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ANTHOCYANIN SYNTHESIS IN CORN ENDOSPERM TISSUE CULTURES. I. IDENTITY OF THE PIGMENTS AND GENERAL FACTORS [1]

JACOB STRAUS [2]

DEPARTMENT OF BIOLOGY, UNIVERSITY OF OREGON, EUGENE, OREGON

Until recently few physiological studies have been made which bear directly on the biosynthesis of anthocyanin. However, a great deal of work has been done on the chemistry and genetics of these pigments. Much of the earlier literature has been summarized by Onslow (7) and more recently Blank (1) has written an extensive review. Many of these physiological studies have been concerned with the effects of environmental factors such as light and temperature. In addition, studies have been carried out to determine the effects of feeding different sugars and sugar concentrations to seedlings of green plants (3).

Recently, Thimann and his associates (4, 16, 17, 18, 19, 20) have entered upon a new approach to the problem of anthocyanin synthesis. They chose to work with the comparatively simple green aquatic plant, *Spirodela oligorrhiza* K., which can be easily grown under known controlled conditions. With this plant, for example, Edmundson and Thimann (4) have been able to demonstrate the probable mediation of a copper-containing enzyme in anthocyanin synthesis. Thimann and Radner (20) have shown that riboflavine plays an important role in the production of these pigments. Siegelman and Hendricks (11) have determined the action spectrum for anthocyanin formation in seedlings of turnip and red cabbage. They suggest that their data strongly support the implication of a copper-containing enzyme. However, in all of those studies as well as in those by many other workers, green plants or plant parts which ordinarily photosynthesize were used and under those circumstances the effects of photosynthesis, whether direct or indirect, can hardly be discounted. Slabecka-Szweykowska (12) used callus cultures of *Vitis vinifera* stem for studying anthocyanin synthesis. These cultures do not contain chlorophyll and she was able to demonstrate conclusively that light was essential for anthocyanin synthesis in these tissue cultures. However, the production of anthocyanin by the Vitis tissue occurs only on media in which the sugar content is 3 % or greater. Thus, anthocyanin synthesis must be induced in this tissue by relatively high sugar concentrations. It would seem desirable to have a tissue devoid of chlorophyll and which synthesizes anthocyanin under ordinary circumstances as an experimental tool with which to investigate anthocyanin synthesis. Therefore, when an anthocyanin-bearing strain of corn endosperm had been isolated as a continuing tissue culture (13, 15), it was thought that this tissue might provide the ideal experimental system for these studies.

METHODS AND MATERIALS

The endosperm tissue cultures were isolated from the maize variety, 'Black Mexican Sweet', according to the methods described by Straus and LaRue (15). The nutrient medium contained White's major elements, Nitsch's trace elements (6), 0.5 % Seitz-filtered yeast extract, 2 % sucrose, and 0.9 % agar unless otherwise noted. The general growth conditions and methods for determining changes in weight were the same as those described by Straus and LaRue. In an attempt to reduce the effect of variations in the initial pigment concentration, tissue was grown in the dark. Such tissue is virtually colorless (see effects of light) and was used as a source of inocula. However, it was soon discovered that the final pigment concentrations of both light- and dark-grown tissues subjected to the same experimental conditions were usually the same with a few notable exceptions. Thereafter, explants were chosen from light-grown tissue in order to simplify handling procedures. One experiment reported here, however, was conducted with dark-grown tissue as will be pointed out later.

Because of the requirement for yeast extract, experiments which would involve extensive manipulation

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of mineral nutrients and many organic compounds in the medium are severely restricted. However, work is progressing toward the development of a synthetic medium which should greatly facilitate experimentation with these cultures.

EXTRACTION AND QUANTITATIVE DETERMINATION OF THE PIGMENT: The tissue is removed from the culture vials, gently blotted with filter paper to remove excess water, weighed and placed in a flask. Two percent concentrated hydrochloric acid in 95 % ethanol (v/v) is poured over the tissue and the mixture permitted to steep for 4 hours at room temperature. At the end of this period, the tissue is removed by filtration and extracted a second time with fresh solvent. This procedure is repeated once more. The volume of the combined extracts is determined and 10 ml are used to determine the relative pigment concentration. A Klett colorimeter employing a green (no. 54) filter is used for this purpose. The Klett reading is substituted in the formula that Thimann and Edmundson (16) used for the pigments in Spirodela to give relative pigment concentration:

$$\text{P.U./g} = \text{Klett reading} \times \frac{\text{ml solvent}}{10} \times \frac{1}{\text{g tissue extracted}}$$

where P.U./g stands for pigment units per gram of tissue in 10 ml of the extract.

The first 2 extractions remove almost all of the free pigment. Steeping the tissue overnight or for several days in the 3rd solvent change removes a very slight additional amount of pigment although the tissue remains a bright pink. Heating or grinding the tissues in the solvent removes a bit more pigment, but the consequent cell disruption results in a suspension which is difficult to clarify and which interferes with colorimetric readings. Because of this, it was decided that a more or less constant error due to unextractable pigment was preferable.

To estimate the amount of pigment produced in absolute units, chromatographically purified chrysanthemin (see below) was prepared and 26.04 mg were dissolved in 100 ml 95 % ethanol containing 2 % HCl (v/v). Appropriate dilutions were made and the solution read in a Klett colorimeter with a green filter. A concentration of 0.13 mg in 10 ml gave a reading of 40. Thus 1 pigment unit is equivalent to 3.25 μg of anthocyanin. This is not entirely accurate as it does not take into account the absorption by other colored substances in the crude extract. However, the figure is probably a close approximation. Further calculations show that the pigment concentration of fresh tissue is about 0.5 %. The percent dry weight of the tissue is 7.5 %, hence anthocyanin represents about 6.5 % of the dry weight of the tissue.

CHROMATOGRAPHY: Extracts for chromatography were prepared by disintegrating the tissues in a Waring blendor with the extraction solvent mentioned above. The debris was removed by filtration and the filtrate evaporated to dryness in an air stream at room temperature. The residue was extracted with a minimum quantity of 95 % ethanol or absolute methanol and filtered. The filtrate was applied to the paper without further treatment unless otherwise noted.

The irrigating solvents used were the organic phases of the following mixtures: *n*-butanol : acetic acid : water (4 : 1 : 5 v/v; hereafter referred to as solvent 1) and *n*-butanol : 2*N* hydrochloric acid (1 : 1 v/v; hereafter referred to as solvent 2). Whatman filter papers no. 1 and no. 3 MM were used. They were washed in *N* hydrochloric acid; then with distilled water until the washings were chloride-free; then in 0.1 % ethylenediamino tetraacetic acid (disodium salt) (w/v) and finally rinsed with a volume of distilled water equal to that needed to wash the paper free of chloride following the acid treatment. The papers were equilibrated with the aqueous phase of the solvents for 8 hours at 30° C.

RESULTS

IDENTITY OF THE PIGMENTS: Extracts prepared as previously described were spotted on Whatman no. 1 paper and chromatographed with solvent 1. Under the conditions described, 3 anthocyanins were discerned at R_f 0.3 (band 1), 0.36 (band 2), and 0.47 (band 3). The spot at R_f 0.36 represents the pigment present in greatest concentration followed in much lower concentration by the pigment at R_f 0.47. A rough estimate of the relative concentrations of these 2 pigments shows a ratio of about 9 to 1. The slowest running pigment is barely visible.

Sando et al (10) demonstrated the anthocyanin present in the husks of purple-husked corn to be chrysanthemin (cyanidin-3-monoglucoside). Coe (2) also showed that a cyanidin-3-monoglucoside was present in the aleurone layer of maize kernels of the genetic constitution, Pr. In addition, he reported the presence of callistephin (pelargonidin-3-monoglucoside) in the same tissues as well as in tissues of pr. Maize aleurone is purple when among other genetic factors, the genetic factor, Pr, is present and is red when homozygous for pr. On the assumption that these same pigments were present in the extracts of the endosperm tissue cultures, authentic chrysanthemin was prepared from extracts of flower heads of a bronze variety of chrysanthemum (7, 8, 9) and callistephin from ripe strawberries (5). Initially, an attempt was made to obtain callistephin from the deep red flowers of a Pelargonium species. However, upon chromatographing the extract, 6 poorly separated bands were found and these flowers were abandoned as a source of pigment.

The chrysanthemum and strawberry pigments were extracted in the same manner as that described for the endosperm tissues. The extracts were streaked across the shorter dimension of whole sheets (18¼ x 22¼ inches) of Whatman no. 3 MM paper and chromatographed with solvent 1. The well separated bands were cut out and eluted with 95 % eth-

anol. The solutions were concentrated and the process repeated.

To establish the identity of these pigments, the qualitative tests devised by Robinson and Robinson (8) were applied to the anthocyanins and their aglycones. The aglycones were prepared by adding an equal volume of concentrated hydrochloric acid to a solution of the pigment and heating in a boiling water bath for 15 minutes. The anthocyanidins were extracted with *n*-amyl alcohol. The Robinsons' tests established the chrysanthemum pigment as the cyanidin-3-monoglucoside; the strawberry pigment as the pelargonidin-3-monoglucoside; and the aglycones as cyanidin and pelargonidin respectively.

The endosperm pigments were purified chromatographically in the same manner as that described for the chrysanthemum and strawberry pigments. The endosperm pigment of band 2 was cochromatographed with chrysanthemin on Whatman no. 1 paper in solvents 1 and 2. The R_f values of the pigments were identical. When the 2 pigments were spotted together and permitted to advance almost to the edge of the paper (about 20 hr) in solvent 1, there was no visible separation of the pigments. The aglycone of the endosperm pigment was prepared and compared chromatographically in solvent 2 with cyanidin obtained from chrysanthemin and again the R_f values of the two were alike. The same procedures were repeated with callistephin and endosperm pigment from band 3 and again identical R_f values and non-separation of the pigments were obtained. Thus, it seems quite reasonable to conclude that the 2 endosperm pigments investigated are chrysanthemin and callistephin. The 3rd pigment is present in such minute amounts that it contributes very little to the total pigmentation and no attempt was made to establish its identity.

Absorption Spectrum: The absorption spectrum of the crude endosperm extract prepared by steeping the tissue was determined with a Beckman Model DU spectrophotometer. The combined extracts were diluted to a convenient color density and readings were taken over the range of 310 to 600 mμ. Two absorption peaks were consistently found: one at 325 mμ and another rather broad one at 530 to 540 mμ. The peak at 325 mμ is probably due to flavone pigments (12, 16). An absorption spectrum of a corn endosperm tissue culture derived from a variety of corn that is normally unpigmented did not show any peaks within the same range of wave lengths. The spectrum of a colorless tissue which arose spontaneously from the anthocyanin-producing tissue, however, showed strong absorption in the same near ultra-violet region as did the pigmented tissue, but no peaks were found at longer wave lengths (14).

The absorption spectra of the purified anthocyanins were also determined. The solvent used for this purpose was 2% hydrochloric acid in 95% ethanol (v/v). Chrysanthemin (band 2) had peaks at 272 and 540 mμ; callistephin (band 3) had peaks at 275 and 520 mμ.

Growth-pigment Curve: Comparison of individual cultures shows variations in the intensity of pigmentation from culture to culture. In preparing pools of tissue for explants, uniformly pigmented tissue was chosen as far as practicable. Newly transferred tissue has a uniformly pigmented surface, but the interior is of a lighter hue. When growth commences in a fresh explant, the new tissue is devoid of any visible anthocyanin, but as time progresses, pigment begins to appear in the older portion of the new tissue. Thus, during a period of active growth, the cultures have the general aspect of a pigmented island surrounded by a non-pigmented peripheral area. However, at the end of the growth period (25 days) all of the tissue is pigmented. Growth of the tissue is very uniform, but pigment production is rather variable. This may be due to the difficulty in providing uniform lighting under the conditions in which the cultures are grown. Because of this variability in pigment concentration, controls were run with each experiment using tissue from the same pool as that used for the experimental series. The results are therefore not corrected for initial pigment concentration. Indeed, corrections for initial pigment concentration do not seem to be necessary because when tissues grown in the dark and therefore virtually colorless, are used as inocula, the resulting cultures produce as much pigment at the end of 25 days as when inoculated from light-grown tissues.

In order to determine the time of maximum pigment concentration, 300 cultures were prepared and the wet weight and pigment content of 50 vials of tissue were determined every 5 days for a total of 30 days. The data are shown in figure 1. After several days growth, there is a general trend toward lower pigment concentration up to 20 days. This is because newly produced tissue is devoid of pigment so that the net effect is one of less pigment per unit of tissue. After 20 days, however, when growth begins to slow down, anthocyanin synthesis continues and there is then a slight net increase in pigment content. All experiments to be described hereafter were terminated on the 25th day after preparation.

Effect of pH: A series of cultures was prepared with the pH of the medium varying from 3.2 to 8.7. The media were prepared by adjusting the pH of the yeast extract solution with HCl or KOH and adding the extract to the rest of the medium. A pH of 5.0 favors pigment production in these tissues (fig 2). These results differ from those of Thimann and Edmundson (16) who found that a pH slightly above neutrality was most favorable for anthocyanin synthesis in Spirodela.

Effect of Light: Only the general effects of light were tested. Cultures grown in complete darkness produce between 180 and 300 pigment units per gram of tissue in contrast to those cultures grown in the diffuse light of the laboratory which produce between 1520 and 1600 pigment units per gram. The pigment in the dark-grown tissue may be due to a dilution of the pigment present at the outset of the

experiment due to the growth of the tissue. However, the possibility of some synthesis in the dark cannot be overlooked (16). The final increases in weight of the light- and dark-grown tissues were not significantly different. Much of the tissue produced in the dark is colorless. When such tissue is exposed to the ordinary light of the laboratory, pigment first becomes visible in about 6 hours. This evidence clearly supports Slabecka-Szweykowska's (12) view of the influence of light on anthocyanin synthesis without the complications of photosynthesis mediated by chlorophyll.

EFFECT OF SUGARS: Table I summarizes the effects of glucose, fructose, and sucrose as well as those of different concentrations of sucrose. Sucrose is the best of the 3 sugars in promoting pigment production. Glucose is least effective and fructose is intermediate in effectiveness. The results for pigment production correspond to those obtained by Thimann et al (17) for Spirodela. However, they reported that glucose supported greatest growth and sucrose least, fructose again being intermediate in effect. As can be seen from the data in table I, the opposite growth effects are attributed to glucose and sucrose for these endosperm cultures. The same relative effectiveness of these sugars in the support of growth for endosperm cultures of a different variety of maize holds true (15).

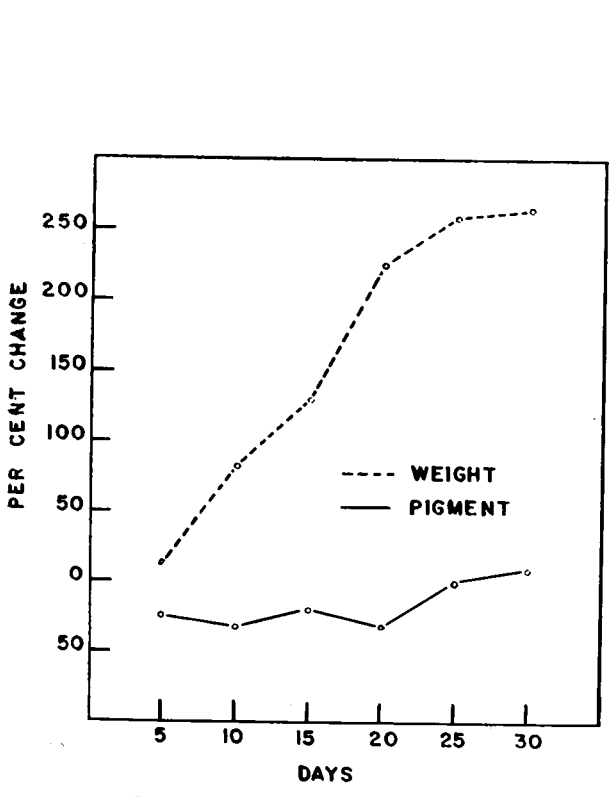
TABLE I

EFFECT OF SUCROSE, GLUCOSE, FRUCTOSE AND DIFFERENT CONCENTRATIONS OF SUCROSE ON ANTHOCYANIN FORMATION AND GROWTH *

SUGAR	CONC M	PIGMENT UNITS PER GRAM TISSUE	% INCREASE FRESH WEIGHT
Glucose	0.05	915	210
Fructose	0.05	1230	284
Sucrose	0.05	1520	268
Sucrose	0.10	1650	270
Sucrose	0.15	1840	260
Sucrose	0.20	1910	240
None	...	302	0

* Inocula for these experiments were taken from tissue grown in total darkness.

Increases in concentration of sucrose in the medium from 0.05 M to 0.2 M result in slight increases in pigment content. The increase in pigment does not appear to be commensurate with the increase in sugar concentration. While this appears to be true for Spirodela also, Thimann et al (17) have shown that there is a direct relationship between pigment content and reducing sugar content of Spirodela. However, the reducing sugar content of the tissue may not necessarily have a relationship to the sugar concentration in the medium.



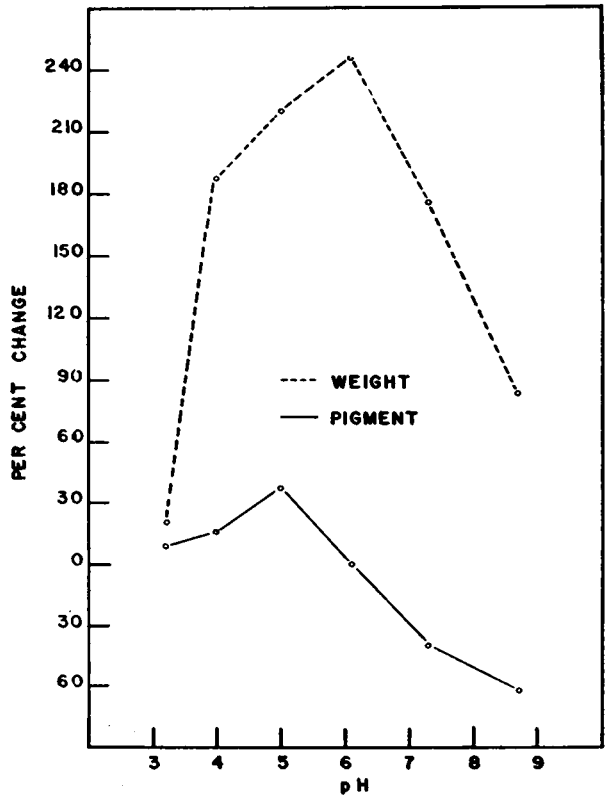


FIG. 1 (*left*). The growth-pigment curve. Pigment concentration changes very little during the growth period.
FIG. 2 (*right*). The effect of pH on pigment production and growth. Alkaline media cause a decided decrease in pigment formation.

Discussion

The effect of pH on pigment content is of considerable interest. With an initial pH of 3.2, growth of the tissue is negligible, yet the pigment content of the tissue has increased somewhat over what one might expect the initial pigment concentration to be (approximately 1500 pigment units per gram of tissue). At pH 8.7 some growth has occurred, yet the pigment concentration is only 570 pigment units per gram. Assuming an initial pigment concentration of 1500 pigment units and further assuming the tissue to have approximately doubled in bulk (87 % increase in wet weight), then the final pigment content of the tissue should be about 750 pigment units assuming no pigment synthesis has occurred. The value of 570 units would certainly indicate that no anthocyanin synthesis has occurred at pH 8.7. Indeed, there may have been some destruction of pigment. Approximately the same observations may be made of the tissue grown at pH 7.3. It would appear then that acid pH values down to at least 3.2 have relatively little effect on pigment production, while alkaline media definitely interfere with pigment synthesis or perhaps accelerate or induce the disappearance of the pigment. It is possible that highly alkaline media interfere with sugar uptake, but the fact that appreciable growth takes place indicates that sugar is entering the tissue. It is more probable that the high alkalinity has caused precipitation of some mineral element in the medium or otherwise restricted the entry of some mineral into the tissue which is needed for anthocyanin synthesis. In view of the work of Edmundson and Thimann (4) which implicated a copper-containing enzyme, this opinion does not seem unreasonable.

It is a general assumption that conditions which lead to the restriction of growth and which permit sugar accumulation are conducive to increased anthocyanin synthesis. The experiment with high pH would seem to indicate that caution should be used in applying such a generalization. Nevertheless, this assumption is borne out by other experiments reported here. If growth of the endosperm is inhibited by lack of sugar, pigment synthesis is very small. On the other hand, if growth is inhibited by low pH or by lack of organic nitrogen (from unpublished data), but sugar is supplied to the cultures, then pigment content reaches and frequently surpasses normal pigment concentration.

The effects obtained with sugars on pigment production are in general agreement with those obtained by most other workers. However, the results obtained with the sugarless medium require some comment.

The data reported in table I were obtained with inocula from tissue grown in the dark. Such tissue has a very low pigment concentration. When this tissue was transferred to a medium devoid of sugar, 302 pigment units per gram of tissue were synthesized in a 25-day experimental period. There are 2 possibilities to be considered as contributing to the pigment synthesis: 1) the tissue may have synthesized the pigment at the expense of stored carbohydrate since these tissues ordinarily store large amounts of starch on normal media, and 2) the concentration of anthocyanin represents pigment synthesized from a precursor formed in the dark but which requires light for the final step(s) in producing anthocyanin. Thus with the possible exhaustion of a precursor and with no carbohydrate available for its further synthesis, pigment production ceased. At any rate, one point which is clearly demonstrated is that light is essential for the synthesis of anthocyanin in these tissues and that the effect of light is not connected with the production of carbohydrates.

Summary

There are at least 3 anthocyanins produced by endosperm tissue cultures derived from 'Black Mexican Sweet Corn'. Two of them have been identified. One is cyanidin-3-monoglucoside (chrysanthemin) and the other is pelargonidin-3-monglucoside (callistephin). The 3rd pigment is present only in trace amounts and was not identified. In general acid media have small promotive effects on pigment synthesis, but alkaline media have a definitely inhibitory effect. It is suggested that the latter is due to the unavailability of copper or other mineral nutrients in alkaline media. Sucrose on a molar basis permits greater pigment synthesis than either glucose or fructose. Increased concentrations of sucrose have rather small effects on increasing pigment production. Light is essential for the synthesis of anthocyanin by these tissues. When grown in the dark, the tissue is virtually colorless. Such tissue shows visible signs of pigmentation after 6 hours exposure to light. It is suggested as one possibility that precursors are produced in the dark which require a photochemical reaction in order to be transformed into anthocyanin.

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AMIDE METABOLISM IN HIGHER PLANTS. III. DISTRIBUTION OF GLUTAMYL TRANSFERASE AND GLUTAMINE SYNTHETASE ACTIVITY [1]

W. D. LOOMIS

DEPARTMENT OF CHEMISTRY, OREGON STATE COLLEGE, CORVALLIS, OREGON [2]

Previous reports (2, 16, 17) described the preparation and properties of a glutamyl transferase from pumpkin seedlings which catalyzes exchange of the amide group of glutamine with either hydroxylamine or isotopic ammonia. The product of the reaction is glutamyl hydroxamic acid or N^{15}-labelled glutamine, respectively. Cofactors for the exchange are manganous ion, adenosine polyphosphate, and either phosphate or arsenate. Similar enzyme preparations have been reported from bacteria, Neurospora, pigeon and mammalian tissues (7, 19, 20), and chick embryos (14). A glutamine synthetase which synthesizes glutamine from glutamic acid and ammonia, or glutamyl hydroxamic acid from glutamic acid and hydroxylamine, with adenosine triphosphate (ATP) as energy donor, has also been reported from various sources, including higher plants (5). Glutamine synthetase preparations from pea seeds have been shown to have transferase activity even after purification of 1000- to 4000-fold (6, 18). Levintow and coworkers (9) subjected a highly purified synthetase preparation from pea seed to ultracentrifugal analysis and showed that both synthetase and transferase activities were associated with a single monodisperse protein component. In preparations from several other sources, including pumpkin seedlings, they found a close association between synthetase and transferase. From this evidence it appears that the synthesis of glutamine and the exchange of the amide group are catalyzed either by a single enzyme or by 2 very closely associated enzymes. In view of the central role of glutamine in plant metabolism, it seemed desirable to learn something of the distribution of these enzymic activities in plants. If glutamyl transferase is of general importance in plant metabolism, or if it is in fact identical with glutamine synthetase, it should be widely distributed among plant species. If, on the other hand, it is found only in certain species, then the distribution might furnish the key to a role in

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