Intermediates in the Folic Acid Biosynthetic Pathway Are Incorporated into Molybdopterin the Yeast, *Pichia canadensis*+

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Bioynthesis of molybdopterin was followed in the yeast, *Pichia canadensis*, using labeled precursors. High performance liquid chromatography analysis of extracts from cells labeled with [U-14C]guanosine showed that the label was incorporated into the molybdopterin oxidation product, dephospho Form A. Dephospho Form A isolated from cells labeled with [U-14C,5'-H]guanosine was devoid of tritium, indicating partial loss of the ribose moiety of guanosine during the synthesis of molybdopterin. *In vivo* labeling of *P. canadensis* using [7-14C]neopterin and [6,7,1-14C]hydroxymethylpterin led to label from both compounds appearing in dephospho Form A as well as in folic acid in wild type cells. When these labeled precursors were incubated with *P. canadensis* mutants blocked in molybdopterin synthesis, only folic acid was labeled. These results suggest a shared pathway in the biosyntheses of molybdopterin and folic acid. [6-14C]Glucose labeling experiments led to exclusive incorporation into the 4'-position of dephospho Form A but not in folic acid. It is proposed that molybdopterin synthesis branches from the folic acid biosynthetic pathway at dihydroxyhydroxymethylpterin and that a 3-carbon phosphorylated compound such as glyceraldehyde 3-phosphate may condense with dihydroxyhydroxymethylpterin to form the 4-carbon side chain precursor to molybdopterin.

The element molybdenum is present in a number of enzymes known as oxidoreductases. It constitutes an essential element for human viability (1). Molybdoenzymes are found in a variety of organisms from bacteria and fungi to higher plants and man (2). A cofactor was first isolated in 1964 from molybdoenzymes by Pateman et al. (3). It was later determined that in all cases except nitrogenase the molybdenum ion is attached to the protein by a pterin moiety known as molybdopterin (4). The cofactor consists of a molybdenum atom attached to a dithiol group on a 4-carbon side chain attached to C-6 of the pterin ring. It functions in the transfer of electrons between substrate and product. The active cofactor is difficult to study because of its extreme lability when removed from the protein. Presence of active cofactor can be determined using a complementation assay with a cofactor mutant of *Neurosora crassa* (5). However, in 1980 Johnson and co-workers (6) were able to isolate a stable, inactive derivative of molybdopterin with strong fluorescence from the purified molybdooenzymes sulfite oxidase and xanthine oxidase. This molecule, termed Form A, was isolated from purified enzyme as well as from whole cells by subjecting the sample to acid hydrolysis. A second derivative, Form B, results from acid hydrolysis without the use of iodine. Similarly, a fluorescent derivative of a precursor molecule was isolated from *E. coli* mutants of *Escherichia coli* which are unable to synthesize active cofactor. The factor responsible for the conversion of the precursor molecule to molybdopterin, termed converting factor, has been isolated and characterized (7-9). Characterization of the precursor molecule and the converting factor have provided insights into the final steps on the biosynthesis of active molybdopterin.

In contrast, very little is known of the initial steps in the biosynthetic pathway of molybdopterin. Guanosine triphosphate (GTP) is the precursor molecule for the pterin ring in the synthesis of folic acid, biopterin, and other known pterinoid compounds. The first step in the biosynthetic pathway is the conversion of GTP to dihydropterin triphosphate via GTP cyclohydrolase I (10, 11). Dihydropterin triphosphate can follow two known pathways at this point, that for the biosynthesis of biopeterin and that for the biosynthesis of folic acid. We explored the possibility that the biosynthesis of molybdopterin shares a part of one of these pathways in eukaryotic cells. The yeast, *Pichia canadensis*, was used for these studies as it is one of a group of organisms able to grow on nitrate as a sole source of nitrogen, and, therefore, synthesizes the molybdoenzyme nitrate reductase. The initial work in this study was done using doubly labeled guanosine with tritium in the 5 position of the ribose ring and 14C throughout. Cells were labeled *in vivo* and dephospho Form A was isolated to determine the fate of both labels. In addition, *P. canadensis* cells were labeled *in vivo* using chemically synthesized [7-14C]neopterin and [6,7,1-14C]hydroxymethylpterin. Dephospho Form A isolated from the cells was assayed for incorporation of label. Although these pterins are biologically active in the dihydro form, *P. canadensis* cells were able to utilize fully oxidized neopterin and hydroxymethylpterin. This indicated the ability of the yeast to reduce those molecules for incorporation into biological products.

**EXPERIMENTAL PROCEDURES**

**Materials**—The following materials were obtained commercially: [U-14C]glyceraldehyde and [1-14C]ribose from American Radiochemicals, Inc., [U-14C]guanosine from Moravek, [9,8,5-3H]adenosine from DuPont-NEN, 2,4,5-triamino-6-hydroxypyrimidine, dihydrofolate reductase, xanthine oxidase, sodium m-periodate, neopterin, hydroxymethylpterin, and alkaline phosphatase from Sigma, and sodium hydroxide.**

**HPLC** solvents from Fisher. HPLC analysis was done using a 255-mm Econosil C18, 5-μm reversed-phase column from Alltech.

**P. canadensis** strains Y11911 5-9A (mating type 5) and Y11912 21-9D (mating type 21) were obtained from Dr. Marjorie Crandall at the UCLA Medical Center.

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*This work was supported by National Institutes of Health Grant GM41003. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 soley to indicate this fact.

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1 The abbreviation used is: HPLC, high performance liquid chromatography.
Isolation of Cofactor Mutants of P. canadensis—Spontaneous mutants for nitrate reductase were selected by their resistance to chlorate. Chlorate is reduced to the toxic chloride by active nitrate reductase and is lethal to the cell (12). Chlorate-resistant colonies were chosen from each mating type. The isolation of cofactor mutants from the chlorate-resistant colonies was done according to Iryby et al. (16). Colonies were selected on the basis of their sensitivity when grown on hypoxanthine media. Cofactor deficiency of selected colonies was confirmed by assays for nitrate reductase partial activities (14). Cofactor-deficient mutants are called cof.

Culture and Labeling of Cells—Yeast cells were grown and stimulated for nitrate reductase activity essentially as in Amry and Rajagopalan (15) with some modifications. One colony of P. canadensis was suspended in 1 ml of minimal media containing 0.5% ammonium sulfate, 2% dextrose, and vitamins, minerals, and trace elements, omitting riboflavin and folic acid. The culture was incubated with agitation overnight. One liter of minimal media was inoculated with this culture and grown for 18 h or until OD₆₀₀ = 0.4–0.7 and the cells were 89% budding. The cells were washed with 1 liter of distilled water and put into 1 liter of nitrate media for 6–7 h. Nitrate media was made with the same components as minimal media but with 1% potassium nitrogen substituted for the ammonium sulfate as the nitrogen source. One liter of nitrate media yielded about 0.2 ml of packed cells. Labeling of cells was performed by the addition of 20 ml of labeled nitrate media to the 2-ml pellets of cells stimulated for nitrate reductase and incubated with shaking at room temperature. Cells were labeled using one of the following compounds: [l-'¹⁴C]-Ribose (5 pCi), 35% aqueous hydrazine (10 ml), or 5 µCi of [U-'¹⁴C]guanosine and various quantities of [5-¹⁴C]neopterin, [2-¹⁴C]glucose, and 10 µCi of [5-¹⁴C]glucose. A sample was removed to determine radioactivity taken up by cells. The entire reaction (approximately 100 µl) was applied to a Silica Gel 60 plate and run with the mobile phase. In the case of labeled samples, unlabeled dephospho compounds used in this study are commercially unavailable. Double-labeled guanosine was prepared by the addition of complete to determine the quantity of radioactivity taken up by cells. The reaction was incubated in the dark at 30°C for 20 min. The tube was centrifuged at 14,000 revolution/min to remove the debris. The supernatant was combined with the previous supernatants. The resulting aqueous solution was brought to pH 6.5 and treated with 50 units of alkaline phosphatase to convert FAD to FMN. The mixture was adjusted to pH 8.5 and treated with 50 units of alkaline phosphatase to convert FMN to riboflavin. The sample was lyophilized and reconstituted in 200 µl of water. All samples were subjected to HPLC analysis, eluting with 10 mM KH₂PO₄, pH 8.5, and 50% ¹⁴C-glucose. A sample was chromatographed with 50 nmol of unlabeled riboflavin as an internal standard. The radioactivity was collected, dried, dissolved in water, and reappied to the HPLC column. Fractions were collected at 30-s intervals and subjected to scintillation counting.

Preparation of Dephospho Form A—Dephospho Form A of molybdenum cofactor was prepared from xanthine oxidase by the method of Hageman and Rajagopalan (20) but without the Sephadex G-25 column. The enzyme was diluted with water to 2 mg/ml and subjected to acid hydrolysis in the presence of iodine. Alkaline phosphatase was added at 50–100 units/10 mg protein and the reaction incubated in the dark at room temperature overnight. The reaction was purified in a QAE-Sephadex column. Fluorescent fractions were pooled and concentrated. This method was also used for isolating dephospho Form A from cells. The fluorescent fractions from the QAE-Sephadex column were pooled and lyophilized. The residue was dissolved in 200 µl of water and analyzed by HPLC using degassed 10% methanol with 10 µM KH₂PO₄, pH 8.5, as the mobile phase. In the case of labeled samples, unlabeled dephospho Form A extracted from purified xanthine oxidase was added as an internal standard. The fluorescent peak corresponding to dephospho Form A was collected, lyophilized, and dissolved in 50 µl of water. The sample was reapplied to the HPLC column, and fractions were collected through the peak area to assure that all radioactivity was collected with the fluorescent fraction.

Assay for Pterin Reductase Activity—One colony of yeast was suspended in 50 ml of nitrate media and grown to OD₆₀₀ of 0.9–1.1. The cells were pelleted and kept at 20°C until use. The cells were passed twice through a French pressure cell at 8,000 pounds/square inch in 1 ml of buffer containing 10 mM Tris, 1 mM EDTA, and 100 mM NaCl, pH 8.5, and centrifuged at 14,000 revolutions/min to remove the debris. 30–60 µl of crude cell extract was added to a tube containing 100 nmol of neopterin or hydroxymethylpterin and 250 nmol of NADPH in a total reaction volume of 3 ml. Reduction of the pterin was monitored by observing the decline in fluorescence emission of neopterin at 454 nm, with excitation set at 356 nm. The reaction was also monitored by observing the decline in absorbance at 340 nm. Methylene blue (1 mM) was added to some samples to determine sensitivity of the enzyme to this compound. Results were corrected with those obtained using pure pterinedihydrololate reductase from chicken liver and those from pterin that was chemically reduced with sodium dithionite.

RESULTS

Formation of Labeled Compounds—Many of the labeled compounds used in this study are commercially unavailable. Double-labeled guanosine was prepared and used to determine the fate of the components of the ribose ring with respect to the molybdenopterin molecule. The purification of [5-¹⁴C]guanosine resulted in a product that was more than 98% pure, as assessed by HPLC analysis. The specific activity of [5-¹⁴C]guanosine was calculated to be 739 µCi/µmol, and specific activity of the [U-¹⁴C]guanosine was 473 µCi/µmol. The two labeled pterin compounds, neopterin and hydroxymethylpterin, were synthesized from commercially available compounds and purified by thin layer chromatography and QAE-Sephadex chromatography. Two fluorescent bands appear on the thin layer chromatography plate in the region of the standard, one of which is detected at 260 nm, and the other the 7-isomer as determined by their UV and fluorescence spectra (21, 22). The bands were scraped from the plate and eluted from the silica gel with 0.1 M NH₄OH. The sample that comigrated with the pterin standard also coeluted on HPLC analysis with authentic pterin. Further purification was carried out on QAE-Sephadex columns to remove the unresolved ribose or glyceraldehyde from the neopterin or

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hydroxymethylpterin preparation, respectively. The final yield of neopterin based on the moles of ribose used for the preparation was 14%. The neopterin is 98% pure, based on HPLC analysis. The yield for hydroxymethylpterin was 9%. Both compounds had a specific activity of 50 μCi/μmol.

Guanosine Labeling Studies—In preliminary studies, cells were labeled with [U-14C,5'-3H]guanosine to measure incorporation into riboflavin. The ratio of 2H to 4C in riboflavin isolated from such a labeling reaction was similar to that added to the media for both wild type and cof- cell cultures (Table I), indicating that the entire guanosine molecule was incorporated into riboflavin. This result shows that the entire guanosine molecule is incorporated into GTP and used in P. canadensis, eliminating the possibility of rearrangement of the molecule during transport or intracellular metabolism. Once it was determined that exogenously supplied guanosine was incorporated into biological products in vivo, incorporation of label from guanosine into molybdopterin was investigated. In each experiment, 10% of the labeled cell pellet was removed for isolation of riboflavin; dephospho Form A was isolated from the remaining 90%. Results (Table I) show that while 14C was incorporated into molybdopterin, no 2H was present, indicating that the 5 hydrogen was lost. No label was incorporated into dephospho Form A isolated from cofactor deficient cof- cells (Fig. 1), although appropriate label was incorporated into riboflavin. Confirmation that the labeled peak was identical to dephospho Form A was supported by results which show that label incorporated into dephospho Form A, upon treatment with sodium m-periodate, relocated to coelute with the periodate-treated dephospho Form A fluorescent peak (Fig. 2).

Pterin Labeling Studies—To explore the possibility that the folic acid biosynthesis intermediates neopterin and hydroxymethylpterin are also intermediates in the biosynthesis of molybdopterin, we synthesized both neopterin and hydroxymethylpterin with 14C in the pterin ring. The physiologically active form of these pterins is the dihydro form; consequently, both pterins were reduced with sodium dithionite before labeling. It was found that the crude cell extracts were capable of reducing neopterin and hydroxymethylpterin in both a time- and enzyme concentration-dependent manner. This reduction process required NADPH but was not sensitive to methotrexate (Fig. 3). As a result of this discovery, oxidized pterins were routinely used for labeling cells to reduce stress on the yeast during the labeling period. Yeast cells were found to take up neopterin and hydroxymethylpterin slowly, as only 1% was removed from the media in 45 min. For this reason, labeling periods were 45–60 min in length. Labeling experiments done using both hydroxymethylpterin and neopterin produced three fluorescent-labeled peaks on HPLC analysis, corresponding to dephospho Form A, folic acid, and the starting compound (neopterin or hydroxymethylpterin). Very little label was incorporated into dephospho Form A in cof- cells labeled with neopterin (Fig. 4) or with hydroxymethylpterin (Fig. 5).

Fig. 1. HPLC analysis of [U-14C]guanosine incorporation into dephospho Form A isolated from wild type and cof- cells. Cells from a 1-liter overnight culture were washed, concentrated to 2 ml, and labeled with 5 μCi of [U-14C]guanosine for 30 min at 30 °C. Reactions were stopped by adjusting the pH to 2.5 and adding 0.3 volumes 1 N/24. After boiling the sample for 20 min, the suspension was neutralized with 1 m NaOH and dephospho Form A isolated as described under “Experimental Procedures.” Unlabeled dephospho Form A was added as an internal standard. A, fluorescence profile (fluorescence settings: excitation — 260. emission — 460. B, 14C incorporation. Wild type cells, — — -; cof- cells . . .

DISCUSSION

In our initial experiments on early steps in molybdopterin biosynthesis, the incorporation of labeled guanosine into molybdopterin and riboflavin was compared. Incorporation of la-
bel from guanosine into riboflavin was used as a positive control for three reasons. First, it is known that the ribose ring is incorporated into riboflavin in its entirety (19, 23, 24), allowing a source of comparison of \(^{14}\)C to \(^{3}\)H in a known product. Second, in many cell types, guanosine is broken down into guanine and ribose 1-phosphate by purine nucleoside phosphorylase upon entry into the cell, and the two portions of guanosine may be metabolized separately (25, 26). Third, the incorporation of guanosine into molecules that use GTP as a precursor relies on the existence of a kinase to phosphorylate guanosine upon entry into the cell. Appearance of both \(^{14}\)C and \(^{3}\)H in riboflavin in a similar ratio to that added to the media indicates use of the entire guanosine molecule supplied in the media, satisfying each of these conditions. The approximate 10% increase in the

Two pathways for the formation of pterins exist in many types of living cells, that for the formation of tetrahydrobiopterin and that for the formation of folic acid. These pathways share a common first step in their biosynthesis. Guanosine triphosphate is subjected to hydrolysis by GTP cyclohydrolase I (11, 27), losing C-8 to formic acid, and forming dihydroneopterin triphosphate. At this point the paths branch, one path leading to the production of tetrahydrobiopterin and continuing on to the formation of folic acid, and the other leading to the production of tetrahydrobiopterin. Prokaryotes and yeast cells have only the pathway for the biosynthesis of folic acid. The pathway for the biosynthesis of tetrahydrobiopterin is found mainly in animal cells, although \(^1\)bioppterin has been found in the flagellate protozoan, Astasia longa (28). Both tetrahydrobiopterin and dihydroneopterin are possible intermediates in the biosynthetic pathway of molybdopterin although bioppterin biosynthesis is not known to occur in yeasts. Tetrahydrobiopterin is an end product which retains the hydrogens from 5 position of the original ribose moiety in GTP, although those from the 3 and 4 positions are lost (29, 30). On the other hand, dihydroneopterin is an intermediate in the pathway for the biosynthesis of folic acid, losing both the 5 and 4 carbons from the ribose during an aldolase reaction by which dihydroneopterin is converted to

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dihydrohydroxymethylpterin. These observations led us to examine the biosynthetic pathway of folic acid as a potential shared pathway for molybdopterin. The synthesis of folic acid was used as a positive control, as both dihydroneopterin and dihydrohydroxymethylpterin are known intermediates in its biosynthetic pathway. The incorporation of labeled neopterin into folic acid indicates that the cell is able to transport neopterin and to utilize it. The fact that label from neopterin does not appear in numerous metabolites in the labeled cell extracts, indicates that neopterin is not incorporated in significant amounts into a large number of products. In addition, 95% of the label remaining in the medium after incubation of the cells coeluted with the neopterin peak. Thus, neopterin is not broken down substantially into other metabolites that are then released from the cell. In the case of guanosine, on the other hand, no label remaining in the medium coeluted with the guanosine peak after 10 min of incubation with cells. A time course of labeling for neopterin incorporation into folic acid and dephospho Form A was also conducted (data not shown). Label was incorporated over an interval of 40 min into both folic acid and molybdopterin to approximately the same degree. However, at later times, label in folic acid remained constant while that in molybdopterin decreased. There are a number of possible explanations for this observation. Molybdopterin may be less stable than folic acid and, as cells stop growing, molybdopterin levels may decrease more rapidly than do folic acid levels. Alternatively, the rate of synthesis of molybdopterin may decline as cells reach stationery phase, so as molecules turn over they are not replaced.

The next step in the biosynthesis of molybdopterin from dihydrohydroxymethylpterin is unknown. Hydroxymethylpterin has a single carbon side chain, while molybdopterin has a 4-carbon, phosphorylated side chain. We propose the addition of a 3-carbon compound (Fig. 7). One likely candidate is glyceraldehyde 3-phosphate, since it is of the correct length and stereochemistry. To examine the possibility that glyceraldehyde 3-phosphate might be the 3-carbon donor, [6-14C]glucose was used to label cells. Addition of glyceraldehyde 3-phosphate derived from [6-14C]glucose to the side chain of hydroxymethylpterin would produce a 4-carbon phosphorylated side chain with 14C in the 4 position. Our results indicate that [6-14C]glucose can be used to label dephospho Form A, and, furthermore, that label is lost upon treatment with NaIO4. These results are consistent with the idea that glyceraldehyde 3-phosphate supplies the 3-carbon addition to dihydrohydroxymethylpterin in the formation of molybdopterin and provides further support for the finding that dihydrohydroxymethylpterin is a precursor of molybdopterin. Other 3-carbon donors are possible, but glyceraldehyde 3-phosphate is an attractive possibility since a simple aldolase type condensation would produce the desired intermediate. Subsequent oxidation of the molecule and cyclization of the phosphate would yield the molybdopterin precursor molecule which, upon air oxidation, becomes Compound Z (31) (Fig. 7). Evidence for a pterin containing a 4-carbon side chain at C-6 exists in Rhodopseudomonas sphaeroides. Seo et al. (32) isolated both phosphorylated and non-phosphorylated C-6-substituted pterin compounds from this species. The compounds were identified as 6-(3-hydroxy-4-phosphonyloxy-1-butylnyl)pterin and 6-(1,2,3,4-tetrahydroxybutyl)pterin, respectively, and appear to be distinct from Compound Z. The former compound may, therefore, be an intermediate in the biosyn-
Molybdopterin Biosynthesis

Fig. 7. Proposed biosynthesis of molybdopterin from dihydrohydroxymethylpterin and glyceraldehyde 3-phosphate.

In higher eukaryotes that do not synthesize folic acid but synthesize tetrahydrobiopterin, regulation of molybdopterin biosynthesis need not include the regulation of tetrahydrobiopterin synthesis. Tetrahydrobiopterin down-regulates its biosynthesis through feedback inhibition. A tetrahydrobiopterin-independent complex forms between GTP cyclohydrolase I and another protein (p35), inhibiting GTP cyclohydrolase I activity (33). The effect, if any, of tetrahydrobiopterin-induced inhibition of cyclohydrolase activity on molybdopterin biosynthesis is not known. Several possibilities exist. First, a small amount of cyclohydrolase activity may remain under conditions of repression by tetrahydrobiopterin, enough to maintain cofactor biosynthetic activity if the regulated step in MPT biosynthesis occurs later in the pathway. Second, the cell may contain sufficient stores of molybdopterin to sustain molybdooenzyme activity during a period of cyclohydrolase inactivity. This theory is supported by studies done by Johnson et al. (34). Data from these experiments showed that in rat liver cells, about 33% of the total cellular content of molybdopterin is located on the outer mitochondrial membrane. In human liver a nearly equal amount of molybdopterin has been detected, although human liver displayed a much lower sulfite oxidase activity than did rat liver. This suggested a greater storage supply of molybdopterin, perhaps not dependent on the synthesis of molybdoenzymes. Therefore, the regulation of molybdopterin biosynthesis in animal cells does not appear to correlate closely with the requirements of the cells for molybdopterin.

The data from this study indicate a shared pathway for molybdopterin and folic acid in P. canadensis. The branch point in the pathway occurs after the formation of dihydrohydroxy-
Animal cells do not synthesize folic acid, but still contain discernable levels of biosynthetic intermediates neopterin and hydroxymethylpterin. The normal breakdown product from tetrahydrofolate is a carboxylic acid rather than an alcohol, making dihydrohydroxymethylpterin an unlikely metabolite. If, however, dihydrohydroxymethylpterin is a breakdown product of tetrahydrofolate, it may serve a specific function. This product could be used for biosynthesis of molybdopterin by a salvage pathway from dietary folic acid. From dihydrohydroxymethylpterin, it may serve a specific function. This argument against the use of dietary folic acid for biosynthesis of molybdopterin in animal cells cultured in a folic acid-deficient environment has been shown to maintain sulfite oxidase activity. As with GTP cyclohydrolase I deficiency, however, a partial deficiency may still provide enough activity for cofactor biosynthesis to occur.

The pathway proposed here for the biosynthesis of molybdopterin in animal cells remains consistent with the presentation of cofactor deficiency in human patients. The cofactor deficiencies documented fall into two complementation groups. Extract from both sets of deficient cells produces active cofactor. A multistep unique pathway for molybdopterin would likely produce more numerous and varied deficiencies in human patients.

All of our data are consistent with the formation of molybdopterin from hydroxymethylpterin and a 3-carbon metabolite. First, from the loss of $^3$H from the ribose moiety of GTP, the tetrahydrobipterin pathway is not implicated. Second, labeling studies in yeast show incorporation of label into folic acid and molybdopterin at a similar rate from the precursors neopterin and hydroxymethylpterin. Thus, a shared pathway for the biosynthesis of molybdopterin has been presented (13). An alternative argument against the use of dietary folic acid for biosynthesis of molybdopterin would likely produce more numerous and varied deficiencies in human patients.

Acknowledgment—The technical advice of Dr. Jean Johnson on the purification of dephospho Form A is gratefully acknowledged.

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