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Voltammetric and amperometric determination of 2,4-dinitrophenol metabolites

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ABSTRACT

Methods for determination of 2-amino-4-nitrophenol and 4-amino-2-nitrophenol, metabolites of 2,4-dinitrophenol, were developed using differential pulse (DP) voltammetry and HPLC with amperometric and spectrophotometric detection. The applicability of these methods was tested by the determination of the analytes in model samples of urine after preliminary separation by solid-phase extraction. Voltammetry enabled parallel determination of both analytes, but its application in real matrix was severely limited due to the interference of other compounds present in urine. HPLC allowed the determination in real urine matrix down to micromolar concentrations; amperometric detection proved to be more sensitive and selective than the spectrophotometric one.

1. Introduction

2,4-Dinitrophenol (2,4-DNP) is used in chemical industry in the synthesis of dyes, explosives, pesticides and other products [1]. Due to its toxic properties and frequent occurrence, it is considered as one of the priority pollutants by US Environmental Protection Agency [2]. Besides the industrial applications, however, it is also a subject of interest in toxicology. In exposed organism, it uncouples oxidative phosphorylation by increasing the basal proton conductance through mitochondria membrane, which results in the increased metabolic rate, oxygen consumption and body temperature, finally leading to the loss of body fat [3]. For this effect, it used to be applied as a weight-reduction drug for a short time [4], but the adverse effects, particularly skin lesions and eye cataract [5] and also the dosage problems forbid this application [1]. Nevertheless, it is still misused as a diet pill and dietary supplement for body builders. One of the dangers of the uncontrolled 2,4-DNP application is that the cause of death is usually hyperthermia corresponding to the desired high metabolic rate. Besides, the personal tolerance varies greatly and the dosage needs to be set individually [6]. As a result, causes of poisoning appear, some of them fatal [7–9].

2,4-DNP is partially excreted unchanged and partially metabolized. Its main metabolites are 2-amino-4-nitrophenol (2A4NP) and 4-amino-2-nitrophenol (4A2NP), less significant is the presence of glucuronide and sulphate conjugates and of 2,4-diaminophenol [10]. The determination of these compounds in body fluids as markers of dinitrophenol intoxication was previously described using techniques of gas chromatography–mass spectrometry [11] and liquid chromatography–mass spectrometry [10]. Detection limit for the latter technique was 1.0 μmol L^{-1} for both 2A4NP and 4A2NP. Several other works concern to the determination of 2A4NP and 4A2NP in cosmetics, in attempt to control their undesired presence [12–14]; due to the differences in the matrix and expected concentrations, they do not represent a suitable comparison for this paper.

Due to the presence of electrochemically active groups, electrochemical techniques offer an interesting possibility for the determination of aminonitrophenols. Their utilization for the determination of 2A4NP was previously described in connection with the determination in hair dyes, employing glassy carbon electrode as the working electrode for amperometric detection in HPLC [13].

For this work, anodic oxidation was employed using carbon paste electrode based on microbeads of glassy carbon. This electrode material possesses the general advantages of carbon paste electrodes, such as easily renewable surface, wide potential window, and low background current [15], and also exhibits stability in media containing organic solvents [16], which is not the case with other carbon paste electrodes. Carbon paste electrodes, however, are not suitable for the reductive determination due to the interference of the oxygen present in the paste [17].

The original aim of this work was to develop a simple differential pulse voltammetric method for simple and inexpensive

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determination of the mixture of 2A4NP and 4A2NP (main 2,4-DNP metabolites) based on their anodic oxidation at a carbon paste electrode, because we have expected different voltammetric peak potentials of both substances. Even though this assumption was confirmed, it was not possible to use the DPV method for the determination of 2A4NP and 4A2NP in urine because of interference of other matrix components. This interference could not be eliminated even by a preliminary separation of 2A4NP and 4A2NP by solid phase extraction. However, we have found that HPLC with amperometric detection at a carbon paste electrode proved to be selective enough for the determination of tested metabolites of 2,4 DNP in urine. Moreover, the possibility of the cathodic DPV determination of tested aminonitrophenols using amalgam-based electrodes is under investigation in our laboratory.

2. Experimental

2.1. Chemicals

2-Amino-4-nitrophenol (2A4NP, CAS Number 99-57-0) and 4-amino-2-nitrophenol (4A2NP, CAS Number 119-34-6) were supplied by Aldrich. The stock solutions ($c = 1$ mmol L^{-1}) were prepared by dissolving the exact amount of the respective substance in methanol and kept at a laboratory temperature. It was proved by UV spectrophotometry that the solutions were stable for at least six months.

Britton–Robinson (B–R) buffers served as supporting electrolytes for voltammetric measurements. The same buffers, diluted 10 times with water, were used as aqueous part of mobile phase for chromatographic measurements. For pH 2, diluted B–R buffer was replaced by 0.01 M phosphate buffer (0.01 mol L^{-1} sodium dihydrogen phosphate adjusted to the desired pH value by concentrated phosphoric acid). All chemicals used for buffer preparation were of analytical grade purity and obtained from Lachema Brno, Czech Republic. Other used chemicals were hydrochloric acid (p.a., Lachema Brno, Czech Republic), acetonitrile (for HPLC, Merck), methanol (for HPLC, Chromservis, Czech Republic) and deionized water (Millipore, USA).

2.2. Apparatus

Voltammetric measurements were carried out using Eco-Tribo-Polarograph, controlled by software Polar Pro 5.1 (both PolaroSensors, Prague, Czech Republic). Differential pulse voltammetry (DPV) was employed with a carbon paste working electrode, a platinum wire auxiliary electrode, and an Ag/AgCl (3 M KCl) reference electrode RAE 113 (Monokrystaly Turnov, Czech Republic), to which all the potential values are referred.

HPLC measurements were performed using high pressure pump Beta 10, injector valve with 20 μL loop, UV/VIS detector Sapphire 800 (all Ecom, Czech Republic) and amperometric detector ADLC 2 (Laboratorní přístroje, Czech Republic) connected in series. LiChroCART 125-4 Purospher STAR RP-18E (5 μm) column (Merck) was used for the separation. The HPLC system was controlled via Clarity 2.3 software (DataApex, Czech Republic). The working carbon paste electrode was adjusted in the overflow vessel against the outlet capillary in "wall-jet" arrangement. Auxiliary electrode and reference electrode were the same as used in voltammetric measurements.

The pH of the solutions was measured with a pH meter Jenway 4330 (Jenway, UK) with a combined glass electrode.

2.3. Procedures

The glassy carbon paste electrode (CPE) was prepared from 100 μL of mineral oil (Fluka) and 250 mg of spherical microparticles of glassy carbon with a diameter from 0.4 to 12 μm (Alfa Aesar, Germany) [16]. Carbon paste was packed in the teflon electrode body with 3 mm inner diameter (geometric area 7.1 mm^2). The surface of the electrode was renewed by pressing ca. 0.1 mm of the paste out of the holder by piston and wiping with wet filtration paper. Renewing of the surface of the electrodes was performed once a day during chromatographic measurements and prior each measurement during voltammetric measurements, unless stated otherwise.

Following parameters were set for the measuring techniques: cyclic voltammograms were recorded at a scan rate of 100 mV s^{-1}, differential pulse voltammograms at pulse width of 100 ms, pulse height of 50 mV and scan rate of 20 mV s^{-1}. HPLC measurements were performed with flow rate of 1 mL min^{-1}. Detection wavelength 300 nm was selected from UV spectra of the analytes, preferring higher wavelengths in order to ensure higher selectivity of the determination.

Height of the peak in DP voltammograms was measured as a distance between the top of the respective peak and a straight line connecting minima before and after the pair of the peaks.

Calibration dependences were evaluated by least squares linear regression method. The quantification limits were calculated as the concentration of the analyte which gave a signal ten times the standard deviation of the lowest evaluable concentration [18].

All measurements were made in triplicate. Concentration 100 μmol L^{-1} was used during optimization measurements, unless stated otherwise.

2.4. Urine samples treatment

Prior the extraction, sample pH was adjusted to pH 4.0 with hydrochloric acid.

Solid phase extraction was performed using poly(styren-divinylbenzene) based solid-phase extraction columns LiChrolut® EN 200 mg/3 mL (Merck). The solid phase was conditioned with 3 mL of acetonitrile, 6 mL of methanol and 2 mL of deionized water, which were allowed to pass through the cartridge without the use of vacuum. After that, 100 mL of spiked urine sample was loaded on the column at the flow rate of approximately 1 mL min^{-1}, the cartridges were washed with 2 mL of deionized water and dried under the vacuum for 1 min. Elution of adsorbed analytes was carried out without the use of vacuum by 2 mL of acetonitrile and 2 mL of methanol containing 1% of glacial acetic acid. Both fractions were mixed and further handled as a single sample. Prior injection to chromatographic system, samples were diluted to contain 33% of water to prevent deformations of chromatographic peaks. For voltammetric determination, 0.5 mL of eluate was added to the mixture containing 3.5 mL of methanol and 1 mL of B–R buffer pH 3.

3. Results and discussion

3.1. Voltammetric determination

Voltammetric measurements are often unable to distinguish between two structurally similar compounds because of the peak overlapping. To find out the optimum peak separation in the case of 2A4NP and 4A2NP, anodic DP voltammograms of both compounds were measured in B–R buffers pH 2–12, containing 10% of methanol (v/v). Both compounds give one well developed and analytically utilizable peak, corresponding to the oxidation of amino group in the molecule; during the stabilization of primarily generated cation radical, further oxidation and deprotonation can lead to imine quinone or coupling with another molecule can occur, giving rise to dimmers or polymers, which might form layer that

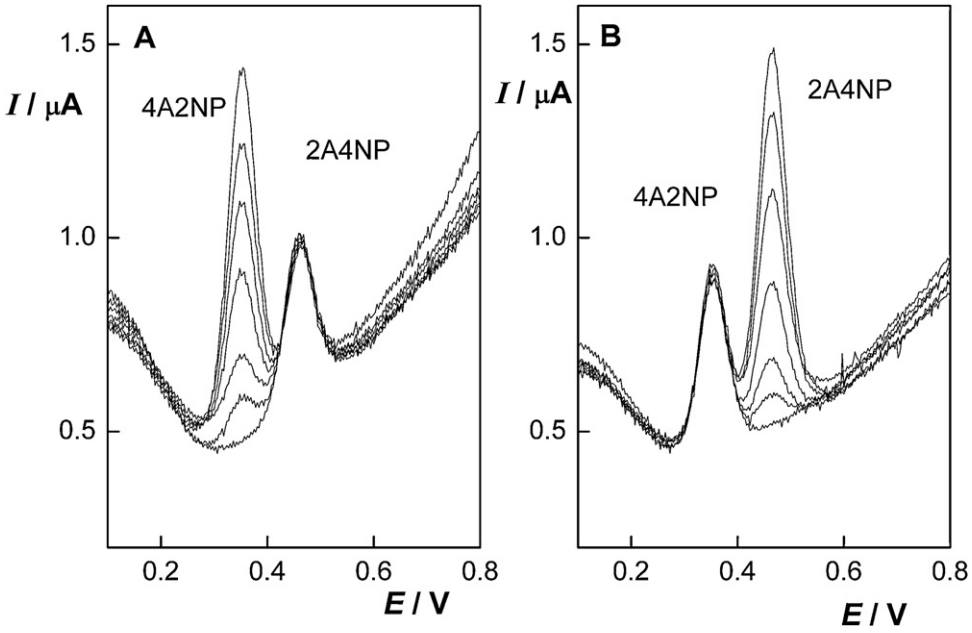

Fig. 1. Voltammograms of mixtures of 2A4NP and 4A2NP (concentrations of analytes 5 μmol L^{-1} of 2A4NP and 0, 1, 2, 4, 6, 8, and 10 μmol L^{-1} of 4A2NP (A) and 5 μmol L^{-1} of 4A2NP and 0, 1, 2, 4, 6, 8, and 10 μmol L^{-1} of 2A4NP (B)). B–R buffer pH 3, 80% of methanol (v/v), pulse width 100 ms, pulse height 50 mV, scan rate 20 mV s^{-1}.

covers the electrode surface. More than one reaction pathways are often observed during this kind of reactions, with preferred products dependent on the substitution of the aromatic ring and the reaction setting [19]. Exact clarification of the mechanism of anodic oxidation of 2A4NP and 4A2NP would require a more detailed study out of the scope of this paper.

The linear potential shift to less negative potential with increasing pH was observed. The distance between the peaks allows their simultaneous evaluation thus enabling simultaneous determination of the two main 2,4-DNP metabolites. With regard to the maximum height of the peaks, B–R buffer pH 3 was chosen as the optimum supporting electrolyte.

Concentration dependences of the response of the respective analyte in the concentration range from 1.0 μmol L^{-1} to 10 μmol L^{-1} in the presence of the other compound at concentration 0; 1.0; 5.0 and 10 μmol L^{-1} were measured to prove independent analytical behavior of both analytes. While the response of 4A2NP did not change with the change of 2A4NP concentration, response of 2A4NP decreased to approximately 50% of the original peak height with increasing concentration of 4A2NP. Together with the shift of 2A4NP peak potential to higher potential values, this effect suggested possible electrode passivation by the first occurring reaction, i.e., the oxidation of 4A2NP. The passivation was successfully suppressed by the measurement in media with higher methanol content, which probably prevent the formation of passivating polymer layer at the electrode surface. The 2A4NP peak height decreased by 20% in media containing 50% of methanol (v/v) and remained stable in media containing 80% of methanol (v/v). The addition of methanol caused also even bigger potential difference between the peaks. Solution consisting of B–R buffer pH 3 and methanol (80%, v/v) was therefore selected as the optimum medium for the determination. The repeatability of the measurements is suitable, with RSD = 4.9% (n = 10). Selected voltammograms obtained under these conditions are shown in Fig. 1.

Obtained concentration dependences are linear, with correlation coefficient between 0.9958 and 0.9998. Parameters of the concentration dependences of the respective compounds measured in mixtures are in good agreement with only very small influence of the concentration of the other analyte, as can be seen in Table 1; parameters of the dependences measured with single compound are slightly different, which can be attributed to the change of the baseline course. Limits of quantification vary between approximately 0.2 μmol L^{-1} and 0.6 μmol L^{-1} for both compounds.

3.2. HPLC determination

Due to their structure, both compounds have relatively low retention in reversed-phase chromatography, which might cause their interference with some substances present in the envisaged real samples. Therefore, the conditions ensuring higher retention and still sufficient resolution of both analytes were sought during

Table 1
Parameters of concentration dependences of 2A4NP and 4A2NP obtained by DP voltammetry in the presence of various concentrations of the other compound.

Analyte (concentration, μmol L^{-1})	Interferent (concentration, μmol L^{-1})	Slope (mA L mol^{-1})	Intercept (nA)	Correlation coefficient	LoQ[a] (μmol L^{-1})
2A4NP (1–10)	–	106.8	−20.0	0.9997	0.22
2A4NP (1–10)	4A2NP (1)	98.9	−49.6	0.9989	0.51
2A4NP (1–10)	4A2NP (5)	96.5	−29.2	0.9981	0.42
2A4NP (1–10)	4A2NP (10)	87.8	−31.9	0.9992	0.25
4A2NP (1–10)	–	93.2	−21.0	0.9998	0.27
4A2NP (1–10)	2A4NP (1)	85.7	−34.6	0.9958	0.53
4A2NP (1–10)	2A4NP (5)	83.9	−18.6	0.9987	0.16
4A2NP (1–10)	2A4NP (10)	85.0	−21.6	0.9995	0.64

[a] Limit of quantification.

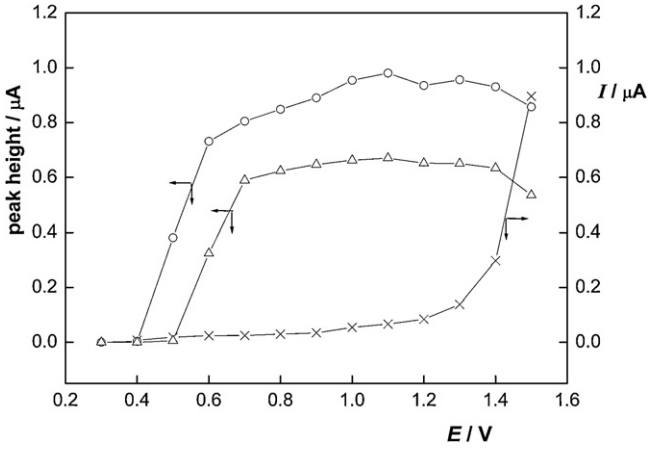

Fig. 2. Hydrodynamic voltammograms of 2A4NP (△), 4A2NP (○), and the background current (×). Column LiChroCART 125-4 Purospher STAR RP-18E (5 μm), mobile phase B–R buffer pH 3 containing 30% (v/v) of methanol, injected 20 μL of the mixture of analytes, $c = 1 \cdot 10^{-5}$ mol L^{-1}.

the optimization of conditions for HPLC determination. Influence of the pH of the aqueous part of the mobile phase in the pH range from 2 to 7 and of the methanol content in the concentration range from 30 to 50% (v/v) was studied. Retention is lower on the both ends of the studied pH range due to the protonization and/or dissociation of the analyte molecules. The requirement for a suitable resolution resulted in selection of pH 3 and methanol content 30% (v/v). Optimum detection potential +0.8 V was selected on the basis of measured hydrodynamic voltammograms (Fig. 2).

The repeatability of the determination was tested by fifteen consecutive injections of the mixture of both analytes in 2-min intervals. RSD of the measurements was found to be 1.1% and 2.2% for 2A4NP and 4A2NP, respectively, using spectrophotometric detection, and 1.3% and 0.7% for 2A4NP and 4A2NP, respectively, using electrochemical detection, which confirms excellent stability and repeatability of the measurements.

Under the optimum conditions, the concentration dependences were measured. Selected parameters of the resulting dependences are summarized in Table 2. The dependences are linear, with correlation coefficients close to one and statistically insignificant intercept. Limits of quantification are lower for the amperometric detection, reaching the values 0.18 μmol L^{-1} and 0.14 μmol L^{-1} for 2A4NP and 4A2NP, respectively. For the spectrophotometric detection, the quantification limits were found to be 0.47 μmol L^{-1} and 0.30 μmol L^{-1} 2A4NP and 4A2NP, respectively.

3.3. Determination in model urine samples

3.3.1. Optimization of the sample preconcentration

Determination in the spiked urine samples was performed in the concentration range, which can be expected in real samples, i.e., in the concentrations lower than 20 μmol L^{-1} [10]. Optimum conditions of the determination of 2A4NP and 4A2NP were used for the determination of both analytes in model urine samples. For the preliminary separation of the analytes, solid-phase extraction (SPE) was employed, using poly(styren-divinylbenzene) based solid-phase extraction columns LiChrolut® EN 200 mg/3 mL (Merck). Due to the high retention of the analytes, particularly 2A4NP, in the solid phase, the combination of two eluting solvents was used, namely acetonitrile and methanol acidified by the addition of 1% glacial acetic acid; the extraction procedure was performed as described in Section 2.2. Under such conditions, recovery of the analytes was 83% and 70% for 2A4NP and 4A2NP, respectively, for 1 μmol L^{-1} analyte concentration in deionized water, and 81% and 96% for 2A4NP and 4A2NP, respectively, for 10 μmol L^{-1} analyte concentration in deionized water.

3.3.2. Voltammetric determination in urine samples

In spite of the preliminary separation with SPE, voltammetric method suffers by high interferences of matrix components and therefore it is not capable to detect 2A4NP in the model urine sample. The presence of 4A2NP can be detected in concentrations over 10 μmol L^{-1}, but the reliable determination is not possible. Therefore, HPLC with amperometric detection on CPE was further investigated.

3.3.3. HPLC determination in urine samples

Chromatographic method enables the determination of the analytes, despite the fact that it does not ensure baseline separation of analytes peaks from other compounds present in the sample. Concentration dependences were measured in the range from 0.5 μmol L^{-1} to 20 μmol L^{-1}; their selected parameters are summarized in Table 2. Although the concentration dependence of 4A2NP is linear, its intercept is markedly negative, probably due to the decrease of analyte recovery with decreasing concentration. Applicable concentration range is therefore limited to the values above approximately 5 μmol L^{-1}. The concentration dependences of 2A4NP are both linear and with statistically insignificant intercept; higher selectivity and sensitivity of the amperometric detection provided the quantification limit of 0.7 μmol L^{-1}, while spectrophotometric detection reached the quantification limit of 1.7 μmol L^{-1}. Chromatograms obtained using both detection techniques are shown and compared in Fig. 3.

Table 2
Parameters of concentration dependences of 2A4NP and 4A2NP, obtained using amperometric and spectrophotometric detection in deionized water and spiked urine samples.

Analyte	LDR[a] (μmol L^{-1})	Slope (AU L mmol^{-1}) or (mA L mol^{-1})	Intercept (mAU or nA)	Correlation coefficient	LoQ[b] (μmol L^{-1})
		Spectrophotometric detection, λ_{DET} = 300 nm			
2A4NP[c]	0.2–100	0.23	−0.15	0.9996	0.47
4A2NP[c]	0.2–100	0.18	−0.13	0.9992	0.30
2A4NP[d]	1–20	0.33	−2.82	0.9625	1.66
4A2NP[d]	5–20	1.5	−6.57	0.9673	6.31
		Amperometric detection, E_{DET} = 0.8 V			
2A4NP[c]	0.1–100	4.59	−1.64	0.9996	0.18
4A2NP[c]	0.1–100	6.09	−1.93	0.9999	0.14
2A4NP[d]	0.5–20	41.9	−18.52	0.9942	0.69
4A2NP[d]	5–20	45.1	−120.47	0.9890	3.68

[a] Linear dynamic range.
[b] Limit of quantification.
[c] Direct determination in MeOH:buffer (1:1, v/v) samples.
[d] In model urine samples after SPE.

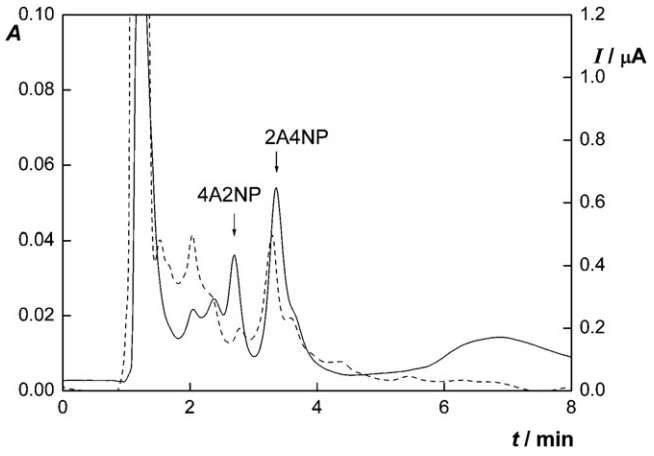

Fig. 3. Chromatograms of urine samples containing 10 μmol L^{-1} of 2A4NP and 4A2NP, obtained using amperometric (full line) and spectrophotometric (dashed line) detection after SPE preconcentration. Column LiChroCART 125-4 Purospher STAR RP-18E (5 μm), mobile phase B–R buffer pH 3 containing 30% (v/v) of methanol, injected volume 20 μL, λ_{DET} = 300 nm, E_{DET} (CPE) = +0.8 V.

4. Conclusions

Several methods for the determination of 2A4NP and 4A2NP, metabolites of 2,4-dinitrophenol, were developed. Applicability of these methods was tested by the determination of studied compounds in model urine samples after preliminary separation by solid-phase extraction. DP voltammetry on CPE allows the parallel determination of both compounds in the media of B–R buffer pH 3, containing 80% (v/v) of methanol. Nevertheless, in model urine samples, interference of the matrix components disables their determination and only detection of 4A2NP is possible. HPLC employed LiChroCART 125-4 Purospher STAR RP-18E (5 μm) column as the stationary phase and B–R buffer pH 3 containing 30% (v/v) of methanol as the mobile phase. UV spectrophotometric detection worked with the wavelength of 300 nm and in series connected amperometric detection on CPE worked with a potential of +0.8 V. These methods were able to determine the studied compounds in model samples in the concentrations, which can be expected in real samples. Electrochemical detection was found to be better than the spectrophotometric one in both selectivity and sensitivity. The best results were obtained in the determination of 2A4NP using amperometric detection, where the quantification limit of 0.7 μmol L^{-1} was reached. These results are comparable to the detection limit of 1.0 μmol L^{-1} obtained earlier by LC–MS method [10] without the necessity to employ such a demanding and expensive technique.

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