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Analytical Chemistry

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Mercury Electrodes as Tools for Voltammetric Determination of Biologically Active Organic Compounds and for Detection of Their Interaction with DNA

Rtuťové elektrody jako nástroje pro voltametrické stanovení biologicky aktivních organických látek a pro detekci jejich interakce s DNA

Dissertation Thesis

Supervisor: doc. RNDr. Vlastimil Vyskočil, Ph.D.

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This dissertation thesis is based on experiments carried-out in the period from 2012 till 2016 at the Charles University in Prague, Faculty of Science, Department of Analytical Chemistry, UNESCO Laboratory of Environmental Electrochemistry.

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I declare that all the results used and published in this dissertation thesis have been obtained by my own experimental work, and that all the ideas taken from work of others are properly referred to in the text and the literature survey. I am conscious that the prospective use of the results, published in this thesis, outside the Charles University in Prague is possible only with a written agreement of the university.

I also declare that neither this thesis nor its significant part has been submitted in any form for another degree or diploma at any university or other institution of tertiary education.

Prague, 30th June 2016 ……..

Mgr. Eva Horáková

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Abstract

The main aim of this work was to use traditional mercury electrodes for the development of voltammetric methods of determination of organic xenobiotics and for the electrochemical study of the interaction between double-stranded deoxyribonucleic acid (DNA) and these compounds.

In relation to my previous research work (conducted in the framework of my diploma thesis), firstly, 4-nitrobiphenyl (4-NBP), the suspected carcinogen, was studied. Interaction of DNA with 4-NBP was studied using differential pulse voltammetry (DPV), cyclic voltammetry (CV), and chronocoulometry at a hanging mercury drop electrode (HMDE), and using CV and alternating current voltammetry at a DNA modified HMDE. Using CV, the reduction mechanism was investigated. The interaction of DNA with 4-aminobiphenyl (4-ABP), a metabolite of 4-NBP, and 4-NBP reduction intermediates was studied. It was found that the interaction of DNA with 4-NBP or 4-ABP results in a formation of a DNA aggregate with these analytes.

The second studied analyte was methyl violet 2B (MV). For determination of MV in a buffered solution were used: direct current tast polarography and differential pulse polarography at a dropping mercury electrode, and direct current voltammetry, DPV, and differential pulse adsorptive stripping voltammetry (DPAdSV) at HMDE. The lowest limit of quantification was reached using DPAdSV at HMDE, i.e., 13 nmol L^{-1}. The developed methods were used for determination of MV in model samples of drinking and river water.

Interaction of DNA with MV in buffered solution was studied using DPV, CV, chronocoulometry, and UV-Vis spectrophotometry. As same as for 4-NBP, the reduction mechanism of MV was studied using CV. It was found that the reduction of DNA with MV results in a formation of a DNA–MV complex. Moreover, based on the decrease of the MV peak current with an increasing concentration of DNA in the measured solution, the method of indirect determination of DNA was developed.

Key Words

Determination, DNA, DNA damage, Interaction, Mercury electrodes, Methyl violet 2B, 4-Nitrobiphenyl, Organic electrochemistry, Voltammetry

Abstrakt

Hlavní cílem této dizertační práce bylo využití pro voltametrickou analýzu tradičních rtuťových elektrod k vývoji elektroanalytických metod stanovení organických xenobiotik a ke studiu jejich interakce s dvouvláknovou deoxyribonukleovou kyselinou (DNA).

V návaznosti na můj předchozí výzkum (vedený v rámci mé diplomové práce) byl prvním zkoumaným analytem 4-nitrobifenyl (4-NBP), který je podezřelý z karcinogenity. Interakce DNA se 4-NBP byla studována diferenční pulzní voltametrií (DPV), cyklickou voltametrií (CV) a chronocoulometrií na visící rtuťové kapkové elektrodě (HMDE), a dále s využitím CV a AC voltametrie (voltametrie se střídavým potenciálem) na DNA modifikované HMDE. Pomocí CV byl studován mechanismus redukce 4-NBP. Dále byla věnována pozornost studiu interakce DNA se 4-aminobifenylem (4-ABP), metabolitem 4-NBP, a s meziprodukty redukce 4-NBP. Bylo zjištěno, že vlivem interakce DNA se 4-NBP a 4-ABP dochází ke vzniku agregátů DNA s těmito analyty.

Druhým studovaným analytem byla methylová violeť 2B (MV). Ke stanovení MV v pufrovaném roztoku byly použity DC tast polarografie a diferenční pulzní polarografie na kapající rtuťové elektrodě (DME) a DC voltametrie, DPV a diferenční pulzní adsorpční rozpouštěcí voltametrie (DPAdSV) na HMDE. Nejnižší mez stanovitelnosti (13 nmol L^{-1}) byla dosažena metodou DPAdSV na HMDE. Vyvinuté metody byly použity ke stanovení MV v modelových vzorcích pitné a říční vody.

Interakce DNA s MV v pufrovaném roztoku byla studovaná pomocí DPV, CV, chronocoulometrie a UV-Vis spektrofotometrie. Stejně jako u 4-NBP, i mechanismus redukce MV byl studován pomocí CV. Bylo zjištěno, že vlivem interakce DNA s MV dochází ke vzniku komplexu DNA–MV. Na základě poklesu proudu píku MV se vzrůstající koncentrací DNA v roztoku byla vyvinuta metoda nepřímého stanovení DNA.

Klíčová slova

DNA, Interakce, Methylová violeť 2B, 4-Nitrobifenyl, Organická elektrochemie, Poškození DNA, Rtuťové elektrody, Stanovení, Voltametrie

List of Symbols and Abbreviations

4-ABP	4-aminobiphenyl
4-NBP	4-nitrobiphenyl
A	adenine
AB	acetate buffer
ACV	alternating current voltammetry
AC	alternating current
BR	Britton-Robinson
c	concentration [mol L^{-1}]
C	cytosine
CAS	chemical abstract services
CV	cyclic voltammetry
D	diffusion coefficient [cm^2 s^{-1}]
DC	direct current
DCTP	direct current tast polarography
DCV	direct current voltammetry
DME	dropping mercury electrode
DNA	deoxyribonucleic acid
DNA–analyte	complex resulting from the interaction of DNA with the analyte
DNA-HMDE	DNA modified hanging mercury drop electrode
DNA–MV	complex resulting from the interaction of DNA with methyl violet 2B
DPAdSV	differential pulse adsorptive stripping voltammetry
DPP	differential pulse polarography
DPV	differential pulse voltammetry
E_p	peak potential [mV]
$E_{p/2}$	half-peak potential [mV]
HMDE	hanging mercury drop electrode
HPLC	high-performance liquid chromatography
IARC	International Agency for Research on Cancer
I_{ac3}	peak 3 current [μA]
I_{CA}	peak CA current [nA]
I_p	peak/wave current [nA]

K	binding constant
k^0	standard rate constant [cm s^{-1}]
L_Q	limit of quantification [mol L^{-1}]
m	binding ratio
MS	mass spectrometric
MV	methyl violet 2B
NAHs	nitrated aromatic hydrocarbons
NPAHs	nitrated polycyclic aromatic hydrocarbons
PB	phosphate buffer
Q	charge [C]
t_{inc}	incubation time [s; min]
t_{int}	interaction time [s; min]
TPM	triphenylmethane
UV-Vis	ultraviolet-visible
v	scan rate [mV s^{-1}]

Contents

1. INTRODUCTION

This dissertation thesis has been submitted as a contribution to a demand for methods of determination of biologically active organic compounds and for the methods of DNA damage detection caused by its interaction with these compounds. It was elaborated under the framework of a long-term research at the UNESCO Laboratory of Environmental Electrochemistry in Prague. In this dissertation thesis, the research follows the diploma thesis Voltammetric Determination of Genotoxic Nitrobiphenyls, and voltammetric behavior and interaction of DNA with another biologically active compound, methyl violet 2B, is investigated.

The presented dissertation thesis is based on the following five scientific publications **[1-5]** which are attached as Appendix parts I – V (Chapters 6 – 10). To distinguish the references referring to these publications in the entire text of the thesis, corresponding numbers in square brackets are in bold.

[1] J. Gajdar, **E. Horakova**, J. Barek, J. Fischer, V. Vyskocil: Recent Applications of Mercury Electrodes for Monitoring of Pesticides: A Critical Review. *Electroanalysis* **28** (2016), DOI: 10.1002/elan.201600239, in press.

[2] V. Vyskočil, M. Blašková, A. Hájková, **E. Horáková**, Z. Krejčova, K. Stávková: *Electrochemical DNA Biosensors – Useful Diagnostic Tools for the Detection of Damage to DNA Caused by Organic Xenobiotics (A Review)*. In Book *Sensing in Electroanalysis*, K. Kalcher, R. Metelka, I. Švancara, K. Vytřas (Eds.), 141-162. University Press Centre, Pardubice 2012.

[3] **E. Horakova**, J. Barek, V. Vyskocil: Voltammetry at a Hanging Mercury Drop Electrode as a Tool for the Study of the Interaction of Double-Stranded DNA with Genotoxic 4-Nitrobiphenyl. *Electroanalysis* **28** (2016), DOI: 10.1002/elan.201600241, in press.

[4] **E. Horakova**, J. Barek, V. Vyskocil: Determination of Methyl Violet 2B Using Polarographic and Voltammetric Methods at Mercury Electrodes. *Analytical Letters* **49**, 56-65 (2016).

[5] **E. Horakova**, V. Vyskocil, J. Barek: Interaction Study of Methyl Violet 2B with DNA and Voltammetric Determination of DNA in Aqueous Solutions. *Monatshefte für Chemie - Chemical Monthly* **147**, 119-126 (2015).

Electroanalytical chemistry represents a very important tool in many different scientific fields. As an example: almost in every chemical laboratory, there is a pH meter; in medical science for screening tests, e.g., glucometers with enzyme electrodes are employed; in a corrosion protection or a jewelry production, the electroplating is used; metal detectors. The greatest advantages of electrochemical analysis are the rapidity and low operating costs.

In this thesis, the research is based on the use of voltammetric techniques at mercury electrodes. Since the polarography was invented by Jaroslav Heyrovský in 1922, wide range of inorganic and organic analytes in various matrices was studied, and a number of different electrode materials was developed. Thanks to the broad knowledge background, the mercury electrodes are still beneficial. For the basic research of the electrochemical behavior and study of the reduction mechanism, mercury still represents the most reliable electrode material. Nowadays, voltammetry is not intended only for the determination of analytes in different samples, but it is also a useful tool for a modern biochemical research and for the detection of the interaction of biomacromolecules with chemical species. It is shown that in the modern research, mercury, the traditional voltammetric material, is still very useful and applicable precisely not only because of its reliability. Moreover, the high affinity of DNA to mercury allows easy preparation of DNA modified electrodes which can be used for the detection of DNA damage caused by its interaction with various analytes.

Presented dissertation thesis is focused on the use of mercury electrodes for the determination of biologically active compounds **[1,4]**, and for the study of the DNA damage caused by the interaction with them **[2,3,5]**. In this thesis, (i) the DNA damage caused by 4-nitrobiphenyl, an environmental pollutant [6], and (ii) the electrochemical behavior of methyl violet 2B, belonging to the group of triphenylmethane dyes inducing many negative effects [7-9], its determination **[4]**, and interaction with DNA **[5]**, are studied in detail.

1.1 References

[**1**] J. Gajdar, **E. Horakova**, J. Barek, J. Fischer, V. Vyskocil: Recent Applications of Mercury Electrodes for Monitoring of Pesticides: A Critical Review. *Electroanalysis* **28** (2016), DOI: 10.1002/elan.201600239, in press.

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2. ANALYTES

2.1 4-Nitrobiphenyl

Under laboratory conditions, 4-nitrobiphenyl (4-NBP, CAS Number 92-93-3) is a white to yellow crystalline solid matter. 4-NBP belongs to the group of nitrated aromatic hydrocarbons (NAHs). Sometimes, it is categorized also to the group of nitrated polycyclic aromatic hydrocarbons (NPAHs), too. Both NAHs and NPAHs are known for their carcinogenic [1-4] and mutagenic effects [5,6]. They are formed by reactions of (polycyclic) aromatic hydrocarbons with atmospheric nitrogen oxides during incomplete combustion processes or in polluted atmosphere [7]. NPAHs occur in ambient [8] and urban [9] air, in fly ash (particular matter-bound NPAHs) [10], diesel exhaust [11], coal combustions [10], and other (reviewed in detail in [12]). The concentration of NAHs/NPAHs in the environment can be determining its pollution.

In living organisms, the genotoxicity of NAHs/NPAHs is induced by forming of free anion-radicals from NAHs/NPAHs by their enzymatic transformation [13]. By the metabolic reduction of the aromatic nitro group, harmful species, e.g., nitroso compounds, hydroxylamines, and amines [14], are formed. These substances can react with the cellular macromolecules. Therefore, the importance of monitoring NAHs and NPAHs in the environment is still increasing [12].

2.1.1 Formation, Sources, Occurrence, and Biological Effects

4-NBP is one of the most volatile NAHs, and in the air, it occurs in the gas phase [15]. As it was mentioned above, the NAHs are formed in the polluted air during incomplete combustion processes, its synthesis is described in [16]. From the air, it can be transferred by rain into natural waters and soil. 4-NBP was found in soil extracts [17], air [18,19], residential honeycomb coal briquette combustion emissions [20], and diesel exhaust particles [21].

According to the International Agency for Research on Cancer (IARC) [22], 4-NBP is listed in group 3 (agents not classifiable as to its carcinogenicity to humans). However, its carcinogenic effect was observed on dogs [23] in which it caused bladder tumors. There are many studies conducting different tissue samples, such as rabbit liver [24], human lung [25], and rat liver fraction [14]. It was found that metabolism of 4-NBP takes place by the cytochrome P450 system.

There are ten reductive metabolites of 4-NBP [26], the major metabolites are 4-aminobiphenyl (4-ABP, CAS Number 92-67-1) and *N*-hydroxy-4-aminobiphenyl, minor metabolites are, e.g., biphenylene and 4-nitrosobiphenyl [14,24]. Carcinogenic effects of 4-ABP on animals and on human are well known as well [27-29]. By IARC, 4-ABP is ranked in group 1 (agents carcinogenic to humans) [30]. *N*-hydroxy-4-aminobiphenyl is suspected carcinogen [25], it is formed by the reduction of 4-NBP to amine by microflora and subsequent hepatic oxidation, by the reduction of 4-NBP in the liver [31], or by the oxidation of 4-ABP. *N*-hydroxy-4-aminobiphenyl can be further activated by O-acylation [32,33] producing electrophilic species (nitrenium ions) which can react with bionucleophiles (e.g., proteins or DNA bases) forming covalent bonds. The DNA adducts can disrupt the replication of DNA and cause mutations [34]. A general overview of the metabolism, DNA damage, and mutagenesis induced by NAHs is figured in Fig. 1 [25], it is expected that 4-NBP undergoes the same metabolic ways.

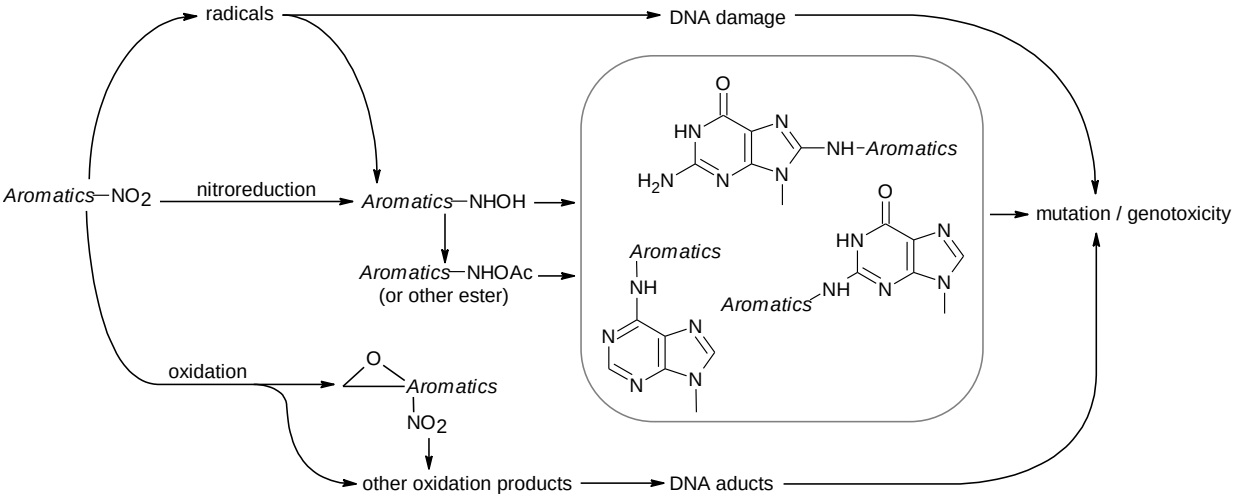

Fig. 1. An overview of the metabolism, DNA damage, and mutagenesis induced by NAHs [25].

2.1.2 Analytical Methods of Determination

For the determination of 4-NBP in the air, chromatographic techniques are very useful and in a routine analysis often used. 4-NBP is volatile, therefore, gas chromatography can be used with mass spectrometric (MS) detection. MS detection with negative chemical ionization was used for determination of PAHs and their derivatives, including 4-NBP, in soil and air particulate matter [35]. MS detection with negative chemical ionization was applied for the detection of NPAHs in diesel exhaust [21]. MS detection with electron ionization or electron-capture ionization was used for determination of PAHs and NPAHs in complex air samples collected using

polyurethane passive air samplers [18]. MS detection with electron capture ionization and negative chemical ionization was used for analysis of NPAHs in urban air particulate matter [6]. For these types of samples, a foregoing extraction (solid phase, Soxhlet, or leaching) and/or sample cleaning are necessary. For 4-NBP, dichloromethane, hexane/dichloroethane, and methanol are suitable solvents.

High-pressure liquid chromatography (HPLC) is often used for the analysis of many different types of matrices. HPLC with normal-phase column with UV detection for analysis of a mixture of biphenyl derivatives is described in [36]. In this work, the reversed-phase and normal adsorption separation were used, too. In [37], the comparison of different detection methods for the reversed-phase HPLC system was made. For the detection of 4-NBP were used these techniques: UV detection, electrochemical detection, and fluorescent detection. In spite of the need to include a foregoing step, the reduction of 4-ABP to 4-NBP (using titanium(III)-based reducing agent), the lowest limit of quantification (L_Q) was achieved using fluorescent detection.

Electrochemical behavior and determination of 4-NBP on mercury electrodes have been already studied [38-40]. The electrochemical reduction of 4-NBP is based on the reduction of nitro group. In dependence on the pH, the mechanism and product of the 4-NBP reduction are different [**41**]. In acidic media, the reduction consists of two steps, and six electrons are exchanged (see Fig. 2A); in neutral media, one step is observed, and four electrons are exchanged (Fig. 2B); and in alkaline media, the mechanism of the reduction is two-step (Fig. 2C), and four electrons are exchanged. There are studies investigating voltammetric behavior and determination of 4-NBP using a mercury meniscus modified silver solid amalgam electrode [42] or a glassy carbon rotating disk electrode [40]. The lowest L_Qs of 4-NBP (at the concentration range of nmol L^{-1}) were obtained at a hanging mercury drop electrode using linear scan adsorptive stripping voltammetry [39] and differential pulse adsorptive stripping voltammetry (DPAdSV) [43].

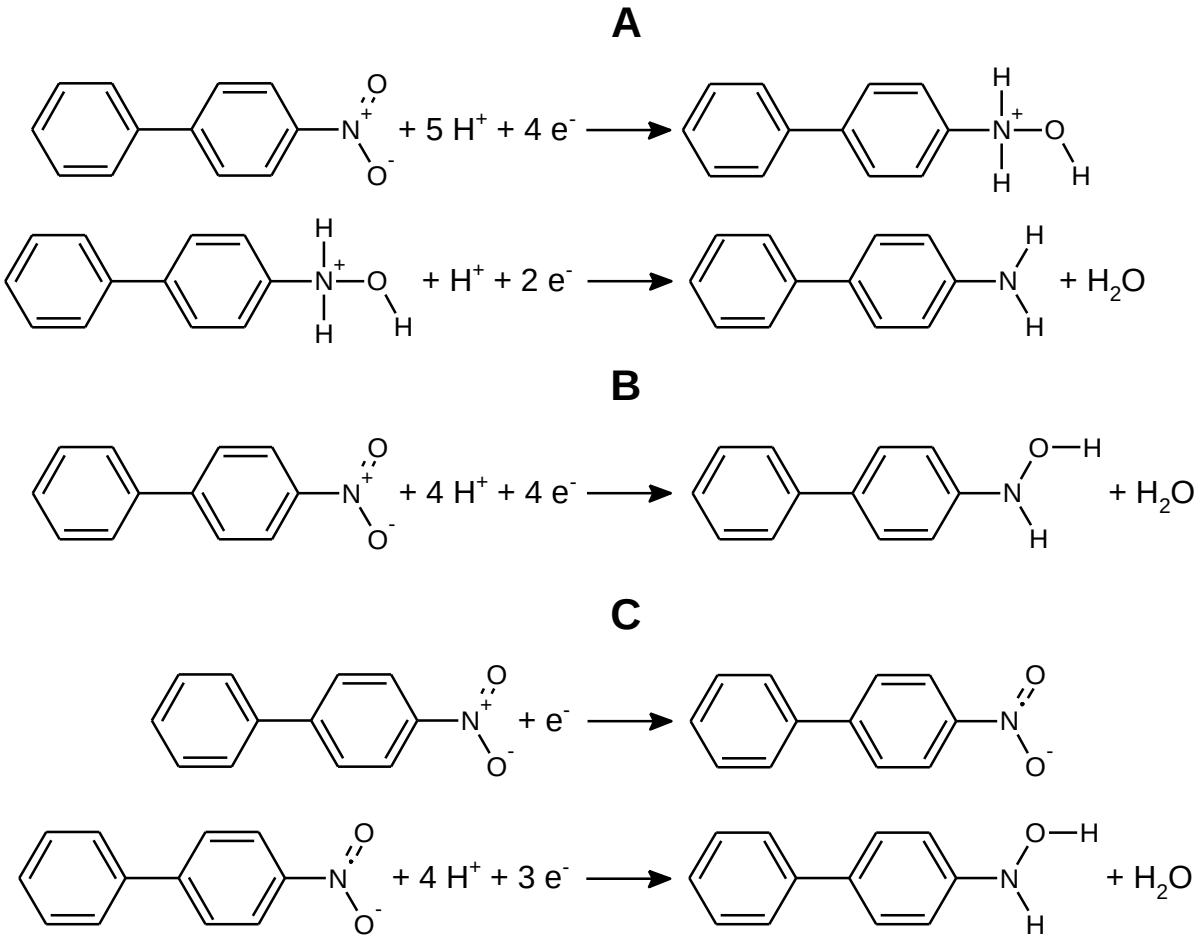

Fig. 2. The mechanism of the reduction of 4-nitrobiphenyl in acidic (A), neutral (B), and alkaline (C) media [**41**].

2.2 Methyl Violet 2B

Under laboratory conditions, methyl violet 2B (MV; CAS Number 8004-87-3; Color Index Number 42535) is dark green crystalline powder. MV belongs to the group of triphenylmethane (TPM) dyes. These organic dyes were used for the vivid depth of their colors, e.g., MV was used for fabrics dying [44,45]. Nowadays, TPM dyes are used in paper dyeing [46], as inks [47,48], as pH indicators [49], and for coloring in food industry [50], pharmacy, and cosmetics. For instance: brilliant blue FCF (E133) is used in the Mtn Dew CODE RED, a beverage produced by PepsiCo Inc., and other beverages [50-52]; erythrosine (E127) is used in the Kaiserkirschen, a fruit compote made by Spreewaldkonserve Golssen GmbH, sweets and beverages [50-52]; green S (E142) is used in sweets and beverages [50-52], and patent blue V (E131) is used for coloring of chewing gums the Airwaves green mint by Wrigley Poland s.p., and in sweets and beverages [50-52].

Because of the bactericidal and fungicidal effects, TPM dyes are used for medical purposes, e.g., brilliant green and crystal violet as local antiseptic agents [53], and in the analysis of Gram's stain [53]. TPM dyes are potentially harmful for humans because of their behavioral toxicity [54], genotoxicity (which is reviewed in detail in [55]), chronic toxicity, mutagenicity, and carcinogenicity [56,57]. They and their metabolites may also accumulate in living organisms [58,59]. Because of these negative effects, the acceptable daily intake of some TPM dyes was established [60], and the use of some TPM dyes is controlled or prohibited [61]. In the European Union, MV is included in the List of Substances Prohibited in Cosmetic Products [62]. Because of the above mentioned effects, studies engaged in the biological effects, and monitoring of their occurrence are required [46,58,63,64], as same as the studies of the treatment of environment and removal of TPM dyes from it [65-67].

2.2.1 Synthesis, Occurrence, and Biological Effects

MV is a synthetic dye, it can be prepared by Friedel-Craft's reaction. The synthesis of MV was firstly described for a mixture of dyes called methyl violet (composing tetra-, penta- and hexamethylated pararosanilines) by Charles Lauth in 1861. Five years later, methyl violet was known as the Violet de Paris, later, the name gentian violet was used, too. MV is formed by the action of a number of oxidizing agents on dimethylaninile [68] (see Fig. 3).

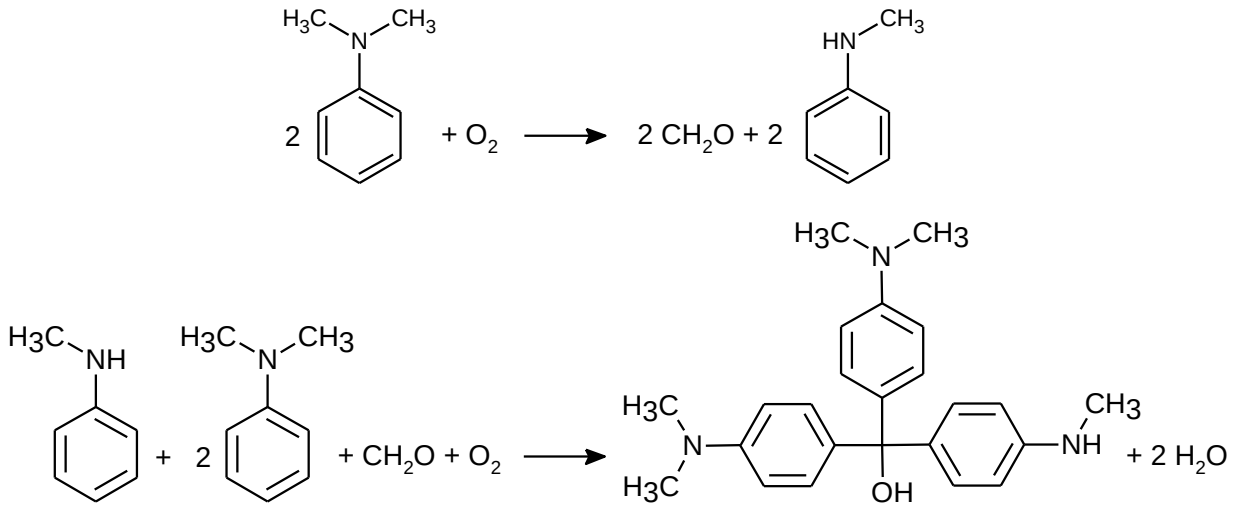

Fig. 3. Scheme of the synthesis of methyl violet 2B.

The interaction of MV with calf thymus double-stranded DNA was studied using spectrometric methods [69], and with fish sperm DNA using voltammetric methods [70]. However, in the work of Sun et al. [70], the experiments were performed in Britton-Robinson buffer at pH 1.5. It is assumed that under these conditions, DNA can be damaged by the effect of the acidic conditions [71,72].

2.2.2 Analytical Methods of Determination

MV can be determined using spectrometric methods. For the detection of TPM dyes used for coloring of a silk yarn, infrared attenuated total reflection or Raman spectroscopy was performed [44].

As it was shown in [44], thin-layer chromatography (following the sample extraction) using the mixture of organic solvents as a solvent system is a useful technique for the separation of the TPM dyes. The separation was finished by the surface enhanced Raman spectroscopy detection. HPLC with diode array detection and with MS detection with electrospray ionization was used for the separation of TPM dyes in historical Chinese textiles [45].

MV is chemically reducible due to the presence of the double bond between nitrogen and carbon atoms (see Fig. 4, the scheme of the reduction of MV) [**41**]. Hence, the investigation of its electrochemical behavior and its determination are possible using electrochemical methods [73,74]. Because of the absence of studies focused on the electrochemical determination of MV, one of the aims of this thesis is the development of sensitive voltammetric methods of determination of MV in deionized, drinking, and river water.

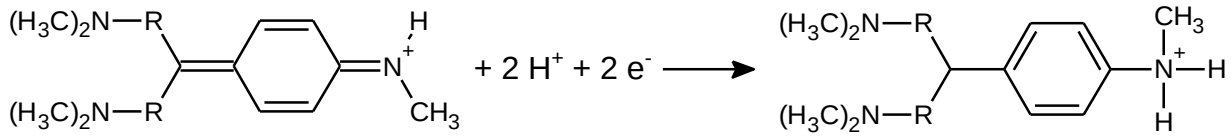

Fig. 4. The mechanism of the reduction of methyl violet 2B [**41**]. *R* represents a phenyl moiety.

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3. MERCURY ELECTRODES AND USED TECHNIQUES

3.1 Working Electrodes

3.1.1 Dropping Mercury Electrode

The main advantages of a dropping mercury electrode (DME) are the inhibited passivation by the products and intermediates of the reduction, the wide potential range, reliability, simplicity, etc. [1,2]. Thanks to these facts, DME is a very useful tool for the basic investigation of electrode reaction, its mechanism, and for the analysis of waste waters, biological fluids, and other matrices containing substances causing the passivation [1,2]. The greatest disadvantages are low robustness and lower selectivity. The usual limits of quatification (L_Q) achieved using differential pulse polarography (DPP) at DME are around 10^{-5} mol L^{-1} [2].

DME was used for the basic polarographic studies of the electrochemical behavior of methyl violet 2B (MV). Determination of MV was performed using direct current tast polarography (DCTP) and DPP. DME was driven by the Eco-Tribo Polarograph controlled by the Polar 5.1 software (both Eco-Trend Plus, Czech Republic). The exact setting and use of DME are given in [**3**], Appx. III.

3.1.2 Hanging Mercury Drop Electrode

A hanging mercury drop electrode (HMDE) is a very useful tool for the environmental analysis [1,**3**,**4**], and for the detection of DNA damage [**5**,6,7,**8**]. There are many advantages of the use of HMDE in comparison to the use of DME, e.g., the reachable L_Qs are lower thanks to the opportunity of the use of adsorptive or electrolytic accumulation (L_Q up to 10^{-10} mol L^{-1}), a consumption of mercury is lower, and it is possible to modify the electrode surface. On the other hand, it can be passivated, and the mechanics and electronics are more complex [2].

HMDE was used for the determination of MV and for the interaction studies of DNA with 4-nitobiphenyl (4-NBP) or MV. For the determination of MV using direct current voltammetry (DCV), differential pulse voltammetry (DPV), and differential pulse adsorptive stripping voltammetry (DPAdSV), HMDE driven by Eco-Tribo Polarograph controlled by the Polar 5.1 software were used. For the interaction studies of 4-NBP or MV with DNA, HMDE driven by

Autolab PGSTAT10 potentiostat/galvanostat connected to a Metrohm 663 VA Stand (Metrohm Autolab, Switzerland) was used. For cyclic voltammetry (CV), DPV, chronocoulometry, and potentiostatic electrolysis, HMDE controlled by the NOVA 1.11 software (Metrohm Autolab, Switzerland) was used; and for CV and alternating current voltammetry (ACV), HMDE controlled by the NOVA 2.0 software (Metrohm Autolab, Switzerland) was used. The exact settings of the parameters and of the use of HMDE are given in corresponding works [**3**,**5**,**8**], Appx. III, IV, and V.

3.2 Polarography and Voltammetry

3.2.1 Voltammetric Techniques

Because of the fact that the molecule is the direct source of analytical signal, the voltammetric techniques are very suitable for analytical chemistry. It is possible to use them for both qualification and quantification of analytes. Thanks to the use of modern digital potentiostats, wide range of voltammetric techniques is available. In analytical chemistry for the determination of analytes, the most frequently used are DCV, DPV, and DPAdSV [**4**,9,10]. As mentioned in Section 3.1.2, the use of adsorptive techniques allows reaching L_Q up to 10^{-10} mol L^{-1}. Another point is the selectivity which is good thanks to the fact that only a percentage of analytes is electrochemically active.

For the purposes of the research on reduction/oxidation mechanisms or DNA damage, these techniques are often employed: CV for the determination of a reversibility of the electrochemical reaction, determination of diffusion coefficient, study of the reduction peak of cytosine and adenine moieties (peak CA); ACV from the determination of the reversibility of the reaction (because flow of an alternating current requires the electrochemical reaction to occur in the forward and reverse directions), study of conformational changes of DNA due to binding of DNA intercalators; and square wave voltammetry which, in comparison to DPV, allows the use of high scan rates and effective suppression of a background current, and for the study of DNA damage investigating the guanine moiety anodic oxidation [**5**,**8**,**11**,12-15].

Nowadays, a wide range of electrode materials is available for the voltammetric analysis, e.g., mercury [**4**,**5**,**8**,16], carbon [17-19], different amalgams [9,20-22], boron-doped diamond [23,24], etc. It is possible to pick up the material in accordance to the appropriate purpose.

3.2.2 Polarographic Techniques

Polarography is a subclass of voltammetry where DME or a static mercury drop electrode is used. The use of mercury has pros (e.g., the wide potential range) and cons (e.g., mercury is oxidized at potentials over ca 0.2 V). DCTP and DPP represent very robust polarographic techniques which are employed in current research and analysis [25,26], reviewed in [**4**], Appx. I. The principle of polarography – using of dropping electrodes, is in the field of voltammetry unsurpassed principle for reliable results. As it was mentioned above in Section 3.1.1 about DME, a dropping of the electrode material prevents the passivation of the electrode surface.

Use of a dislodging valve allows creating mercury drop with the exact lifetime. The possibility to sample the current at a time long enough after the drop has started to emerge from the capillary, the capacitance current is discriminated against to the faradaic current. This is how the tast polarography is utilized [2]. Moreover, use of more advanced pulse waveforms is possible (normal pulse polarography, DPP, square wave polarography, and staircase polarography).

3.3 Electrochemical Detection of DNA Damage

Damage to DNA arises from the interactions of DNA with chemical or physical agents occurring in the environment and may involve a variety of entities [12]. There are several types of consecutive DNA structural changes. Two basic groups can be distinguished, i.e., (i) strand breaks: double (DNA fragmentation) or single, and (ii) damage to DNA bases: release of bases, chemical alterations of bases [27]. Formation of DNA double-strand breaks frequently leads to chromosome aberrations and mitotic faults. Accumulation of mutations and/or other kinds of DNA damage represent carcinogenic or teratogenic risk [12].

Non-covalent DNA interactions with small molecules, e.g., potentially genotoxic agents, can be monitored using electrochemical measurements. Formation of the non-covalent DNA–analyte complexes may lead to the changes of electrochemical signals of either DNA [**8**,**11**,13] or the analytes [**5**,**8**,**11**].

For the investigation of the interactions between DNA and organic compounds and for the DNA damage detection, spectrometric [28,29] and electrochemical [**11**,12,13,30-32] techniques are the most frequently used. Electrochemical techniques represent in these studies very useful tools, and utilization of mercury [**5**,6,**8**,13,33], amalgam [10,34], and carbon [18,19,31] working electrodes is suitable for this purpose. The DNA structural changes reflect in the chemical,

physicochemical, and structural properties of DNA. These changes influence electrochemical behavior of DNA. DNA biosensors are useful tools for the detection of DNA damage. They are constructed for the detection, induction, and control of DNA damage.

3.3.1 Preparation and Use of Biosensor

In general, a biosensor is a device incorporating a biological sensing element either intimately connected to or integrated within a transducer. The usual aim is to produce a digital electronic signal which is proportional to the concentration of a specific chemical or set of chemicals [35]. The transducer transforms the signal resulting from the interaction of the analyte with the biological sensing element into electric signal that is easily measured and quantified. For the DNA biosensor, the transducer (detector element) consists of electrode, usually made of carbon [18,36,37] or mercury [**5**,**8**,38,39]. DNA serves as the biological sensing element.

In comparison to preparation of DNA modified carbon electrode biosensor (which can take several minutes or hours), for the preparation of DNA modified mercury electrode, no additional procedures are required, and the consumption of DNA is lower. For this purpose, HMDE and a mercury film electrode are suitable. In the case of HMDE, the mercury is simply dropped into the DNA stock solution. After flushing with deionized water, the DNA modified HMDE (DNA-HMDE) biosensor is ready to use, see Fig. 5. The exact procedure of the preparation of DNA-HMDE is described in detail in [**8**], Appx. III.

The use of other electrode materials for the preparation of DNA biosensors is described in [**11**], Appx. II.

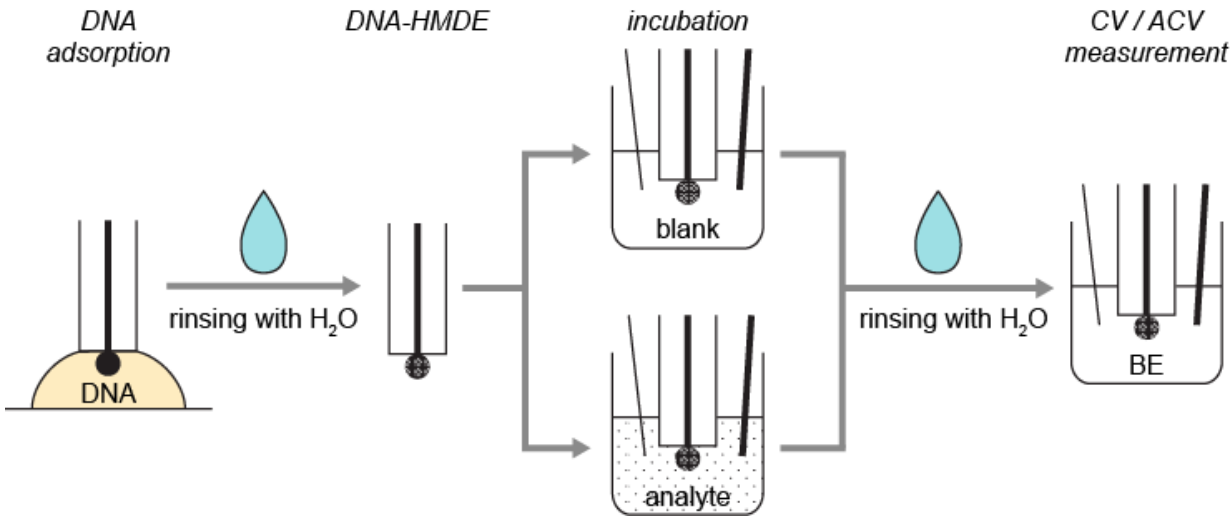

Fig. 5. Scheme of the preparation of DNA modified hanging mercury drop electrode (DNA-HMDE). *BE* stands for a base electrolyte.

3.3.2 DNA Damage Detection Techniques

For DNA damage detection, CV, DPV, square wave voltammetry, and chronopotentiometry are the most commonly used techniques [**11**]. Other electrochemical techniques used are electrochemical impedance spectroscopy [40] and ACV [**8**,13]. The DNA detection techniques can be categorized into the following groups: (i) label-free [**5**,**8**,13]; (ii) using redox indicators non-covalently bound to DNA [30]; and (iii) employing electrochemically active labels covalently bound to DNA. All three groups are described in [**11**], Appx. II.

The use of CV in the label-free mode allows the study of the peak CA at ca −1.45 V in a reduction scan, and of the oxidation of 7,8-dihydroguanine moiety at ca −0.3 V (the reduction product of a guanine moiety transformation) in an anodic scan. An increase of the peak currents (I_p) after incubation of biosensor in the solution of a damaging agent corresponds to the disruption of the double-stranded DNA structure leading to its opening. Causes of the changes of the DNA CV peak currents are explained in detail in [**8**,**11**], Appx. II and III.

Using ACV in the label-free mode, DNA structural changes and DNA damage are studied via the surface changes of DNA-HMDE. In AC voltammograms of DNA, three peaks can be distinguished: peak 1 (ca −1.2 V) caused by the reorientation or desorption of DNA adsorbed via DNA sugar-phosphate backbone, peak 2 (ca −1.3 V) caused by native (double-stranded) DNA form, and peak 3 (at ca 70 mV more negative potential than peak 2) caused by denatured (single-stranded) DNA form. Causes of the changes of the DNA ACV peak currents are explained in detail in [**8**], Appx. III, and in [13].

Using DPV in the label-free mode, the study of the analyte peak is possible, too. If the analyte can serve as a redox indicator and the interaction is non-covalent, it is possible to predict how the analyte interacts with DNA from the peak potential shift [**11**]. If the analyte is reduced and the intercalative mode occurs, the electron density changes in behalf of the easier reduction, so that the analyte peak potential shifts to more positive values. On the other hand, if the analyte is reduced and the electrostatic attraction occurs, the analyte peak potential shifts to more negative values. Moreover, from the change of I_p of the analyte, it is possible to calculate the values of the binding constant and binding ratio [**5**].

3.4 References

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4. RESULTS AND DISCUSSION

4.1 Study of the Interaction of DNA with 4-Nitrobiphenyl

4.1.1 Study of the Interaction of DNA with 4-Nitrobiphenyl in Solution Investigating the 4-Nitrobiphenyl Voltammetric Peak

Experiments were performed in the solution of 4-nitrobiphenyl (4-NBP, 10 μmol L^{-1}) in 0.01 mol L^{-1} phosphate buffer pH 7.0 (PB)–methanol (8:2) in the absence or in the presence of DNA at laboratory temperature. The interaction time (t_{int}) was 5 min. The optimization of the interaction conditions is described in detail in [**1**], Appx. III.

Firstly, the interaction was studied using differential pulse voltammetry (DPV) at a hanging mercury drop electrode (HMDE). The 4-NBP concentration did not affect the trends in changes of the 4-NBP peak current (I_p) or the 4-NBP peak potential (E_p) shift. The presence of DNA in the solution of 4-NBP resulted (i) in a decrease of I_p of 4-NBP; (ii) in a peak of cytosine and adenine moieties (peak CA) formation at ca −1500 mV; and (iii) in a significant shift of E_p of 4-NBP to less negative values at the DNA concentrations over 100 μg mL^{-1}. At the DNA concentrations up to 100 μg mL^{-1}, the shift of E_p was not influenced by the interaction of DNA with 4-NBP. The peak potential shift at the DNA concentrations over 100 μg mL^{-1} is suggesting the formation of a DNA aggregate with 4-NBP and/or an immediate strong sorption of DNA on the electrode surface. It can be concluded that during the interaction of DNA with 4-NBP, the DNA structure is changed. However, a DNA–analyte complex is not formed. Causes of the decrease of I_p for both DNA concentration regions (up to and over 100 μg mL^{-1}) is explained in detail in [**1**], Appx. III.

Secondly, cyclic voltammograms of 4-NBP in the absence and in the presence of DNA (5 and 100 μg mL^{-1}) in PB–methanol (8:2) were measured. At t_{int} from 0 to 30 min in the presence of DNA (5 μg mL^{-1}), the change of I_p of 4-NBP was not significant. On the other hand, the interaction resulted in the peak CA current (I_{CA}) decrease to ca 75% (after 5 min). After 30 min, the decrease was the same. The decrease of I_{CA} can be caused by the DNA aggregation with 4-NBP, leading to the DNA structure changes. The resulting DNA behavior can be affected by the presence of 4-NBP, and vice versa. The observed peaks and corresponding reactions are described in detail in [**1**], Appx. III.

The dependences of the logarithm of the negative value of I_p (log ($-I_p$)) of 4-NBP (10.0 μmol L^{-1}) on the logarithm of the scan rate (log *v*; applied *v* were 5, 10, 20, 50, 100, 200, 500, and 1000 mV s^{-1}) were investigated in the absence and in the presence of DNA (5 and 100 μg mL^{-1}) using cyclic voltammetry (CV). From the slopes of plotting log ($-I_p$) of 4-NBP vs. log *v* (*v* from 20 to 1000 mV s^{-1}), it can be concluded that the electrochemical reduction of 4-NBP in the presence and in the absence of DNA is controlled by both adsorption and diffusion [2].

4.1.2 Determination of Kinetic and Thermodynamic Parameters of the Reduction of 4-Nitrobiphenyl in the Absence and in the Presence of DNA

The solutions of 4-NBP in the absence and in the presence of DNA (5 and 100 μg mL^{-1}) in PB–methanol (8:2) were studied at HMDE.

Values of diffusion coefficients (*D*) were calculated using Anson's equation [3] using data from chronocoulometry. The values of *D* of 4-NBP were calculated as: $(2.3 \pm 0.1) \times 10^{-5}$ cm^2 s^{-1} in the absence of DNA, $(1.9 \pm 0.1) \times 10^{-5}$ cm^2 s^{-1} in the presence of DNA (5 μg mL^{-1}), and $(2.0 \pm 0.1) \times 10^{-5}$ cm^2 s^{-1} in the presence of DNA (100 μg mL^{-1}).

The values of standard rate constant (k^0) were determined from Eq. 1 using the data form CV [4]:

$$k^0 = 1.11 \left[Dv / (E_p - E_{p/2}) \right] \qquad \text{(Eq. 1)}$$

where *D* is the diffusion coefficient, E_p and $E_{p/2}$ are the peak potential and the half-peak potential, respectively, k^0 is the standard rate constant, and *v* is the scan rate (20 mV s^{-1}). The values of k^0 of the reduction of 4-NBP were calculated as: $(1.5 \pm 0.1) \times 10^{-5}$ cm s^{-1} in the absence of DNA, $(1.0 \pm 0.1) \times 10^{-5}$ cm s^{-1} in the presence of DNA (5 μg mL^{-1}), and $(1.3 \pm 0.1) \times 10^{-5}$ cm s^{-1} in the presence of DNA (100 μg mL^{-1}). It is concluded that the reaction is electrochemically irreversible [2].

Neither the value of *D* of 4-NBP nor the value of k^0 of 4-NBP reduction is influenced by the presence of DNA. The DNA–analyte complex is not formed, and the competitive sorption of 4-NBP and DNA on the electrode surface occurs.

4.1.3 Study of the Interaction of DNA with 4-Nitrobiphenyl at the Electrode Surface Using a DNA Modified Hanging Mercury Drop Electrode

The DNA damage was investigated after incubation in the 4-NBP solution using CV and alternating current voltammetry (ACV) investigating I_{AC} and the peak 3 current (I_{ac3}), respectively. The optimal v of CV 200 mV s^{-1} was chosen. ACV measurements were performed in a solution comprising of 0.3 mol L^{-1} NaCl with 0.05 mol L^{-1} NaH_2PO_4 (adjusted to pH 8.5 by titration with 0.2 mol L^{-1} NaOH), with v 20 mV s^{-1}. The optimization of the interaction conditions is described in detail in [**1**], Appx. III.

Firstly, the influence of DNA denaturation processes of double-stranded DNA on I_{CA} measured using CV and the peak 2 and peak 3 currents measured using ACV was studied. The DNA stock solution (1 mg mL^{-1}) used for the HMDE modification underwent different DNA damaging processes [5,6]: (i) a physical by the increased temperature; (ii) a chemical by dimethyl sulfoxide; and (iii) a chemical by NaOH. The influence of the DNA solution used for the HMDE modification described in detail in [**1**], Appx. III. In comparison to other works studying DNA damage, our AC voltammograms are similar to the voltammograms of single-stranded DNA [7]. It is concluded that CV is a more sensitive and reliable tool for the study of DNA damage in both cases – denaturation and degradation.

Using CV with v 200 mV s^{-1} in PB at DNA modified HMDE (DNA-HMDE) after incubation in 4-NBP, it was found that the interaction between DNA and 4-NBP was not affected neither by the 4-NBP (0.5, 1.0, and 5.0 μmol L^{-1}) concentration nor by the interaction time (t_{inc}, from 0.5 to 10 min).

4.1.4 Interaction of DNA with Reduction Intermediates of 4-Nitrobiphenyl

Using CV (from −300 mV to −800 mV or from 0 to −800 mV, both with v 50 mV s^{-1}) or potentiostatic electrolysis (at −800 mV) during the incubation of DNA–HMDE in the solution of 4-NBP in PB (all without bubbling and stirring), the reduction intermediates of 4-NBP were generated. After that, changes of I_{CA} measured using CV or I_{ac3} measured using ACV were investigated. The relative change of I_{CA} was the highest for the measurements following the electrolysis ((228 ± 13)% of I_{CA} of the blank); when cycling from −300 to −800 mV was applied, the relative change of I_{CA} was not significant; and, when cycling from 0 to −800 mV was applied, I_{CA} decreased. At ACV, I_{ac3} decreased after cycling from 0 to −800 mV and after electrolysis, and increased after cycling from −300 to −800 mV. Peak 1 was not influenced by the generation

of the reduction intermediates of 4-NBP. The possible causes of the peak current changes are explained in detail in [**1**], Appx. III.

From the observed data, the interaction between DNA and reduction intermediates of 4-NBP can be confirmed but it is not possible to predict the interaction mechanism and its effect on DNA.

4.1.5 Interaction of DNA with 4-Aminobiphenyl

4-Aminobiphenyl (4-ABP), metabolite of 4-NBP, was chosen to compare its interaction with DNA to the interaction of DNA with 4-NBP. Interaction of DNA with 4-ABP was studied using CV at HMDE and using CV and ACV at DNA-HMDE (all at the same experimental conditions as those used for 4-NBP).

The solution of 4-ABP (10 μmol L^{-1}) with DNA (5 μg mL^{-1}) in PB–methanol (8:2) was investigated using CV at HMDE. I_{CA} decreased to ca 60% (after 5 min). After 30 min, the decrease was the same.

Using CV and ACV at DNA-HMDE with t_{inc} 10 min, the interaction of 4-ABP with DNA was studied in PB, investigating the relative changes of I_{CA} and I_{ac3}, respectively. I_{CA} decreased, the relative change of I_{ac3} was not significant.

Because of the similarity in the 4-NBP and 4-ABP structures, formation of the DNA aggregate with 4-ABP is supposed, too. The competitive sorption in the solution of DNA and 4-ABP on the electrode surface also occurs. The interaction between 4-ABP and DNA was confirmed.

4.1.6 Long-Term Study of the Interaction of DNA with 4-Nitrobiphenyl and 4-Aminobiphenyl Using a Hanging Mercury Drop Electrode

The solutions of 4-NBP (5 μmol L^{-1}) in PB and 4-ABP (5 μmol L^{-1}) in PB, both with DNA (5 μg mL^{-1}), and DNA (5 μg mL^{-1}) in PB (as a blank) were studied using CV (investigating I_{CA}) and ACV (investigating I_{ac3}) at HMDE for 7 days 16 hours. The decrease of I_{CA} was observed immediately after preparation. No significant changes after 19 hours and after 7 days 16 hours were observed, and the DNA damage was not confirmed. As mentioned above, the DNA aggregates with the analyte can be formed and influence the voltammetric behavior of DNA.

4.2 Polarographic and Voltammetric Determination of Methyl Violet 2B

4.2.1 Determination of Methyl Violet 2B Using Direct Current Tast and Differential Pulse Polarography at a Dropping Mercury Electrode

Firstly, the influence of pH on the electrochemical behavior methyl violet 2B (MV) (0.1 mmol L^{-1}) was investigated using direct current tast polarography (DCTP) and differential pulse polarography (DPP) in Britton-Robinson (BR) buffer (pH 2.0 – 12.0). One well-developed wave/peak was observed. In dependence on pH, the reduction involves a two-electron/two-proton transfer (at pH 2.0 – 7.0) or only a one-electron transfer (at pH 8.0 – 12.0) [8]. For pH ≥ 8, a second wave/peak is observed (at ca −1300 mV), corresponding to a reduction of the previously formed semiquinone free radical through a one-electron/two-proton transfer. For analytical purposes, only the first wave/peak was evaluated.

For the determination of MV using DCTP and DPP, the optimum medium BR buffer pH 4.0 was chosen. The current dependences on the MV concentration were linear across the whole calibration range, i.e., from 1 to 100 μmol L^{-1} for DCTP and from 0.1 to 100 μmol L^{-1} for DPP. The obtained limits of quantification (L_Qs) were 1.7 μmol L^{-1} for DCTP and 0.16 μmol L^{-1} for DPP.

4.2.2 Determination of Methyl Violet 2B Using Direct Current, Differential Pulse, and Differential Pulse Adsorptive Stripping Voltammetry at a Hanging Mercury Drop Electrode

For measurements of MV (0.1 mmol L^{-1}) using both direct current voltammetry (DCV) and differential pulse voltammetry (DPV) in BR buffer (at pH 2.0 – 12.0), two peaks were observed. At pH from 6.0 to 12.0, the potential of the first peak was not dependent on the pH value, in accordance with the results described in [8]. For pH ≥ 6.0, the two peaks are merged into one.

The calibration dependences were evaluated from the second peak using both DCV and DPV in BR buffer at pH 4.0. The current dependences on the MV concentration were linear from 0.2 to 10 μmol L^{-1} for DCV and from 0.1 to 10 μmol L^{-1} for DPV. The obtained L_Qs were 65 nmol L^{-1} for DCV and 45 nmol L^{-1} for DPV.

In order to achieve a lower L_Q of MV, differential pulse adsorptive stripping voltammetry (DPAdSV) at HMDE was used, with the optimum accumulation time 600 s at an accumulation potential −500 mV. The optimization of the method is described in [**9**], Appx. IV. The current

dependence on the MV concentration was linear from 20 to 100 nmol L^{-1}. Using DPAdSV at HMDE, the achieved L_Q was 13 nmol L^{-1}.

The voltammetric methods of determination of MV are sensitive tools, with L_Qs comparable to or lower than the value obtained by UV-Vis spectrophotometry, L_Q = 0.5 μmol L^{-1} (measured at the maximum absorbance (λ = 584 nm) of MV in BR buffer pH 4.0).

4.2.3 Determination of Methyl Violet 2B in Water Using Differential Pulse Adsorptive Stripping Voltammetry at a Hanging Mercury Drop Electrode

The developed method of determination of MV using DPAdSV at HMDE was verified on the determination of MV in models samples of drinking and river water. The preparation of model water samples is described in detail in [**9**], Appx. IV. The current dependences on the MV concentration were linear from 0.04 to 1.00 μmol L^{-1} in drinking water and from 0.02 to 1.00 μmol L^{-1} in river water. The achieved L_Qs were 40 nmol L^{-1} in drinking water and 20 nmol L^{-1} in river water.

4.3 Study of the Interaction of DNA with Methyl Violet 2B

4.3.1 Study of the Interaction of DNA with Methyl Violet 2B in Solution Investigating the Methyl Violet 2B Voltammetric Peak

Experiments were performed in the solution of MV (10 μmol L^{-1}) in 0.1 mol L^{-1} acetate buffer pH 4.0 (AB) in the absence and in the presence of DNA at laboratory temperature, and with t_{int} 5 min. The optimization of the interaction conditions is described in detail in [**10**], Appx. V.

Firstly, the interaction was studied using DPV at HMDE. The MV concentration did not affect the trends in changes of I_p of MV or the MV peak potential shift. The presence of DNA in the solution of MV resulted in (i) a decrease of the I_p of MV, and (ii) a shift of E_p to more positive values, reaching the most positive potential at the concentration of DNA 50 μg mL^{-1}, suggesting that the interaction occurs by DNA intercalation or binding into the DNA minor or major groove, followed by the electrostatic interaction which is supported by the low ionic strength [11].

Secondly, cyclic voltammograms of MV in the absence and in the presence of DNA (5 and 100 μg mL^{-1}) in AB were measured. It can be assumed that the reduction of MV, as well as of the DNA–MV complex, is chemically irreversible.

The dependences of the log ($-I_p$) of MV (0.5, 1.0, 5.0, and 10.0 μmol L^{-1}) on log v (applied v were 5, 10, 20, 50, 100, 200, 500, and 1000 mV s^{-1}) were investigated in the absence and

in the presence of DNA (5 and 100 μg mL^{-1}) using CV. From the slopes of plotting log ($-I_p$) of MV vs. log v (v from 20 to 1000 mV s^{-1}), it can be concluded that in the absence of DNA the electrochemical reduction is controlled by both adsorption and diffusion [2]. On the other hand, the values of the slopes in the presence of DNA in the MV solution show the reduction is diffusion controlled, suggesting the formation of the DNA–MV complex formation.

4.3.2 Spectrometric Study of the Interaction of DNA with Methyl Violet 2B in Solution

The UV-Vis spectrophotometric behavior of MV (10 μmol L^{-1}) in AB in the absence and in the presence of DNA (1 – 1000 μg mL^{-1}) was investigated. A shift of the MV spectral band maximum was not significant. However, the hypochromic effect was observed, reaching the absorbance minimum at the DNA concentration 50 μg mL^{-1}. Results and conclusions obtained using UV-Vis spectrophotometry are the same as those obtained using voltammetric methods.

4.3.3 Determination of Kinetic and Thermodynamic Parameters of the Reduction of Methyl Violet 2B and Its Complex with DNA

The solutions of MV in the absence and in the presence of DNA (5 and 100 μg mL^{-1}) in AB were studied at HMDE. Values of D were calculated Anson's equation [3] using data from chronocoulometry. Values of k^0 were determined from Eq. 1 (in Section 4.1.2) using data from CV.

The values of D were calculated as: $(2.3 \pm 0.4) \times 10^{-5}$ cm^2 s^{-1} of the free MV, $(1.4 \pm 0.1) \times 10^{-5}$ cm^2 s^{-1} of the DNA–MV complex with DNA (5 μg mL^{-1}), and $(1.5 \pm 0.4) \times 10^{-5}$ cm^2 s^{-1} for the DNA–MV complex with DNA (100 μg mL^{-1}). The values of k^0 of the reduction were calculated as: $(2.8 \pm 0.4) \times 10^{-5}$ cm s^{-1} for the free MV, $(9.2 \pm 1.0) \times 10^{-5}$ cm s^{-1} in the presence of DNA (5 μg mL^{-1}), and $(9.6 \pm 2.4) \times 10^{-5}$ cm s^{-1} in the presence of DNA (100 μg mL^{-1}). It is concluded that the reaction of both the free from of MV and the DNA–MV complex is electrochemically irreversible [2]. It can be concluded that the decrease of I_p is not caused by the influence on the kinetics.

Under assumption that the decrease of I_p of MV is caused by the decrease of the concentration of free MV, and that the DNA–MV complex is a single complex, the values of the binding constant (K) and the binding ratio (m) of MV and DNA base pairs were evaluated using DPV with the constant concentration of DNA and the changing concentration of MV. The value of K was determined as $(4.6 \pm 0.3) \times 10^4$, and from the value of m, it is estimated that MV and DNA base pairs formed the complex in the ratio 1:1. The value of K was determined using a titration of the solution of MV with the DNA solution, too. The value of K evaluated using this

procedure was $(6.5 \pm 0.1) \times 10^4$. Using UV-Vis spectrophotometry, the value of K was determined as $(6.0 \pm 1.4) \times 10^4$. All equations used for the calculations are in [**10**], Appx. V.

4.3.4 Determination of DNA in Aqueous Solutions Using the Decrease of the Peak Current of Methyl Violet 2B

The decrease of I_p of MV caused by the addition of DNA into the solution was used for the construction of calibration curves of DNA (1 – 1000 $\mu g\ mL^{-1}$) in aqueous solution. The procedure of optimization is described in detail in [10], Appx. V. The optimal concentration of MV was 10 $\mu mol\ L^{-1}$, with L_Q of DNA 0.77 $\mu g\ mL^{-1}$.

4.4 References

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5. CONCLUSION

In this dissertation thesis, it was shown that mercury electrodes represents a very useful and reliable tool for the environmental analysis, interaction studies of double-stranded DNA with organic compounds, and DNA damage investigation. Voltammetry and polarography are suitable for these purposes. For the determination of methyl violet 2B (MV), a triphenylmethane dye, dropping mercury electrode (DME) and hanging mercury drop electrode (HMDE) were used. HMDE was used for the investigation of the interaction between DNA and MV of 4-nitrobiphenyl (4-NBP). Moreover, HMDE was modified by DNA (DNA-HMDE) which served as a biosensor for the study of the interaction between DNA and 4-NBP.

The first studied analyte, 4-NBP, was chosen in order to continue in the research of the diploma thesis which was focused on nitrobiphenyls, their electrochemical determination and behavior in the presence of DNA. Using cyclic voltammetry (CV), differential pulse voltammetry (DPV), and alternating current voltammetry (ACV), the interaction of 4-NBP with DNA was studied at HMDE and at DNA-HMDE. The interaction was investigated in 0.01 mol L^{-1} mol L^{-1} phosphate buffer (PB)–methanol (8:2), studying the 4-NBP peak using cyclic voltammetry (CV) and DPV at HMDE. The interaction resulted in the 4-NBP peak current decrease and the peak of cytosine and adenine moieties (peak CA) decrease. It was found that the electrochemical reduction of 4-NBP is a quasi-reversible process controlled by both adsorption and diffusion in the absence of DNA as well as in the presence of DNA. Using CV in PB and ACV in 0.3 mol L^{-1} NaCl with 0.05 mol L^{-1} NaH_2PO_4 (pH 8.5), the peak CA and peak 3 currents were investigated at DNA-HMDE. After incubation in 4-NBP solution, no significant changes of peak CA or peak 3 were observed. However, the interaction of DNA with reduction intermediates of 4-NBP was confirmed.

Moreover, the interaction of 4-aminobiphenyl (4-ABP), the metabolite of 4-NBP, was studied at the same conditions as 4-NBP. A long-term study of the solutions of DNA with 4-NBP or 4-ABP was performed using CV and ACV, too.

It can be concluded, that the interaction of DNA with 4-NBP or 4-ABP results in a formation of DNA aggregate with the analytes.

The second analyte, MV, was firstly studied using polarographic techniques at DME and voltammetric techniques at HMDE. The optimum medium for its determination was found to be

Britton-Robinson buffer pH 4.0. The lowest limit of quantification (L_Q) 13 nmol L^{-1} was observed using differential pulse adsorptive stripping voltammetry (DPAdSV) at HMDE. Using DPAdSV at HMDE, MV was determined in model samples of drinking and river water. Values of the obtained L_Qs are comparable or lower than the one obtained using UV-Vis spectrophotometry.

Secondly, the interaction of DNA with MV in acetate buffer was studied using CV and DPV at HMDE and UV-Vis spectrophotometry. The interaction of MV with DNA resulted in the formation of a supramolecular complex. The electrochemical reduction of both the free form of MV and the DNA–MV complex is an irreversible process. Using UV-Vis spectrophotometry and results from DPV, the values of the binding constant and the binding ratio of the DNA–MV complex were calculated.

The decrease of the MV peak current caused by the increasing of DNA in the solution was used for the development of a method for the indirect determination of DNA in aqueous solutions, with L_Q 0.77 μg mL^{-1}.

It was shown that the employment of HMDE for the investigation of the interaction of DNA with biologically active compounds is possible using several approaches and is able to confirm different types of interactions. Thus, mercury electrodes still represent reliable and modern tools for the basic in vitro studies.