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FLUORESCENCE-BASED QUANTIFICATION OF PEPTIDE ADSORPTION ON TITANIUM DIOXIDE

Iryna V. Prokipchuk*,[1] Ihor M. Mykytyn[1], Dmytro M. Danyliuk[2], Mariana V. Bedrii[1], Mariia Ya. Pidhirna[1]

[1]Department of Chemistry, Vasyl Stefanyk Precarpathian National University, 57 Shevchenko Str., Ivano-Frankivsk, 76018, Ukraine

[2]Department of Biochemistry and Biotechnology, Vasyl Stefanyk Precarpathian National University, 57 Shevchenko Str., Ivano-Frankivsk, 76018, Ukraine

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Abstract

Titanium dioxide is widely used as a white colorant in medicines, cosmetics, and in the food industry due to the absence of taste and smell, chemical stability in combination with excellent ultraviolet protection properties. However, its safety as a food additive was recently questioned by the European Food Safety Authority (EFSA). There is a need to deeper study the interaction of TiO_2 with the human body and biological tissues. Proteins are the main type of biological molecules that can interact with TiO_2 after oral administration of TiO_2-containing drugs. The classical approach to measuring adsorption of organic molecules on TiO_2 is based on spectrophotometry. However, the sensitivity of this method is not sufficient for measurements of the adsorption of peptides and proteins. This problem can be bypassed using peptides covalently labeled with fluorescent organic dyes possessing relatively high molar absorption coefficients and long absorption wavelengths. In this work, we decided to test the applicability of fluorescence-based quantification of peptide adsorption on titanium dioxide. Namely, we used 11 amino acids peptide labeled with fluorescein and quantified its adsorption on TiO_2 at different ionic strengths of the solution. There are several advantages of using the fluorescence method to study the binding of peptides to a sorbent surface. The use of low peptide concentrations and small sample volumes allows for efficient use of resources. Reliable readout, fast measurement time, and cost-effectiveness make this method attractive for the future peptide binding studies and could potentially find application in other areas of peptide research.

Keywords: titanium dioxide; adsorption; fluorescence; labeled peptide.

ФЛУОРЕСЦЕНТНЕ КІЛЬКІСНЕ ВИЗНАЧЕННЯ АДСОРБЦІЇ ПЕПТИДІВ НА ДІОКСИДІ ТИТАНУ

Ірина В. Прокіпчук,[1] Ігор М. Микитин[1], Дмитро М. Данилюк[2], Мар'яна В. Бедрій[1], Марія Я. Підгірна[1]

[1]Кафедра хімії, Прикарпатський національний університет імені Василя Стефаника, вул. Шевченка, 57, м. Івано-Франківськ, 76018, Україна

[2]Кафедра біохімії та біотехнології, Прикарпатський національний університет імені Василя Стефаника, вул. Шевченка, 57, м. Івано-Франківськ, 76018, Україна

Анотація

Діоксид титану широко використовується як білий барвник у ліках, косметиці та харчовій промисловості завдяки відсутності смаку і запаху, хімічній стабільності в поєднанні з відмінним захистом від ультрафіолету. Однак його безпечність як харчової добавки нещодавно була поставлена під сумнів Європейським агентством з безпеки харчових продуктів (EFSA). У цій суперечливій ситуації виникає необхідність глибшого вивчення взаємодії TiO_2 з організмом людини та біологічними тканинами. Білки є основним типом біологічних молекул, які можуть взаємодіяти з TiO_2 після перорального прийому препаратів, що містять TiO_2. Класичний підхід до вимірювання адсорбції органічних молекул на TiO_2 базується на спектрофотометрії. Однак чутливість цього методу недостатня для вимірювання адсорбції пептидів та білків. Цю проблему можна обійти, використовуючи пептиди, ковалентно мічені флуоресцентними органічними барвниками, що мають відносно високі молярні коефіцієнти поглинання і велику довжину хвилі поглинання. У цій роботі було вирішено перевірити застосовність флуоресцентного кількісного визначення адсорбції пептидів на діоксиді титану. А саме, було використано 11-амінокислотний пептид, мічений флуоресцеїном, і кількісно визначено його адсорбцію на TiO_2 за різної іонної сили розчину. Як результат, використання флуоресцентного методу для вивчення зв'язування пептидів з поверхнею сорбенту має кілька переваг. Використання низьких концентрацій пептидів і малих об'ємів зразків дозволяє ефективно використовувати ресурси. Надійність зчитування, малий час вимірювання та економічність роблять цей метод привабливим для майбутніх досліджень зв'язування пептидів і потенційно можуть знайти застосування в інших сферах дослідження пептидів.

Ключові слова: діоксид титану; адсорбція; флуоресценція; мічений пептид.

**Corresponding author: e-mail: iryna.prokipchuk@pnu.edu.ua*

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Introduction

Titanium dioxide (TiO_2) is extensively used as a white colorant in medicines, in the food industry, in sunscreens and cosmetics. It acts as an ultraviolet (UV) absorbent and has excellent UV-resistant qualities and chemical stability. However, its safety was recently questioned by the European Food Safety Authority (EFSA) which recommended not to use it as a pigment food additive due to possible cancerogenic influence [1]. Meanwhile, TiO_2 is a component of thousands of drugs where it is used to protect acting substances from degradation under exposure to sunlight and to improve visual acceptability by patients due to its white color. Taking into account that its replacement is costly and cannot be done fast, authorities did not recommend removing it from usage [2]. In this controversial situation there is a need to deeper study the interaction of TiO_2 with the human body and biological tissues. Proteins are the main type of biological molecules that can interact with TiO_2 in the human body after oral administration of TiO_2-containing drugs.

The mechanism of protein and peptide adhesion on the TiO_2 surface is poorly understood but it depends mainly on the ionic forces and hydrogen bonding. Therefore, the conditions under which peptide adsorption experiments are performed strongly influence the adhesive behavior of biomolecules. Properties of the media such as pH, ionic strength, and temperature affect the net charge and conformation of peptides that influences the kinetics and equilibrium of the adhesion [3]. In addition, adhesion is influenced by surface properties.

The point of zero charge for titanium dioxide in water is 5.35 [4]. Therefore, under physiological conditions (pH ≈ 7.4) its surface is negatively charged and the interaction with cationic peptides is stronger. Most of the proteins present in tissues and blood are quite large (10–40 kDa) and bear relatively low net charges, like 1–2 per every 10 amino acids of the sequence [5]. The density of charges on the protein or peptide surface is low compared to one of the small organic molecules that results in weaker electrostatic interactions and, consequently, lower adhesion [6].

Typically, the sorption of organic compounds onto TiO_2 is measured using a spectrophotometric method [7]. It is applicable to compounds with distinct light absorption properties like dyes Congo red [8], Methylene blue [9]. Such compounds efficiently absorb visible light showing high extinction coefficients, for example, 74 000 M^{-1} cm^{-1} at 665 nm for Methylene blue, and can be efficiently measured in the concentration range 5 to 100 mg/L that makes them very suitable models for quantification of sorption on TiO_2.

However, in the case of peptides, the sensitivity of the spectrophotometric method can be not sufficient. Peptides absorb light only in the range 220–280 nm that creates a serious problem with light scattering in the presence even of traces of TiO_2 and require special instrumentation capable for work with UV light. Moreover, absorbance at 280 nm is due only to one amino acid, tryptophan that is quite rare and characterized by a relatively low molar absorption coefficient (~5900 M^{-1} cm^{-1}) that makes the concentration determination much less precise than in the case of organic dyes like Congo red. This problem can be bypassed using peptides covalently labeled by organic dyes possessing relatively high molar absorption coefficients and long absorption wavelengths. However, since molecules of peptides are about ten-fold larger than organic dyes, attachment of one organic dye to peptide would still be not sufficient to achieve the same precision of concentration detection using the spectrophotometric method as in the case of organic dye molecules.

Therefore, we decided to use a more sensitive detection method, fluorescence, and rely on peptides covalently modified with fluorescent dyes.

To develop and validate the methodology of fluorescence-based quantification of peptide sorption on TiO_2 we decided to prepare a model peptide covalently labeled with a fluorescent dye Fluorescein and study its adsorption. We observed good sensitivity of the new detection method that allowed us to quantify the binding of the protein to the TiO_2 surface and evaluate the degree of the surface coverage under physiological conditions.

Experimental

Preparation of sorbent. Samples of TiO_2 were prepared using the liquid-phase approach starting from the chloride-acid solution of the titanium tetrachloride [10]. Concentrated hydrochloric acid (1.17 g/mL) cooled to 0 °C was added in small portions to titanium tetrachloride $TiCl_4$ (1.4 : 1 by weight). The temperature of the reaction mixture did not exceed 25 °C. The density of the resulting precursor solution was 1.515 g/mL (at 20 °C). 50 mL of the precursor

solution was poured into a 800 mL glass beaker and 250 mL of distilled water was added. Then, the beaker was heated to a boiling (112 °C) for 5 hours. During boiling, as the volume of the reaction mixture decreased by about 10 %, due to the evaporation of water and HCl, distilled water was added to the beaker to compensate for the evaporation. At the final stage of this process, the boiling point of the reaction medium decreased to 106–108 °C. After 20–30 minutes of boiling, the solutions become white due to the formation of TiO_2. The process of boiling was accompanied by intense acoustic cavitation. Gas bubbles that emerge during boiling are destroyed and cause powerful hydraulic shocks directed into the volume of the reaction mixture that intensifies the course of reactions. The synthesized TiO_2 nanoparticles were removed from the reaction medium by vacuum filtration, washed with distilled water, dried for 2 hours at a temperature of 105 °C, and then calcined for 2 hours in an electric furnace at 350 °C. Then it was ground in a porcelain mortar [11].

Preparation of fluorescently labeled peptides. Peptide Fl–GKVVIKIAKIA (11 amino acids, molecular weight 1573 g/mol) was kindly provided by the synthesis facility of the Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences. It was synthesized by solid phase peptide synthesis using standard side-chain protected fluorenylmethoxycarbonyl (Fmoc)-amino acids, HBTU/HOBt coupling protocol and LL-HMP resin as solid support.

Two equivalents of the label (5(6)-carboxyfluorescein) were dissolved in N-methyl-2-pirrolidone (NMP), mixed with two eq. of HBTU/HOBt coupling solution (in DMF) and added to Fmoc-deprotected peptidyl resin. After a few minutes of shaking, five eq. of diisopropilethylamine (DIEA) solution was added. Then, the reaction mixture was stirred overnight at 40 °C. The resin was filtrated and washed with NMP, methanol and dichloromethane.

Cleavage and deprotection of the peptidyl resin were performed for 2 hours using a 10 mL trifluoroacetic acid (TFA) solution containing water (5 %), TIS (iPr_3SiH, 2.5 %), phenol (1 %) and ethanedithiol (2.5 %). The solution was concentrated in vacuo and the peptide was precipitated by using ice-cold diethyl ether and then pelleted by centrifugation. The peptide was solubilized with 0.1 % aqueous TFA and purified by HPLC on a C8 column in water/acetonitrile mixture containing 0.1 % TFA with a linear gradient of 10 to 70 % of acetonitrile for 30 minutes [12].

Peptide purity was checked before adsorption experiments by reverse-phase TLC in a water-acetonitrile 1:1 mixture (Fig. 1a).

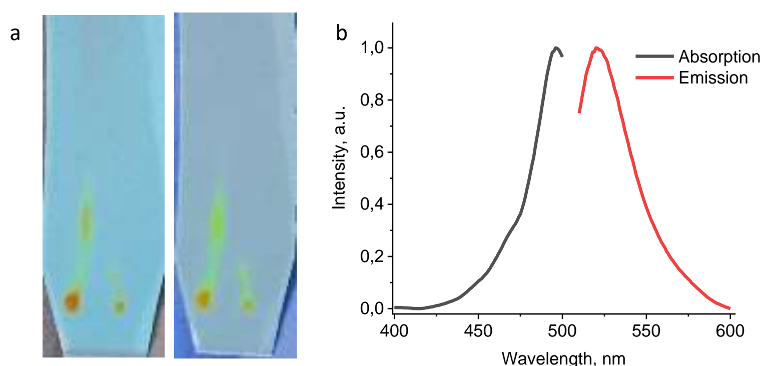

Fig. 1. Peptide characterization: a) RP-TLC visualized under visible and UV light (365 nm); b) Normalized absorption and emission spectra

Measurements of peptide concentration using light absorbance. The concentration of fluorescently labeled peptide was determined based on the light absorption of the label (5(6)-carboxyfluorescein, ε = 76000 M^{-1} cm^{-1}, λ = 480 nm) using a DS-11 FX+ spectrophotometer (DeNovix Inc., Wilmington, DE, USA).

Measurements of fluorescence. Fluorescence intensity was measured on RF-5301 PC spectrofluorophotometer Shimadzu (Shimadzu Co., Kyoto, Japan) using 470 nm excitation (slit 5 nm) and recording emission spectra in the range 500-650 nm (1 nm step, slits 5 nm) (Fig. 1b). The samples with a volume of 500 μL were measured in a 4×10 mm plastic cuvettes. The emission intensity was corrected for the baseline (signal from water in the same cuvette) and, if necessary, for light scattering. The fluorescence intensity used for calculations was read at 530 nm (slightly red-shifted from the emission maximum in order to minimize the influence of the light scattering from the residual TiO_2 particles in the solution). We used a calibration curve obtained for peptide solutions with known concentrations ranging

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from 10 to 100 mg/L) for conversion of the fluorescence intensity to the concentration of the labeled peptide [13].

Adsorption quantification. The adsorption of the peptide was quantified in neutral solutions (10 mM phosphate buffer, pH = 7) containing 0, 150, 300 mM NaCl. Peptide was dissolved in a corresponding buffer to concentration (C_0 = 1, 2, 4, 6, 10, 15, 20, 25, 40, 60 and 100 mg/L). 1 mL of peptide solution was incubated with 5 mg of TiO_2 in microcentrifuge tubes ("Eppendorfs") for 4 hours with periodic shaking every 10 minutes and then for 15 more hours without shaking. After that tubes were centrifuged (10000×g, 10 min) and supernatant solutions containing not adsorbed peptide were moved to empty tubes. Peptide concentrations in those solutions were determined using fluorescence intensity as described below. The fraction of adsorbed peptide (E) and the adsorption capacity (q_e) were calculated with formulas (1) and (2), respectively:

$$E(\%) = \frac{C_0 - C_e}{C_0} \cdot 100\% \quad (1)$$

$$q_e = \frac{(C_0 - C_e) \cdot V}{m} \quad (2)$$

where q_e is adsorption capacity, in mg of peptide per 1 g of dry adsorbent (mg/g); C_0 and C_e are the initial and equilibrium concentration of peptide in the solution (mg/L); V is the volume of the peptide solution (1 mL); m is the mass of the adsorbent (5 mg) [14].

Results and discussion

Design of experiment. To measure adsorption of peptides on TiO_2 we decided to use peptide covalently labeled with organic fluorophore. It will allow to precisely measure the concentrations even in diluted solutions and to minimize the influence of light scattering on the residual amounts of titanium dioxide. As the fluorescent dye we selected 5(6)-carboxyfluorescein, an easily accessible fluorophore that contains a reactive carboxylic group suitable for coupling to the N-terminus of peptides during the solid phase synthesis. Fluorescein is a relatively bright fluorophore (ε = 76000, quantum yield 50–75 %) with green excitation (~480 nm) and emission (~520 nm) that is frequently used for peptide labeling.

For the experiment, we require a water-soluble peptide that show no tendency to self-aggregation. To achieve this we decided to use peptide containing a significant fraction of cationic amino acids and, therefore, bearing a positive charge at neutral pH. Since the charge of peptides strongly depends on the pH we will need to control the pH during the experiment. To minimize the effect of the label on peptide sorption on TiO_2 we need to use a relatively long peptide. We decided to use 11 amino acid peptide that combine relatively large size with sufficient synthetic accessibility. As the labeling of peptide has to be uniform and controllable, we decided to attach the label covalently to the N-terminus of peptide during the solid phase synthesis.

The sequence of peptide was Fl–GKVVIKIAKIA where Fl stands for carboxyfluorescein attached to the N-terminus.

Adsorption isotherms. To quantify the adsorption of peptide on the surface of TiO_2 we incubated the sorbent with peptide solutions of different concentrations and different ionic strengths for 2 hours at room temperature. Then, the solution was separated from the sorbent by centrifugation and the fraction of adsorbed peptide was quantified using the fluorescence intensity of the labeled peptide left in the solution.

We performed adsorption experiments under three conditions that differ by the concentration of NaCl in the solution: 0, 150, and 300 mM. They were chosen to vary the ionic strengths of the solution from the physiological value (~150 mM NaCl) to higher and lower values [15]. In all three cases, we observed a gradual increase in the number of adsorbed peptide on the TiO_2 surface with its increasing concentration in the solution (Fig. 2a).

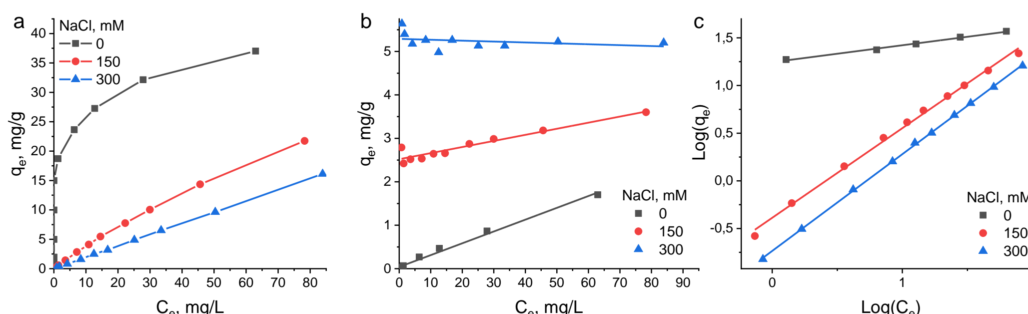

Fig. 2. Linear forms of Langmuir and Freundlich models for adsorption of peptide to TiO_2 in aqueous solutions

The sorption of the peptide on the surface of TiO_2 can be possibly described using Langmuir and Freundlich models (Table 1).

Langmuir adsorption isotherm model assumes reversible sorption-desorption of molecules on independent binding sites and is characterized by two parameters [16]: K_L – Langmuir isotherm constant determining the enthalpy of adsorption [L mg^{-1}] and q_{max} – a maximum mass of a substance that can be adsorbed per unit mass of adsorbent [mg g^{-1}]. According to this model, the mass of adsorbed substance at equilibrium (q_e, [mg g^{-1}]) hyperbolically depends on the equilibrium concentration of a substance in solution (C_e, [mg L^{-1}]):

$$q_e = \frac{q_{max} K_L C_e}{1 + K_L C_e} \quad (3)$$

In the low concentration region, the adsorption of the analyte increases proportionally to the concentration and then reaches a constant value due to the saturation of the active centers of the adsorbent. The constants K_L and q_{max} can be determined from the experimental data either using non-linear fitting or by transforming Equation (3) to a linear form:

$$\frac{1}{q_e} = \frac{1}{q_{max} K_L C_e} + \frac{1}{q_{max}} \quad (4)$$

Adsorption of the tested peptides is satisfactorily described by the Langmuir model as can be seen from the linear dependences of the q_e/C_e on the equilibrium concentration of the peptide in solution (C_e) (Fig. 2b).

We fitted the data to the Langmuir model in order to determine the binding parameters. However, for the 300 mM NaCl samples, the uncertainty of q_{max} value was really high, most likely because of relatively low affinity that did not allow to reach the saturation at a reasonable concentration range. It is logically to assume that the difference in solution ionic strength would affect the affinity but not the maximal capacity. Therefore, we repeated the fit to the Langmuir model assuming that q_{max} for all samples is the same and is equal to the value obtained for the 0 mM NaCl sample where the saturation was the most pronounced (Fig. 2a). Such fit provided more reliable data and narrower confidence intervals.

The q_{max} value obtained for the peptide was about 50 % higher than one for the small organic molecule Congo red studied under similar conditions [17]. It can be explained by the relatively large size of the peptide that can cover TiO_2 forming a thicker layer than smaller molecule of the dye. The value of Langmuir isotherm constant K_L reflecting the binding enthalpy for studied peptide is about 3-fold larger than for Congo red. Very likely, it is a result of the formation of multiple hydrogen bond with TiO_2 surface by a single peptide molecule that is completely in line with the presence of multiple polar groups (amide, amino groups of lysine side chains) in the peptide molecule.

The presence of multiple polar groups in peptide molecule can also result in the interaction between peptide molecules on the sorbent surface that would result in cooperativity of binding. To test this hypothesis, we decided to use the Freundlich model that account for such type of interactions.

Table 1

Isotherm parameters of Langmuir and Freundlich models for adsorption of peptides to TiO_2 in aqueous solutions

Model parameters	Sample		
	0 NaCl/TiO_2	150 NaCl/TiO_2	300 NaCl/TiO_2
Langmuir isotherm			
q_{max}, mg/g	36.7±1.2	71.3±7.8	483±530
K_L, L/mg	0.68±0.34	0.0055±0.0007	0.00039±0.00043
R^2	0.992	0.996	0.999
Langmuir isotherm, fixed q_{max}			
q_{max}, mg/g	36.7±1.2	36.7[a]	36.7[a]
K_L, L/mg	0.68±0.34	0.0108±0.0005	0.0051±0.0002
R^2	0.992	0.992	0.992
Freundlich isotherm			
K_F, (mg/g)(L/mg)$^{1/n}$	17.6±0.4	0.41±0.02	0.184±0.003
n	5.62±0.26	1.06±0.02	0.99±0.01
R^2	0.992	0.996	0.999

[a]was fixed to the value obtained for 0 NaCl sample

In the case of the Freundlich isotherm model, we are dealing with physical adsorption, and the equation describing the model is an empirical equation [18]:

$$q_e = K_F C_e^{1/n} \quad (5)$$

where K_F – Freundlich isotherm constant describing the adsorption capacity [(mg/g)(L/mg)$^{1/n}$]; n – Freundlich isotherm

constant describing the adsorption intensity, related to the energy inhomogeneity.

The model assumes that no saturation of the adsorbent surface occurs. The adsorbed particles of the first layer interact with each other and with the particles forming the subsequent layers through Van der Waals or hydrogen bonding interactions. The constants of the Freundlich isotherm can be determined by transforming equation (5) to a linear form by two-sided logarithmization [19]:

$$\log q_e = \log K_F + \frac{1}{n} \log C_e \qquad (6)$$

Our data show good compliance with the Freundlich model resulting in almost straight lines after linearization (Fig. 2c). The fit yields n values of 5.6 for low ionic strength solution conditions (0 NaCl) and of ~1 for solutions with 150 and 300 mM NaCl. Both values are significantly larger than in the case of the small organic dye Congo red (~0.15).

In the Langmuir model, the K_L constant is related to the adsorption energy and the affinity between the peptide and the adsorbent surface. The value of this constant, obtained by linear estimation, is 0.68 L/mg. The higher the value of the K_L constant, the higher the adsorption energy and the higher the affinity. The 1/n constant in the Freundlich model, on the other hand, tells us about the heterogeneity of the surface. The smaller the value of 1/n (closer to 0), the greater the heterogeneity of the sorbent surface [20]. In the case of the present study, the 1/n value obtained by linear estimation was 1 to 5.6 which indicates a relatively low heterogeneity of the adsorbent surface.

Conclusions

We found that the increase of the ionic strength strongly decreases the adsorption of peptides on titanium dioxide. The presence of ions in the solution screens the charges on peptide and TiO_2 surface weakening electrostatic interactions and limiting the adhesion of peptides and proteins to TiO_2 surfaces.

The adhesion of peptide to the TiO_2 surface is relatively weak and comparable to one of small molecules. However, the surface coverage reached under physiological conditions approaches half of the maximal value at high peptide concentrations.

The fluorescence-based method for investigating the peptide binding to sorbent surface allows to work at relatively low concentrations of peptides and low sample volumes. Namely, to measure sorbtion at one condition we used approximatwelly $9.43 \cdot 10^{-5}$ mol of peptide while adsorption-based measurements described in previous works [17] approximately 100-fold larger volumes and larger molar concentrations were used that resulted in consumption of about 0.07 mol of dye per one experimental conditions. We believe that a fluorescence-based approach would be very useful for further studies of peptide adhesion since it combines robust read-out, fast measurement times and cost-efficiency.

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