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The Development of Isocitric Lyase Activity in Germinating Cotton Seed[1]

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ROBERTA H. SMITH,[2] A. MICHAEL SCHUBERT, AND C. ROY BENEDICT
Department of Plant Sciences, Texas A&M University, College Station, Texas 77843

ABSTRACT

In cotyledons of germinating cotton (*Gossypium hirsutum* L. var. Stoneville 213) seedlings, in the dark, isocitric lyase (EC 4.1.3.1) activity peaks after 2 days and thereafter slowly declines to a negligible value after 8 days. The maximum activity of this enzyme in cotyledons of 2-day-old seedlings was 16.2 μmoles of glyoxylate formed/15 min·10 cotyledon pairs. Actinomycin D at a concentration of 10 μg/ml, if added to the imbibing solution, completely prevents the development of isocitric lyase activity in these germinating seed. In cotyledons of germinating cotton seedlings, in the light, isocitric lyase activity peaks after 2 to 3 days and sharply declines to a negligible value after 4 days. The maximum activity of this enzyme in cotyledons of 2- to 3-day-old seedlings was 13.2 μmoles of glyoxylate formed/15 min·10 cotyledon pairs. Actinomycin D at a concentration of 10 μg/ml, if added to the imbibing solution, severely inhibits the development of enzyme activity.

In germinating seed, in the light, the synthesis of chlorophyll and glyceraldehyde-3-P dehydrogenase is also limited by the addition of low concentrations of actinomycin D. The new synthesis of fructose-1,6-diP aldolase, which is detectable after 1 to 2 days of germination, is inhibited by 10 μg/ml of actinomycin D. We, therefore, conclude that the synthetic events leading to the development of chlorophyll, some glyoxysomal and chloroplast enzymes in germinating cotton seedlings depend on newly transcribed mRNA.

Isocitric lyase (EC 4.1.3.1) activity increases several fold during the first few days of germination of fatty seed (4). Isocitric lyase and malate synthetase do not pre-exist in an inactive form in the cotyledons but are synthesized *de novo* during the germination of peanut seed (7, 13). Isocitric lyase has been localized in the glyoxysomes of germinating castor beans (3) and recently, the development of this enzyme has been studied in relation to the development of glyoxysomes and peroxisomes (12, 16).

There is little understanding of the control of the synthesis of isocitric lyase in germinating fatty seed (7, 8, 11). Hock and Beevers (8) conclude that isocitric lyase is inhibited by Act D[3] but only if the antibiotic is added early in the period of imbibition. They suggest the mRNA for this enzyme may be synthesized for only a limited period but is functional for several days. Gientka-Rychter and Cherry (7) have shown that the development of this enzyme is inhibited 50 to 60% by Act D during 4 days of germination in peanut seed. Ihle and Dure (9, 10) have shown the presence of a long lived mRNA in the cotyledons of germinating cotton seed which directs the synthesis of carboxypeptidase. Recently, Ihle and Dure (11) have provided evidence indicating carboxypeptidase and isocitric lyase activity in germinating cotton seed arise *de novo* from a stable mRNA synthesized during embryogenesis, yet not translated until 24 hr of germination. These 2 enzymes may be representative of an entire group of germination enzymes which are controlled in a similar fashion (11). The purpose of the work in this paper was to determine whether the transcription and translation of isocitric lyase (and other enzymes active during germination) in cotyledons of germinating cotton seed are separated in time.

MATERIALS AND METHODS

Materials. DL-Isocitric acid trisodium salt, glutathione reduced form, glyoxylic acid sodium salt, cycloheximide, and actinomycin D (isolated from *Actinomyces antibioticus*) were obtained from Sigma Chemical Co.

Germination and Seedling Growth. Cotton seed (*Gossypium hirsutum* L. var. Stoneville 213) was soaked in H_2O for 3 hr and the seedcoats removed. The embryos were imbibed for an additional 3 hr in either H_2O, CH, or Act D solutions. In separate experiments, we have shown that the imbibition period for cotton seed is 6 to 7 hr. The Act D solution was made just before use, and at a concentration of 10 μg/ml, the pH of the deionized H_2O was not altered. The embryos were germinated in the dark at 30 C or in the light, 1000 ft-c, at 30 C. The imbibed embryos were germinated on top of five layers of Whatman No. 1 filter paper in Petri dishes containing 10 ml of H_2O or the antibiotic test solution. The zero time of the germination period in all of these experiments was 6 hr after the initiation of seed imbibition.

Enzyme Analysis. For the preparation of crude enzyme extracts, the cotton cotyledons from germinating seedlings were harvested, washed with deionized H_2O, blotted, and weighed. The plant material was ground in a chilled mortar in 0.05 M potassium phosphate buffer, pH 7.6, and sand in a ratio of 1 ml buffer-1 g fresh weight cotyledon. The homogenate was centrifuged at 15,000 rpm in a refrigerated Sorvall centrifuge for

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[2] Present address: Department of Biology, Sam Houston State University, Huntsville, Texas 77340.

[3] Abbreviations: Act D: actinomycin D; CH: cycloheximide.

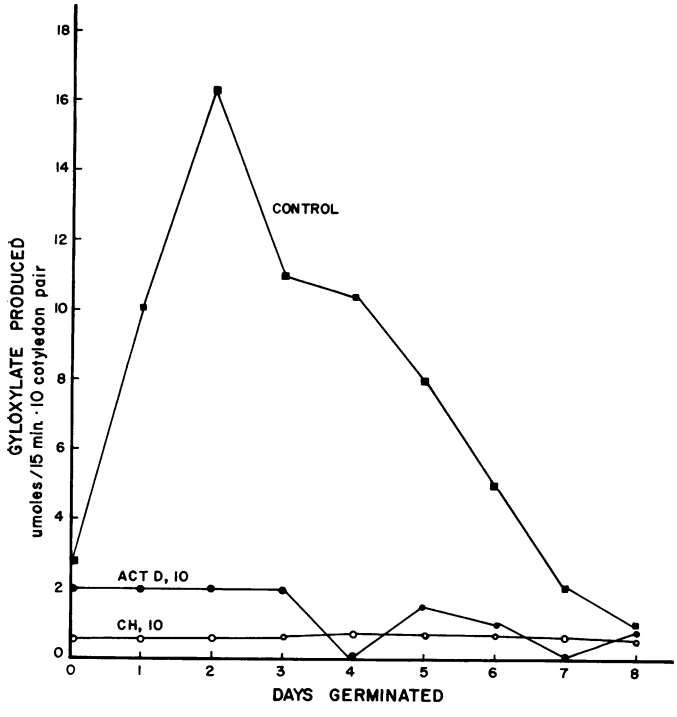

FIG. 1. Effect of actinomycin D and cycloheximide on the development of isocitric lyase activity in cotyledons of dark-grown cotton seedlings. The concentrations of Act D and CH were 10 μg/ml.

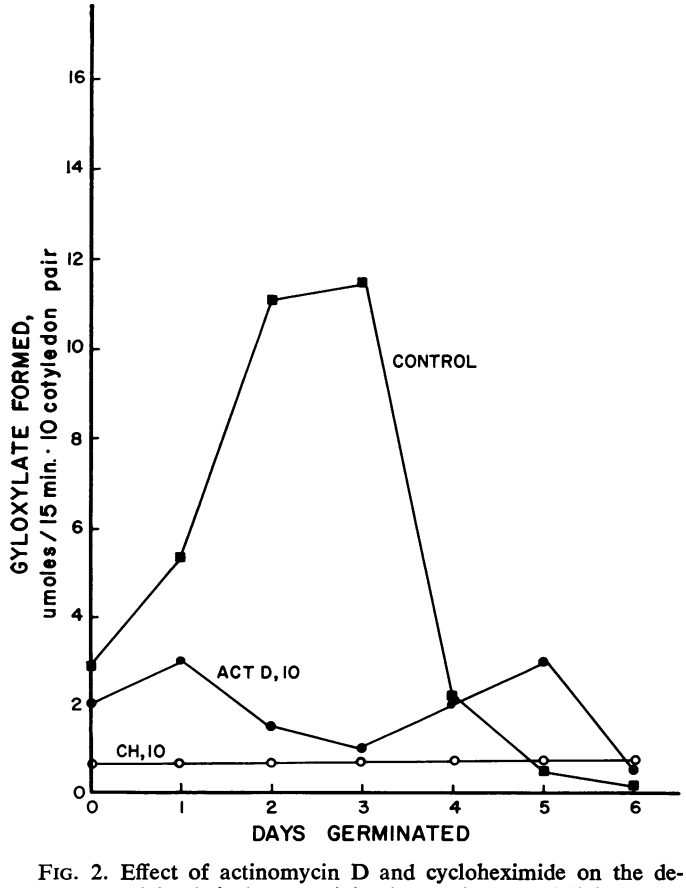

FIG. 2. Effect of actinomycin D and cycloheximide on the development of isocitric lyase activity in cotyledons of light-grown cotton seedlings. The concentrations of Act D and CH were 10 μg/ml.

20 min. An aliquot of the soluble supernatant fraction was used as the source of enzymes.

Isocitric lyase activity in the homogenate was assayed by a modified procedure of Carpenter and Beevers (4) and Gientka-Rychter and Cherry (7). Originally, our assay procedure was similar to that described by Carpenter and Beevers (4). The enzyme activity was linear for 15 min. Rather than developing a kinetic curve for each development time, we modified this original assay according to the procedure as described by Gientka-Rychter and Cherry (7). The test reaction mixture contained in μmoles: 200, potassium phosphate buffer (pH 7.6); 5, GSH; 5, $MgSO_4$; 10,DL-isocitrate; 0.2 ml of protein extract, and H_2O to 1.0 ml. The blank reaction mixture contained enzyme and all of the reagents minus the isocitrate. The test and blank reaction mixtures were incubated 15 min at 30 C. The reactions were terminated by adding 0.2 ml of 100% trichloroacetic acid to the tubes. The denatured protein was removed by centrifugation of the reaction tubes at 10,000 rpm for 10 min. To form the glyoxylate 2,4-dinitrophenylhydrazone, 0.33 ml of 0.1% 2,4-dinitrophenylhydrazine·2 N HCl was added to 0.5 ml of the above protein-free supernatant. This mixture was incubated for 15 min at 30 C. At the end of this second incubation period, 1.67 ml of 2.5 N NaOH was slowly added to each tube, and the absorbance of the glyoxylate 2,4-dinitrophenylhydrazone was recorded at 445 nm in a Beckman Acta C III recording spectrophotometer. For each determination of isocitric lyase activity, the absorbance of the blank reaction mixture, containing no isocitrate, was subtracted from the absorbance of the test mixture.

Glyceraldehyde-3-P dehydrogenase and fructose-1,6-diP aldolase activity in the cotyledonary extracts and the Chl $a+b$ content of the intact cotyledons were determined by a previously described procedure (1).

RESULTS

A study of the cofactor requirement of isocitric lyase activity in cotyledon extracts of 2-day-old germinating cotton seedlings in the dark showed the complete reaction mixture had 0.032 A_{445nm} units/min·mg protein of enzyme activity. In the presence of boiled extract or in the absence of DL-isocitrate, there was no enzyme activity. In the absence of $MgSO_4$, the enzyme activity was 0.021 A_{445nm} units/min·mg protein. The enzyme activity in the complete reaction mixture is comparable to the activity in cotton seedlings originally reported by Carpenter and Beevers (4).

The development of isocitric lyase activity in cotyledons of dark-grown cotton seedlings is shown in Figure 1. The enzyme activity reaches a maximum after 2 days of germination and slowly declines to a negligible value 8 days after germination. In dry, unimbibed cotton seed the amount of enzyme activity is 1.2 μmoles of glyoxylate formed/15 min·10 cotyledon pairs. Cotton embryos which had been imbibed for 3 hr in Act D, 10 μg/ml or CH, 10 μg/ml did not develop any isocitric lyase activity throughout 8 days of germination. The amount of isocitric lyase activity after 2 days of germination of the seedlings in H_2O is 16.2 μmoles of glyoxylate formed/15 min·10 cotyledon pairs (or 0.06 μmole of glyoxylate formed/min·cotyledon). The amount of isocitric lyase activity in 4-day-old cotyledons of dark-grown watermelon seedlings is 0.16 μmole of isocitrate consumed/min·cotyledon (12). There is a 3-fold greater activity in watermelon cotyledons compared to cotton cotyledons. Carpenter and Beevers (4) have shown that there is a 3-fold higher amount of isocitric lyase activity in pumpkin cotyledons compared to cotton cotyledons.

The development of isocitric lyase activity in cotyledons of light-grown cotton seedlings is shown in Figure 2. The enzyme

Table I. *Development of Isocitric Lyase Activity in Cotyledons of Light-grown Cotton Seedlings*

The test reaction mixture for each assay of isocitric lyase was the same as described in "Materials and Methods." The blank reaction mixture for each enzyme determination contained all of the reagents in the test reaction plus 0.2 ml of 100% trichloroacetic acid.

Germination Time	Isocitric Lyase Activity		
	Control	1 μg/ml Act D	10 μg/ml Act D
days	*μmoles of glyoxylate formed/15 min·10 cotyledon pairs*		
1	1.8	0.0	0.5
2	14.0	9.1	0.0
3	17.6	11.5	3.8
4	0.0	4.0	0.0

activity reaches a maximum level after 2 to 3 days of germination and sharply declines to a negligible value after 4 days of germination. The maximum enzyme activity developed in cotyledons of 2- to 3-day-old seedlings is 13.2 μmoles/15 min·10 cotyledon pairs. Cotton embryos which have been imbibed in 10 μg/ml Act D, or 10 μg/ml CH, and germinated in these solutions do not develop any significant amount of isocitric lyase activity throughout 6 days of germination.

The development of isocitric lyase activity in cotyledons of light-grown seedlings in solutions of 1 μg/ml and 10 μg/ml Act D is shown in Table I. The enzyme activity in the H_2O control reaches a maximum in 2- to 3-day-old seedlings and again falls to a low value after 4 days of germination. Act D at 10 μg/ml nearly prevents the development of all lyase activity. Isocitric lyase activity in cotyledons of seedlings imbibed and growing in solutions of 1 μg/ml Act D, are inhibited only 35% after 2 and 3 days of germination. This is an important point because it demonstrates that 1 μg/ml Act D, although inhibiting some enzyme development, allows for development of 65% of the enzyme activity. It further demonstrates that while imbibing and germinating cotton seed in 1 μg/ml Act D, the cotyledons do not accumulate abnormally high concentrations of Act D which are toxic to all development.

In order to determine the effectiveness of Act D on a number of synthetic activities, which are prominent during germination and seedling growth of cotton seed, we tested the effect of this antibiotic on the light-dependent development of Chl, glyceraldehyde-3-P dehydrogenase and fructose-1,6-diP aldolase activity. Figure 3 shows that the development of Chl in cotyledons of germinating cotton seedlings is nearly linear after a 24-hr lag period. Act D at 10 μg/ml nearly prevents the development of Chl. Act D at 1 μg/ml inhibits Chl development by only 10 to 15% through 4 days of germination. Figure 4 shows the development of NADP-linked glyceraldehyde-3-P dehydrogenase in germinating seedlings. There was no detectable quantity of this enzyme activity in dry seed, and there was a 24-hr lag period in the development of this activity in the light. After 4 days of growth in the light, the activity had not plateaued. Act D at 10 μg/ml severely limits the appearance of this activity in the cotyledons, but a major amount of this enzyme activity develops in the presence of 1 μg/ml of Act D. Figure 5 shows the development of fructose-1,6-diP aldolase activity in germinating seedlings. There is a substantial amount of enzyme activity in the dry seed and after 4 days of germination in the light the new synthesis of this activity is still increasing. Act D at 1 μg/ml does not interfere with the development of this enzyme activity. Act D at 10 μg/ml does not interfere with the basal level of enzyme activity but does prevent the further development of this activity in the cotyledons.

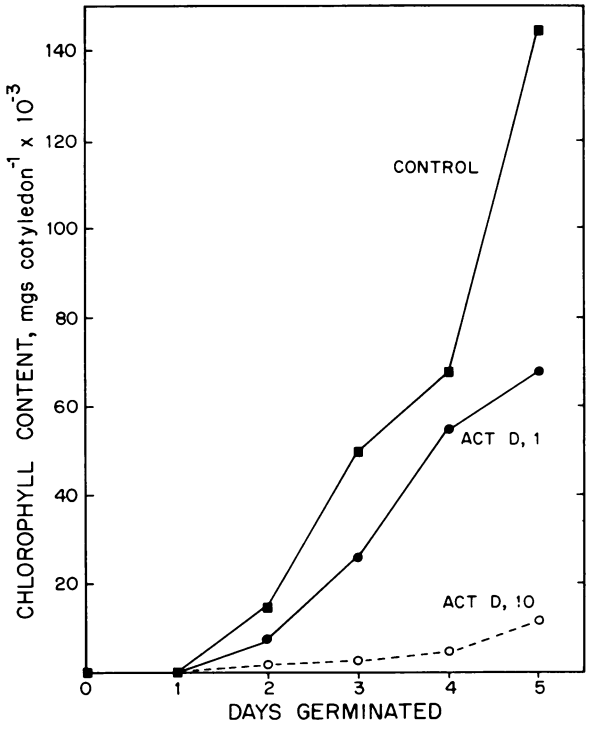

FIG. 3. Effect of actinomycin D on chlorophyll development in cotyledons in light-grown cotton seedlings. The concentrations of Act D were 1 and 10 μg/ml.

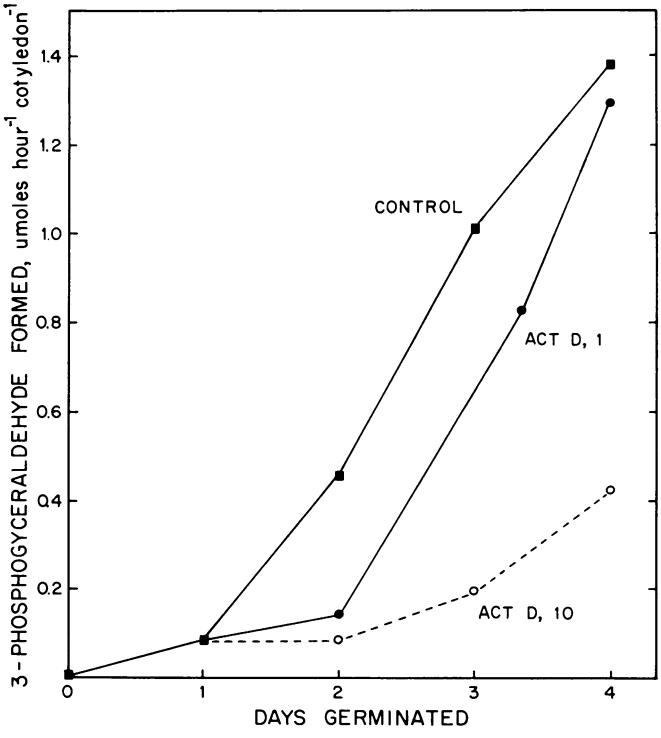

FIG. 4. Effect of actinomycin D on the development of NADP-glyceraldehyde-3-P dehydrogenase in cotyledons of light-grown cotton seedlings. The concentrations of Act D were 1 and 10 μg/ml.

DISCUSSION

The development of isocitric lyase activity has been determined in cotyledons of dark-grown or light-grown cotton seed-

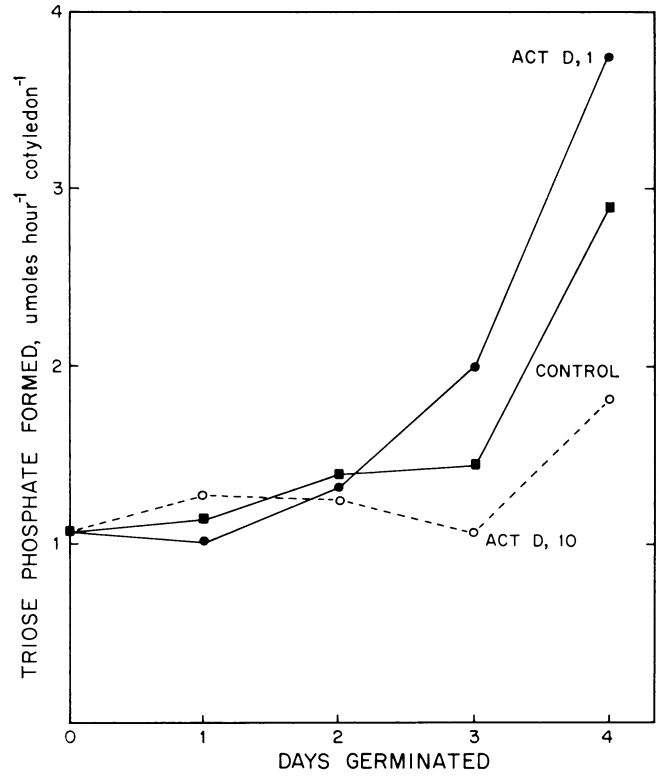

FIG. 5. Effect of actinomycin D on the development of fructose-1,6-diP aldolase in cotyledons of light-grown cotton seedlings. The concentrations of Act D were 1 and 10 μg/ml.

lings. In the dark-grown seedlings, the enzyme activity peaks after 2 days of germination and declines slowly. In the light-grown seedlings, the enzyme activity peaks after 2 to 3 days of germination and then declines sharply. This enzyme is an established component of the glyoxylate cycle in fatty seed (2, 4), and the appearance of activity on germination represents *de novo* synthesis (7, 13). The purpose of this work was to determine whether the transcription and translation of isocitric lyase (and other enzyme activities) in cotyledons of germinating cotton seed are separated in time. We have shown that low concentrations of Act D (10 μg/ml), if added during the soaking period (imbibition period), prevents the development of isocitric lyase in germinating cotton seed. These results generally agree with Hock and Beevers (8) and Gientka-Rychter and Cherry (7). These results differ from Ihle and Dure's finding (11) that the development of isocitric lyase activity in cotyledons of 3-day-old dark-grown cotton seedlings is insensitive to 20 μg/ml of Act D if added during the soaking period. In our experiments, the cotton seed was imbibed for 3 hr in H_2O followed by 3 more hr of imbibition in the antibiotic solutions. The embryos were germinated in the antibiotic solution. Ihle and Dure (9, 11) have generally imbibed the cotton embryos for 30 min in Act D or CH followed by germination in agar containing the antibiotic. We do not feel that our procedures lead to an abnormal accumulation of toxic amounts of Act D, because in all of our experiments 1 μg/ml of Act D resulted in only 0 to 35% inhibition of all the enzymes tested. In one instance, 1 μg/ml of Act D stimulated the development of fructose-1,6-diP aldolase activity in the cotyledons. Ihle and Dure (11) conclude the insensitivity of the development of isocitric lyase activity to Act D is due to the presence of a stable mRNA which is transcribed during embryogenesis and stored in the mature seed. This mRNA would be translated 1 to 2 days after seed germination. This type of control mechanism has also been postulated to be operable in the development of a number of enzymes during the germination of cotton seed (11).

We have tested the effect of Act D on synthetic events developing in cotton seedlings 1 to 2 days following the soaking period. The light-dependent synthesis of both Chl and glyceraldehyde-3-P dehydrogenase shows a 24-hr lag leriod in germinating seed and is very sensitive to low concentrations of Act D. The new synthesis of fructose-1,6-diP aldolase activity which is detectable after 1 to 2 days of germination is inhibited by 10 μg/ml of Act D. The effect of Act D on the development of these light-dependent enzyme activities is similar to its effect on the development of isocitric lyase activity during germination. We can find no evidence for the presence of stable mRNAs for the development of any of these enzymes in germinating cotton seed.

Marcus and Feeley (15), Dure and Waters (6), and Marcus (14) have shown previously that the protrusion of the radicle in germinating peanut and cotton seed is insensitive to Act D. Our experiments agree consistently with these findings. Act D does not interfere with the germination of cotton seed. The growth of the radicle requires 24 hr in germinating cotton seed. These results show the presence of a stable mRNA which is translated during germination. There is evidence that new mRNA is synthesized for subsequent growth of seedlings; in wheat embryos the new mRNA is synthesized 12 hr after the imbibition period. Our present work shows that, although cotton seed germination is not affected by Act D, subsequent synthetic events leading to the development of Chl, and some glyoxysomal and chloroplast enzymes 24 hr after imbibition is inhibited by Act D. The control mechanism for the synthesis of these enzymes and pigment (which appear in the seedling considerably later than radicle protrusion) is dependent on the translation of newly transcribed mRNA.

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