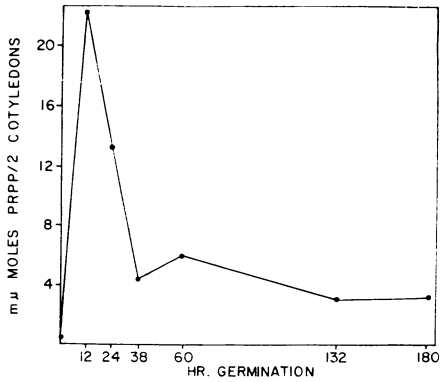

FIG. 5. Changes in amounts of PRPP in pea cotyledons during germination. Values are means of two analyses with 0.1 ml of cotyledon extract.

DISCUSSION

Interpretation of the effects of CH upon development of the four enzymes studied requires analysis of its influences upon growth and synthesis of protein, since it can have additional effects upon plant metabolism (5, 14). CH prevented embryonic axis growth, while protein synthesis in the cotyledons was strongly inhibited but apparently not completely stopped. No influences upon respiration were observed after exposure to 50 μg of CH per ml. Harwood and Stumpf (8) studied the influence of various concentrations of CH up to 1 mg/ml upon water absorption, fatty acid synthesis, and leucine-^{14}C incorporation into protein of whole pea seeds during the first 24 hr of germination. CH had no effect on imbibition, consistent with our unpublished data, but leucine incorporation during the first 24 hr of germination was inhibited about 78% by 10 or 25 μg of CH per ml. Our apparently comparable results at 12 hr indicate about 85% inhibition of phenylalanine incorporation into protein by 50 μg of CH per ml.

Although CH did not significantly influence $^{14}CO_2$ release from phenylalanine at 12 hr, it inhibited it at 84 hr. If enzymes catabolizing phenylalanine to CO_2 are synthesized from amino acid precursors in control seeds during this time, CH would be expected to interfere with such synthesis, and this might account for the observed results. The increasing ability of pea cotyledons to degrade an amino acid observed here is apparently not restricted to phenylalanine (but note contrasting results with phenylalanine and pea seeds in Ref. 12), since similar results were noted for leucine catabolism (3). Degradation of orotic acid and uridine increased only slightly in similar studies (15), while adenine breakdown decreased with time (3). Increased catabolic capacity during the first few days of germination is thus not a general characteristic of pea cotyledons.

A major objective of these studies was to determine how pyrimidine nucleotide metabolism is regulated in pea cotyledon storage tissues during germination, with particular interest in explaining the increased anabolism of orotic acid observed previously (15). OMP pyrophosphorylase became much more active until imbibition was completed, yet the activity then decreased at times when orotate was previously found to be more rapidly converted into UMP and RNA. The results of Brown and Wray (4) suggested that the supply of PRPP might increase in the cotyledons with time, thus accounting for increased conversion of orotate to uridine nucleotides. However, our analyses of PRPP levels in the cotyledons gave a time curve very similar to that of OMP pyrophosphorylase activity. We therefore cannot explain the continuous increase in orotate anabolism observed previously. Perhaps the levels of extractable PRPP and activities of orotate-metabolizing enzymes do not reflect those *in vivo*.

Activities of the four enzymes studied showed some developmental similarities and differences. The activity of each was relatively low in dry seeds. This might be due to incomplete extraction, yet the amount of total protein reacting in the Lowry test (10) was usually highest for dry seeds. If CH indeed penetrated and inhibited protein synthesis in the same cells synthesizing these enzymes, the increases in activities of OMP pyrophosphorylase, OMP decarboxylase, and probably of cytidine deaminase during germination are likely due to activation rather than actual synthesis. Enzymes capable of fatty acid formation (8) and an amylopectin-1,6-glucosidase (11) similarly increase in activity in cotyledons of germinating peas without being synthesized *de novo*. However, assuming the only important effect of CH upon increase of uridine hydrolase activity resulted from inhibition of protein formation, this enzyme probably arises at least in part from *de novo* synthesis.

Decreases in activities of the OMP-metabolizing enzymes after the maxima were reached in control cotyledons might be due to the accumulation of a protein, perhaps proteolytic, capable of destroying or inactivating them. Evidences for increased protease activity during germination were presented for cotton cotyledons by Ihle and Dure (9) and for pea cotyledons by Beevers (2). Regardless of the mechanism of enzyme inactivation, this process is less active in the presence of CH. Engelsma (6), Zucker (19), and Travis *et al.* (17) previously observed decreased rates of plant enzyme inactivation in the presence of CH. CH also prevents losses in activities of certain animal enzymes, but its interference with protein degradation is not a general phenomenon (7). As yet there is apparently no convincing evidence that hydrolysis rather than a more subtle cause of inactivation accounts for these decreases in enzyme activities (19).

The functions of cytidine deaminase and uridine hydrolase

in the cotyledons are not yet understood. They might participate in catabolizing cytidine and uridine released after ribonuclease action upon storage RNA. We (15) found, however, that considerably more uridine-2-^{14}C is converted into RNA than is degraded to $^{14}CO_2$, suggesting that cytidine deaminase may function primarily as part of a salvage pathway for reutilization of cytidine (via uridine nucleotides) in RNA synthesis. As a final alternative, both enzymes might participate in providing uridine and uracil for transport to the embryonic axis. Our data indicating a role for uridine hydrolase rather than uridine phosphorylase in uridine degradation are consistent with those of Achar and Vaidyanathan (1). We are not aware of any direct demonstrations of uridine phosphorylase activity in higher plants, which may indicate (*a*) that incorporation of uracil into RNA normally involves the direct conversion of this pyrimidine into UMP by UMP pyrophosphorylase and (*b*) that the first step of uridine degradation is hydrolytic rather than phosphorolytic.

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