S elektrodami na biomakromolekuly:

uplatnění elektrochemických metod v moderní biochemii, molekulární biologii a biomedicíně

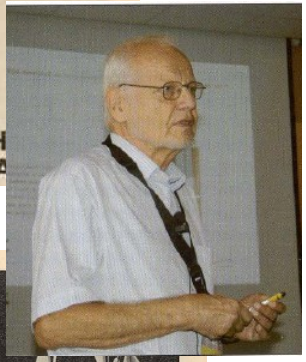
doc. RNDr. Miroslav Fojta, CSc.

MU, 4. května 2010

50-letá výročí

(Reprinted from *Nature*, *Vol.* 188, *No.* 4751, *pp.* 656–657, *November* 19, 1960)

Oscillographic Polarography of H
Polymerized Deoxyribonucleic A



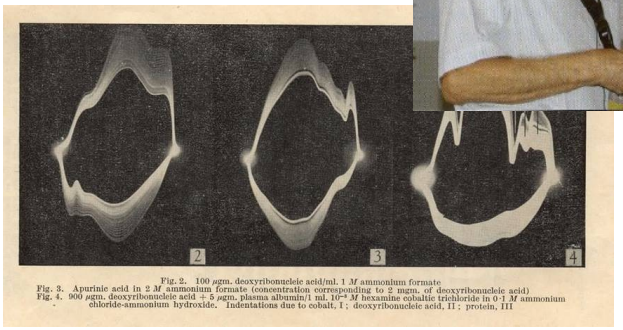
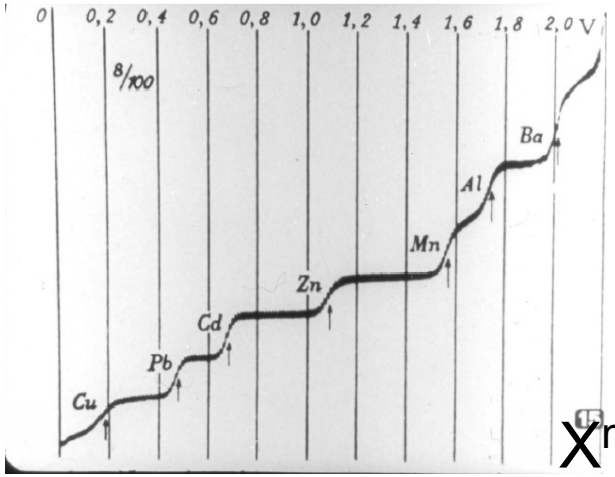
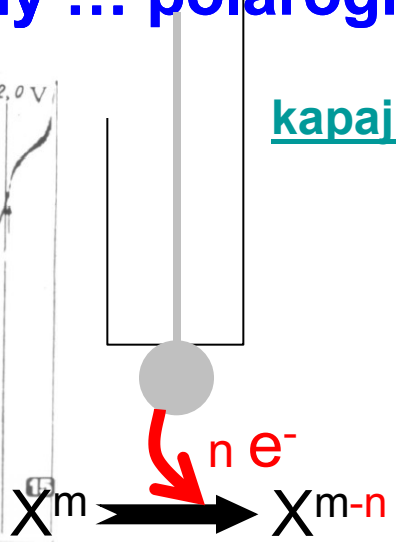


Fig. 2. 100 μgm. deoxyribonucleic acid/ml. 1 *M* ammonium formate.
Fig. 3. Apurinic acid in 2 *M* ammonium formate (concentration corresponding to 2 mgm. of deoxyribonucleic acid)
Fig. 4. 900 μgm. deoxyribonucleic acid + 5 μgm. plasma albumin/1 ml. 10^{-3} *M* hexamine cobaltic trichloride in 0·1 *M* ammonium chloride-ammonium hydroxide. Indentations due to cobalt, I; deoxyribonucleic acid, II; protein, III

Elektrochemické metody … polarografie



kapající rtuťová elektroda



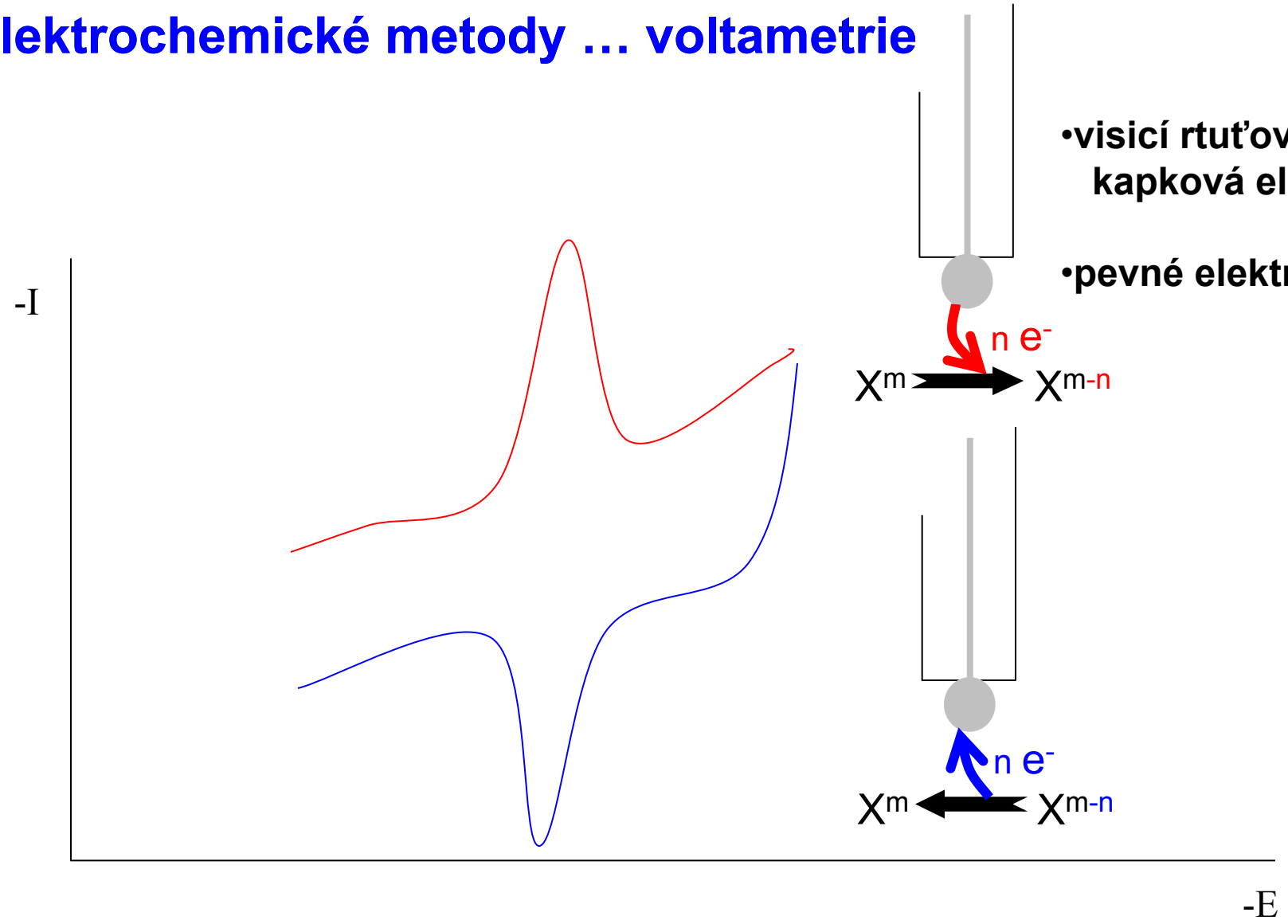
-I

kvantifikace
(jaká je koncentrace)

-E

identifikace elektrochemicky aktivní látky
(co to je)

Elektrochemické metody … voltametrie



- visicí rtuťová kapková elektroda
- pevné elektrody

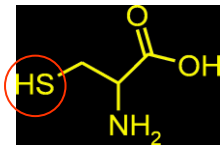
Jak TOHLE souvisí s DNA, bílkovinami, experimentální biologií, biomedicínou?

- víte, co se děje s elektrony v molekulách luminoforů, když měříte fluorescenci (nebo „jen“ fotíte gel značený ethidiem)? Víte, co to je molekulový π-orbital? Víte, co se děje na CCD detektoru?
- víte, proč nemáte nanášet na PAGE vzorky DNA s velkou koncentrací solí, jinak dostanete „hnusný“ gel?
- víte, proč glycerol indukuje hvězdičkovou aktivitu restriktáz?
- atd.
- pořád (fyzikální) chemie, pořád fyzika!!!

elektrochemie proteinů

Elektrochemie proteinů

od dvacátých let XX. století (J. Heyrovský, R. Brdička)



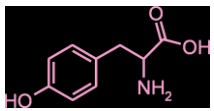
redukce vazby S-Hg

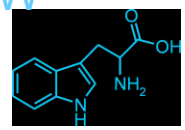
redukce vazby
S-S (cystin)

prenatriová vlna
pík H

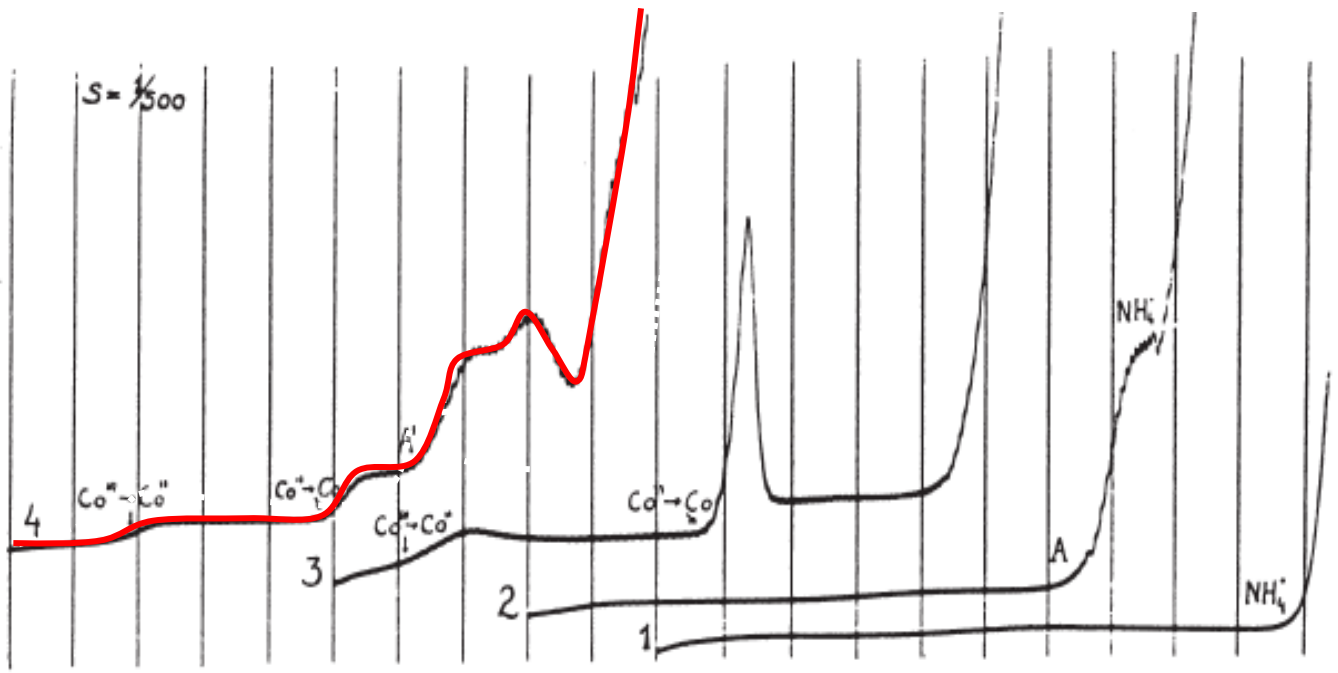
Brdičkova reakce
(v přítomnosti Co)

Y W





Brdičkova reakce



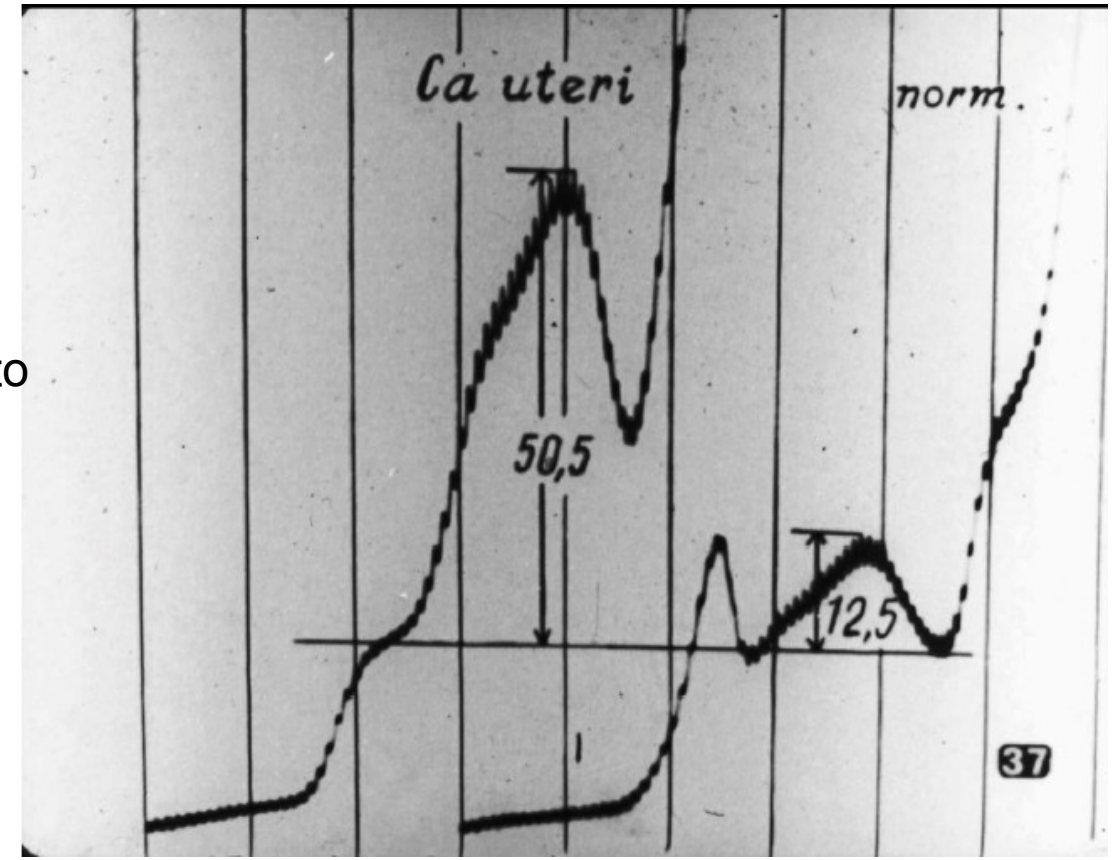
Brdička, 1933: polarografické vlny v přítomnosti sérových proteinů a solí kobaltu

Brdičkova reakce a diagnostika rakoviny

-kdysi poměrně rozšířený diagnostický test

-dnes renesance tohoto přístupu

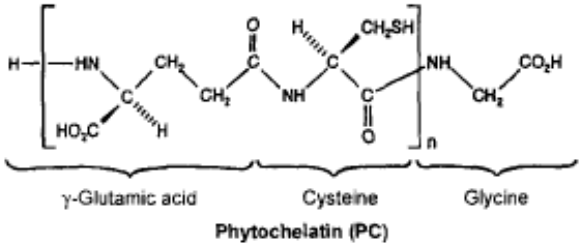
-R. Kizek a spol (MENDELU)
-rakovina a metalothioneiny

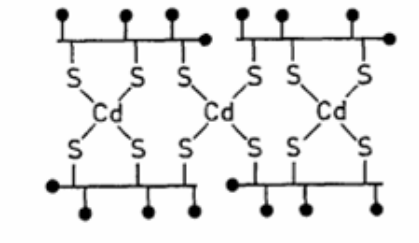


Stanovení fytochelatinů v rostlinných buňkách

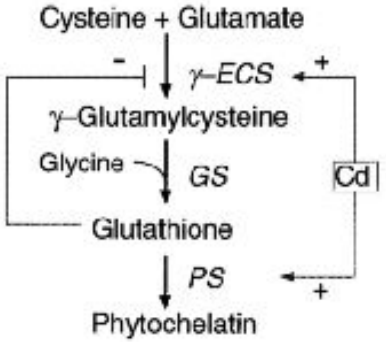
Fytochelatiny: „rostlinné metalothioneiny“

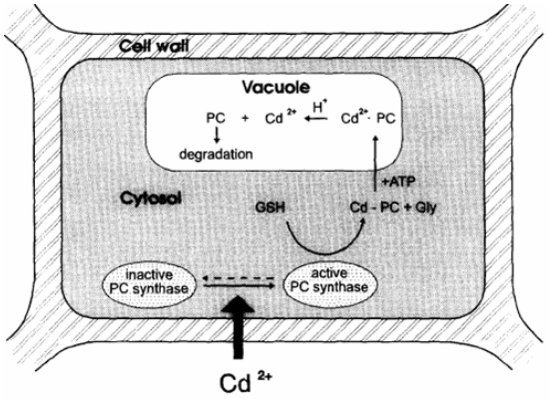
- detoxifikace těžkých kovů v rostlinných buňkách





- syntéza indukována těžkými kovy (kadmium)

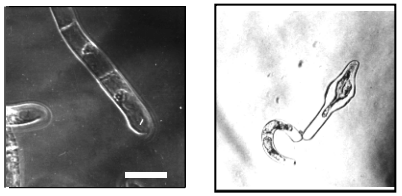




Kadmium navozuje u buněk TBY-2 apoptózu (programovanou buněčnou smrt)

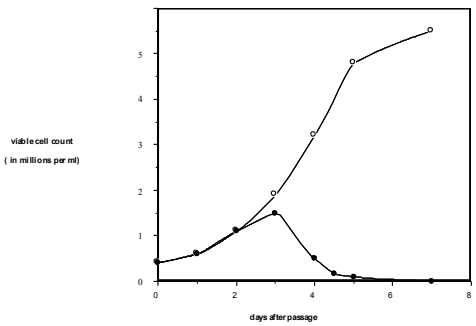
M. Fojtova, and A. Kovarik, **Genotoxic effect of cadmium is associated with apoptotic changes in tobacco cells**, Plant Cell Environ. 23 (2000) 531-537.

M. Fojtova, J. Fulneckova, J. Fajkus, and A. Kovarik, **Recovery of tobacco cells from cadmium stress is accompanied by DNA repair and increased telomerase activity**, J. Exp. Bot. 53 (2002) 2151-2158.

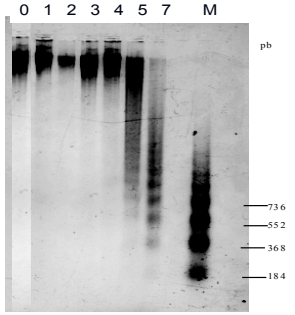


TBY-2

TBY-2 po 5 dnech v 50 μM $CdSO_4$



ztráta viability



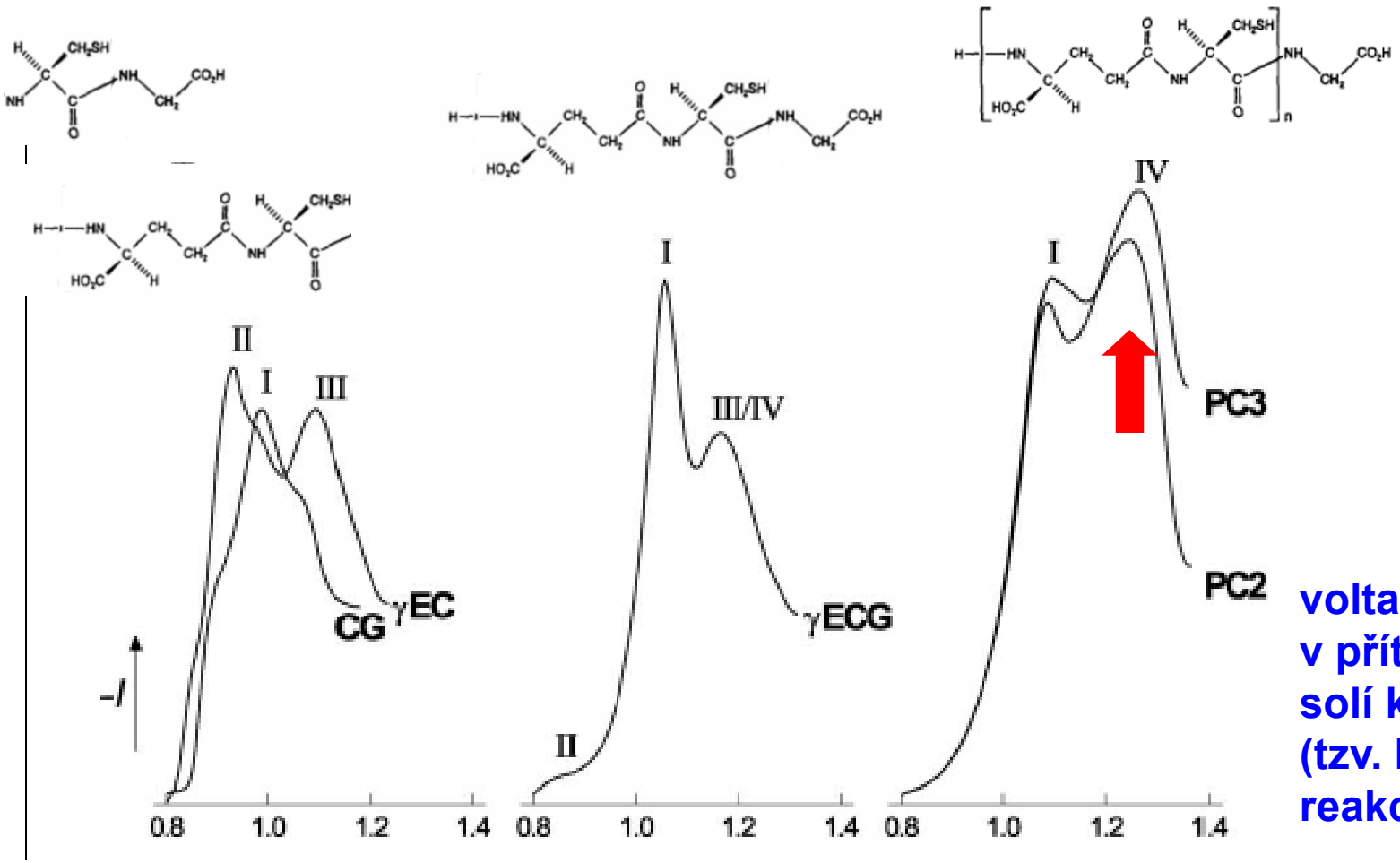
fragmentace DNA

- s 10 μM kadmiem si buňky „poradí“
- 100 μM kadmium indukuje apoptózu dříve než 50 μM kadmium

Co se děje s hladinou PC v průběhu těchto změn?

Jak souvisí s apopotózou/přežitím?

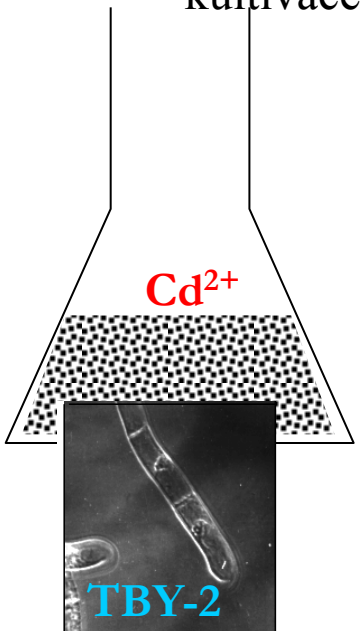
Dokážeme PC v extraktech z buněk jednoduše stanovit elektrochemicky?

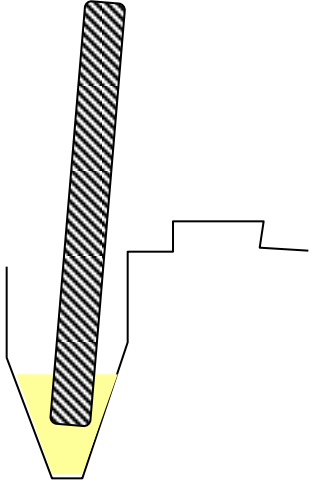


voltametrie v přítomnosti solí kobaltu (tzv. Brdičkova reakce)

na základě poměru intenzit jednotlivých signálů (tvaru voltamogramu) lze PC odlišit od CG, γEC i od glutathionu (Dorčák a Šestáková, 2005)

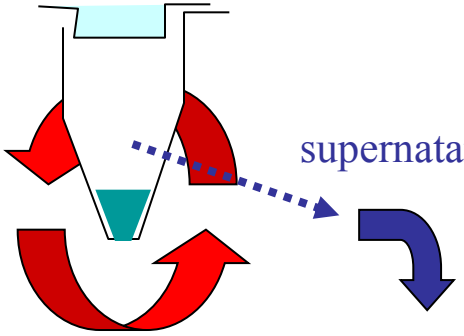
kultivace buněk



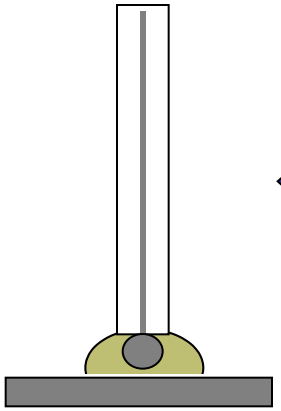


homogenizace

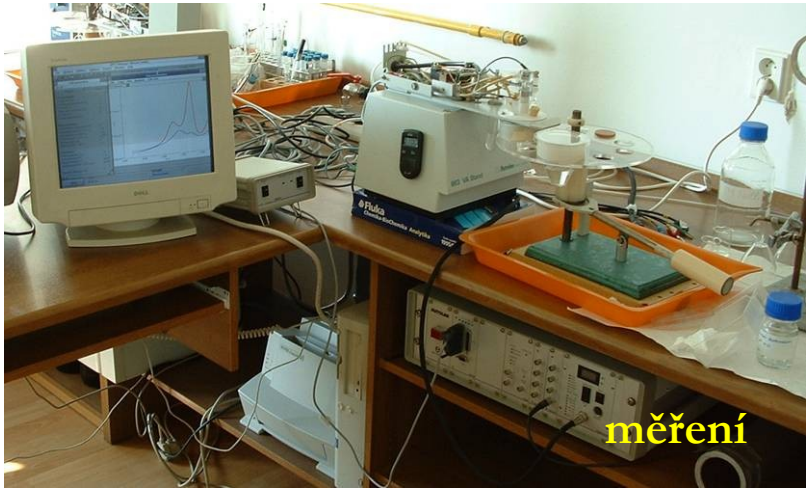
centrifugace

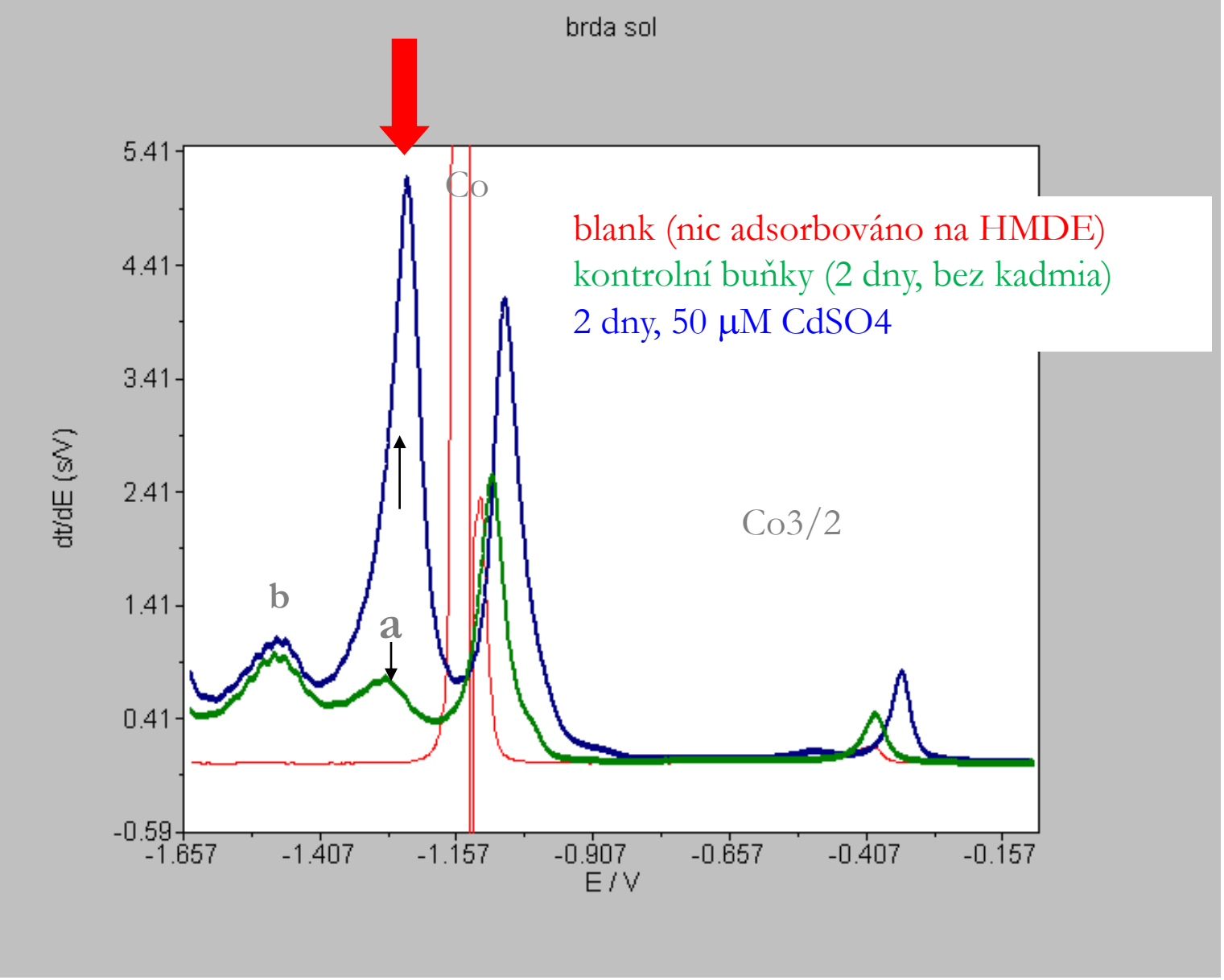


naředění na jednotnou koncentraci celkového proteinu

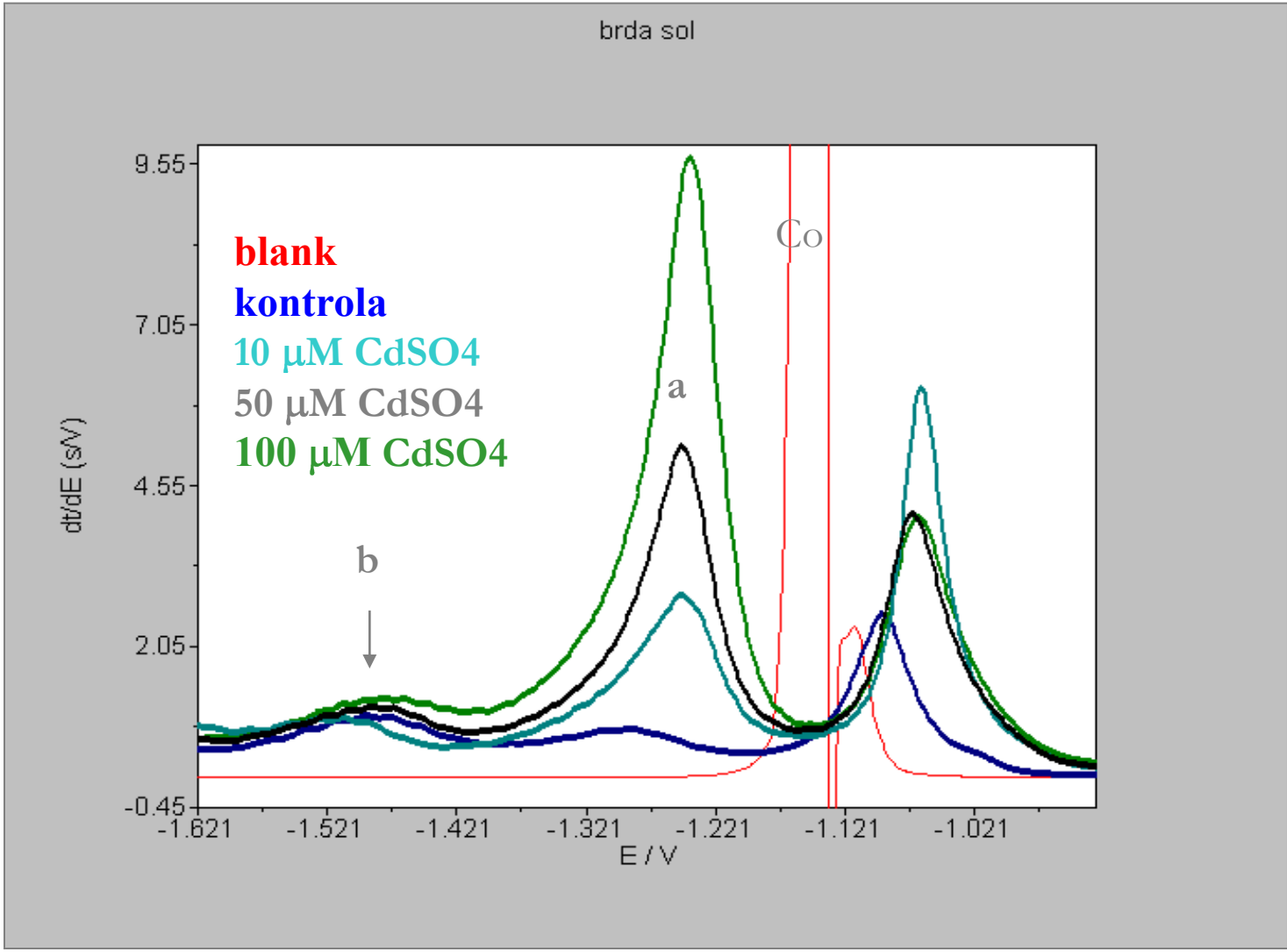


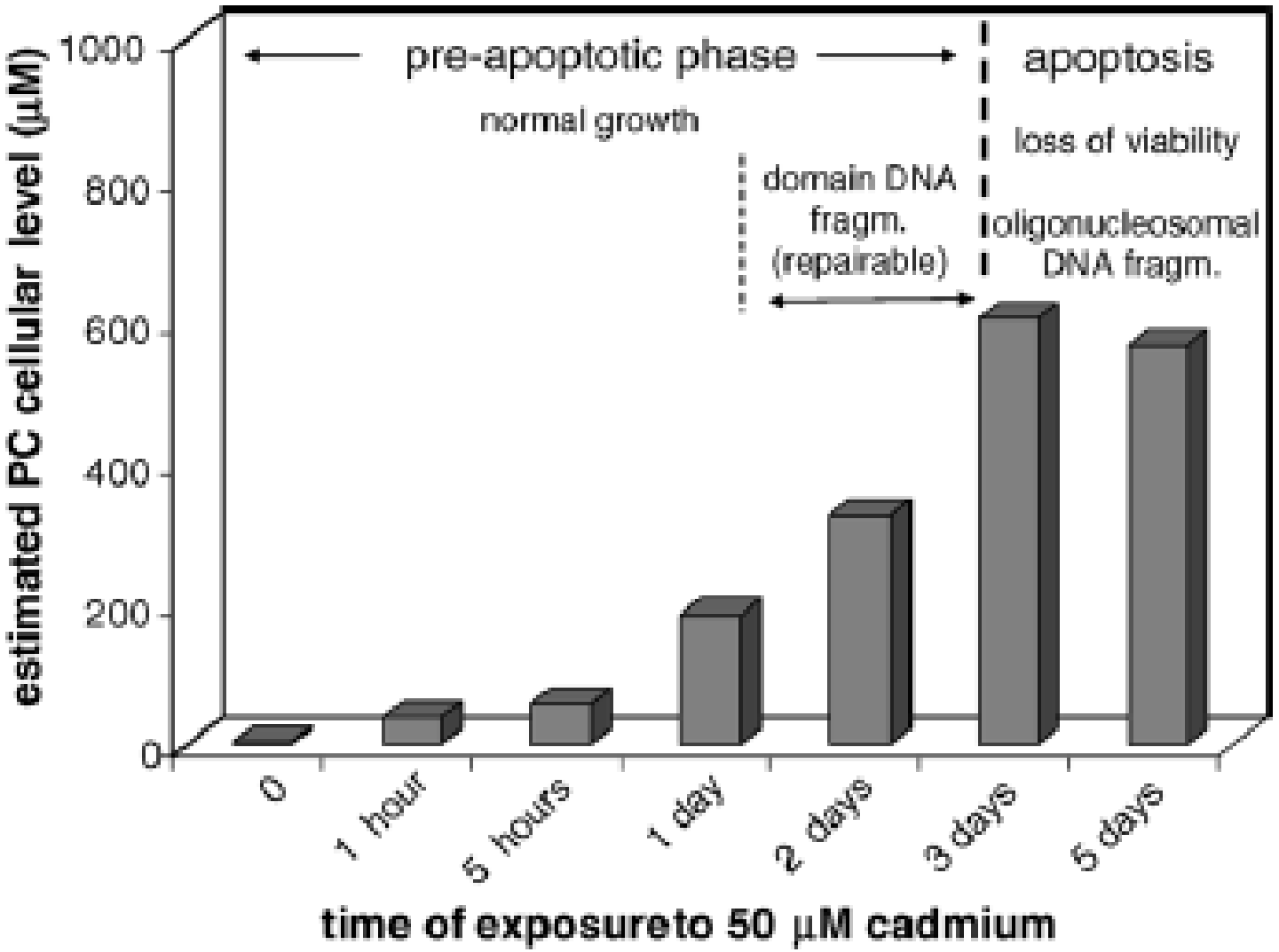
adsorpce na rtuťovou elektrodu




brda sol
Co
blank (nic adsorbováno na HMDE)
kontrolní buňky (2 dny, bez kadmia)
2 dny, 50 µM CdSO4
Co3/2
b
a
dt/dE (s/V)
5.41
4.41
3.41
2.41
1.41
0.41
-0.59
-1.657
-1.407
-1.157
-0.907
-0.657
-0.407
-0.157
E / V

Výška **píku „a“** reprodukovatelně reaguje na přítomnosti kadmia v médiu, a to jak v závislosti na jeho koncentraci, tak na době kultivace



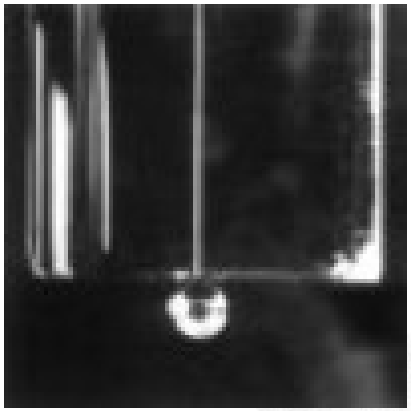

pre-apoptotic phase
apoptosis
normal growth
loss of viability
domain DNA fragm. (repairable)
oligonucleosomal DNA fragm.
estimated PC cellular level (µM)
1000
800
600
400
200
0
0
1 hour
5 hours
1 day
2 days
3 days
5 days
time of exposureto 50 µM cadmium

Biofyzikální ústav AVČR, Brno
Laboratoř biofyzikální chemie a molekulární onkologie
Centrum biofyzikální chemie, bioelektrochemie a bioanalýzy

Monitorování hladiny fytochelatinů v rostlinných buňkách pomocí elektrochemických metod

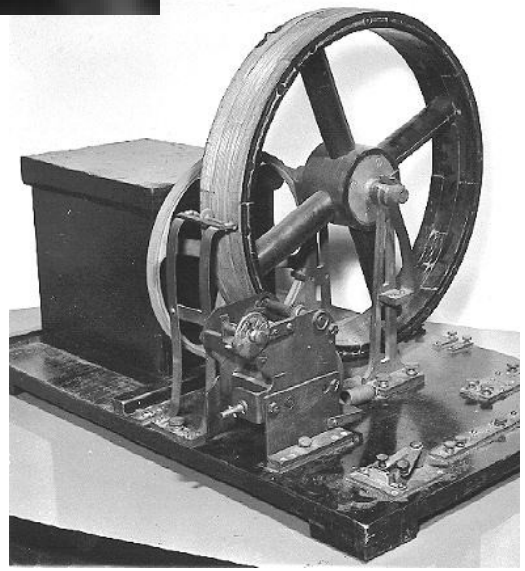
4.10. 2006 Srní, 4. metodické dny

Dotaz od jednoho posluchače po přednášce:



TO SE JEŠTĚ DĚLÁ??

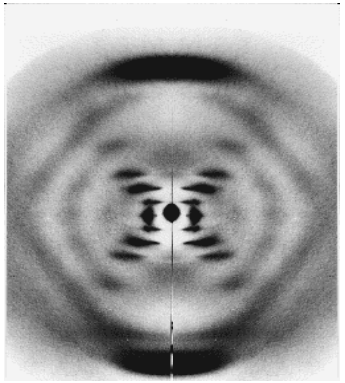
Já myslel, že to je už k vidění jenom v muzeu…



?

A co DNA?

Struktura DNA…

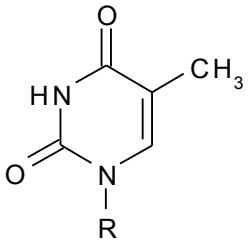


1953: James Watson, Francis Crick, Rosalind Franklin, Maurice Wilkins: dvoušroubovice DNA

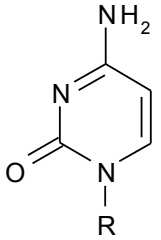
1962: Nobelova cena (JW, FC, MW)

vysvětlení základních principů uchování, předávání a exprese dědičné informace

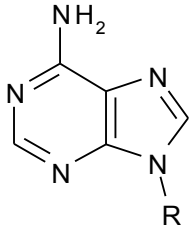
pyrimidinové báze **purinové báze** **dvoušroubovice**



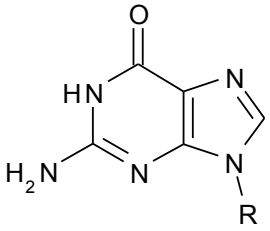
thymin (T)



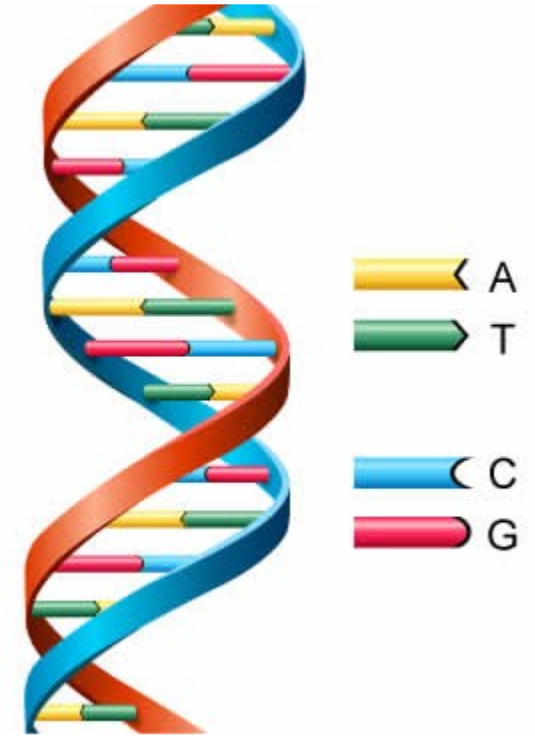
cytosin (C)

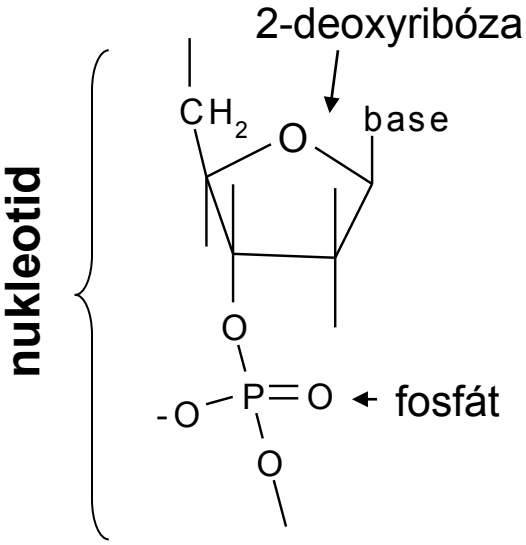


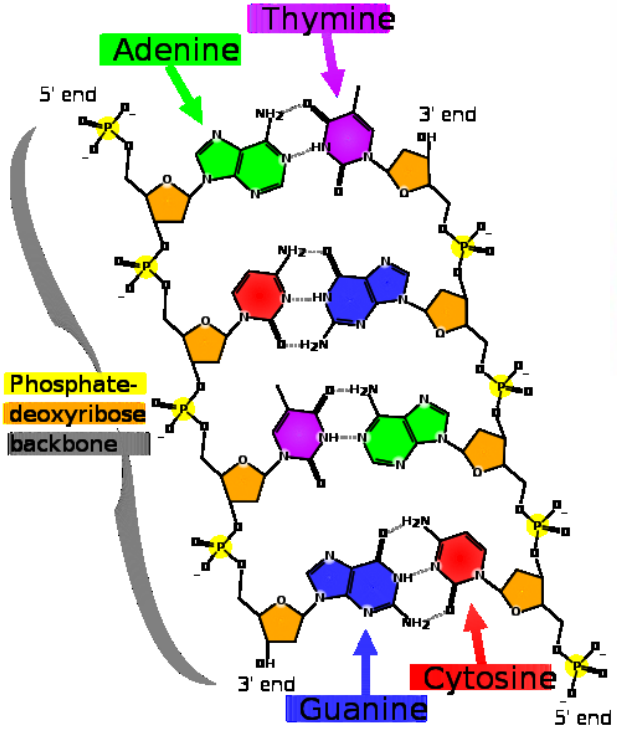
adenin (A)



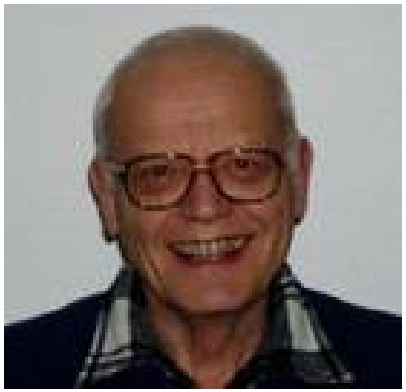
guanin (G)



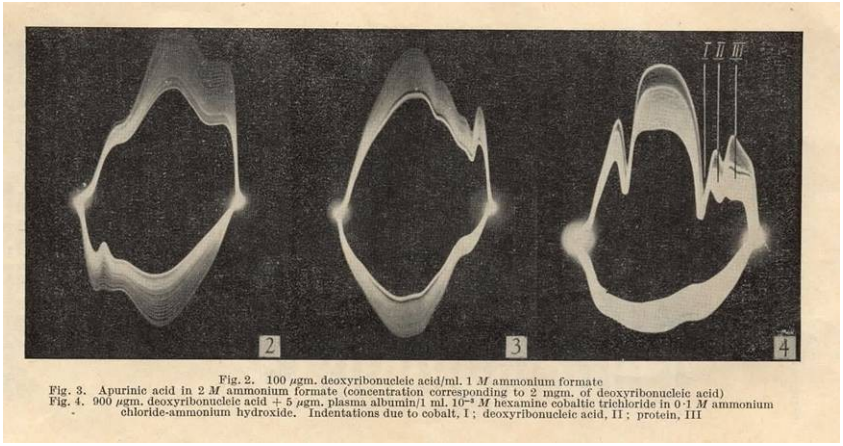




párování bazí v řetězcích DNA



1958 – 1960 Emil Paleček: polarografie DNA



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(*Reprinted from Nature, Vol.* 188, *No.* 4751, *pp.* 656-657, *November* 19, 1960)

Oscillographic Polarography of Highly Polymerized Deoxyribonucleic Acid

PROCEEDING from my finding[1,2] that nucleotides, nucleosides and the bases of nucleic acids can be analysed by alternating current oscillographic polarography[3-5], I have also tried to study polymerized deoxyribonucleic acid by this method.

The apparatus used was a Polaroskop *P* 524 (Křižík, Praha). With this apparatus it is posssible to plot dE/dt against E (Fig. 1). The analysis was carried out by means of the dropping mercury electrode in the same electrolytes as were used in my previous work[1,2]. All measurements were carried out with specimens of deoxyribonucleic acid from calf thymus.

I have established that in a medium of molar ammonium formate, deoxyribonucleic acid shows an anodic indentation at the same potential as deoxyguanylic acid (Fig. 2). Other characteristics of both indentations are also analogous (dependence on direct voltage, temperature, concentration of the electrolyte), which appears to indicate that that due to

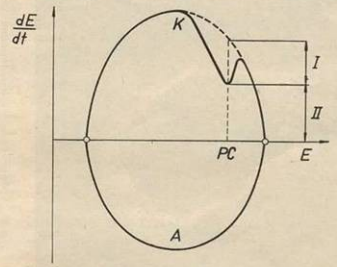


Fig. 1. Graph of dE/dt against E. The nature of the material analysed is characterized by the potential of the indentation (*PC*), which is somewhat similar to the polarographic half-wave potential. The quantity of the material is characterized by the depth of the indentation. For qualitative analysis, the height II, which can be measured much more easily, is generally measured. *K*, Cathodic part; *A*, anodic part

Institute of Biophysics
Department of Biophysical Chemistry and Molecular Oncology
Centre of Biophysical Chemistry, Bioelectrochemistry and Bioanalysis

Effects of DNA structure on its electrochemical behavior. Detecting DNA damage.

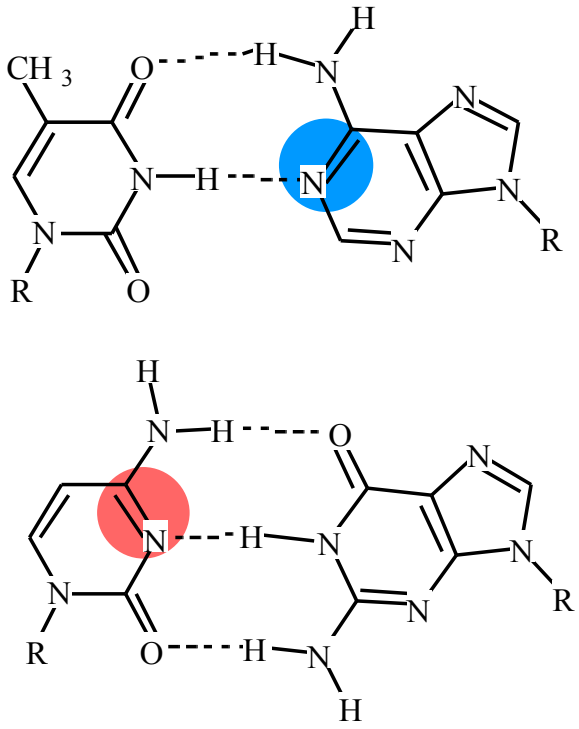
nucleic acids are electroactive

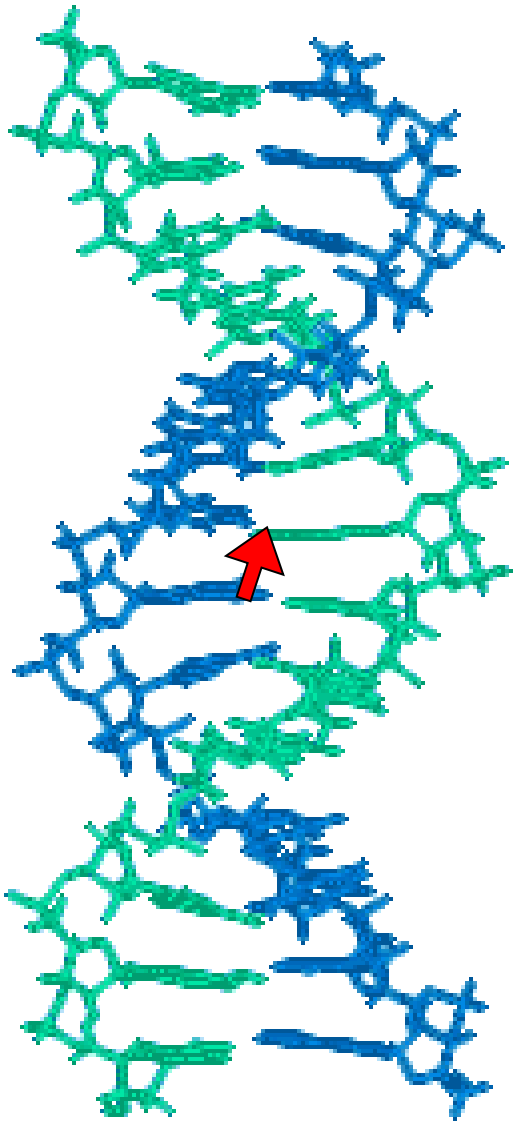
- at mercury electrodes, bases A,C and G undergo redox processes

- at carbon electrodes, nucleobases can be oxidized

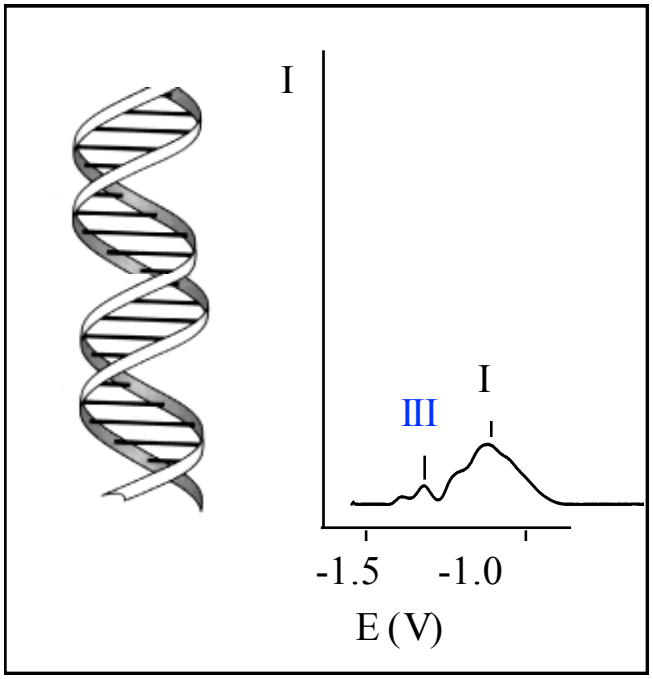
Reduction DNA signals at the
are strongly influenced by

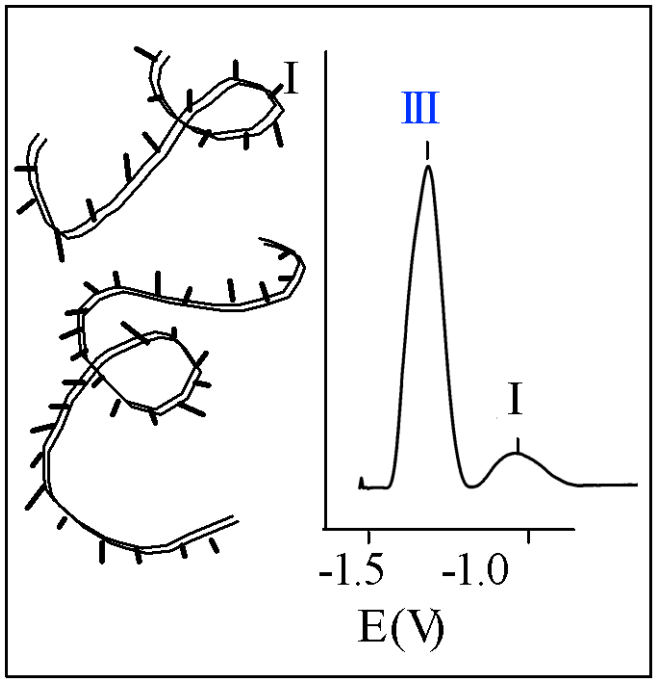
- this is due to location of the A and C electroactiv
within the Watson-Crick hydrogen bonding syste





Reduction DNA signals at the mercury electrodes are strongly influenced by DNA structure

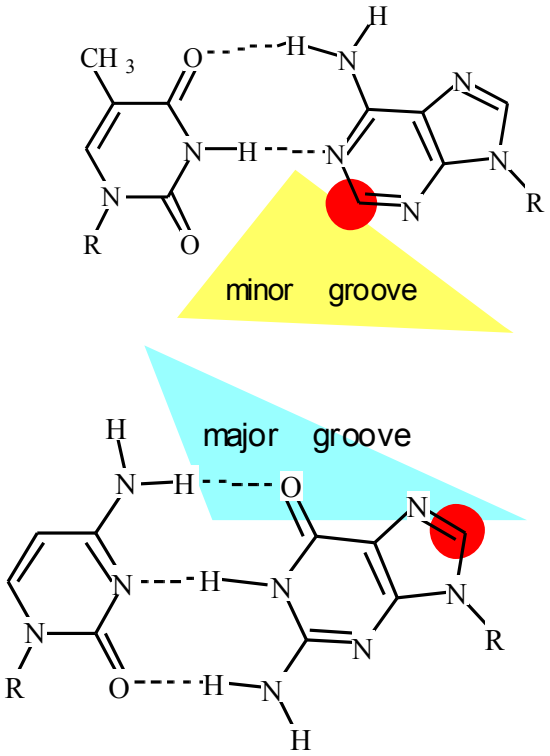


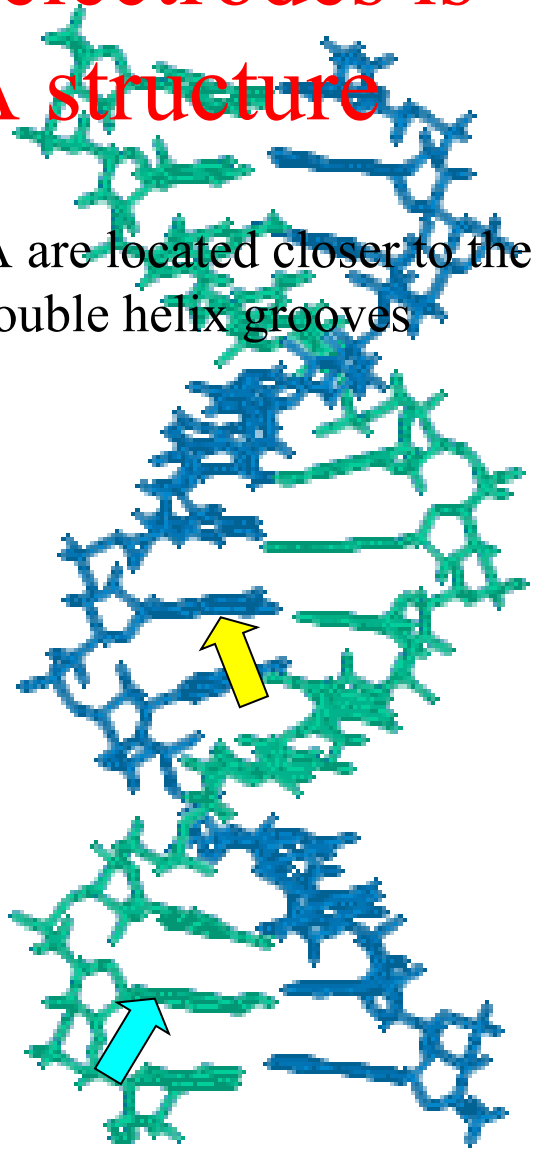


square-wave voltammetry

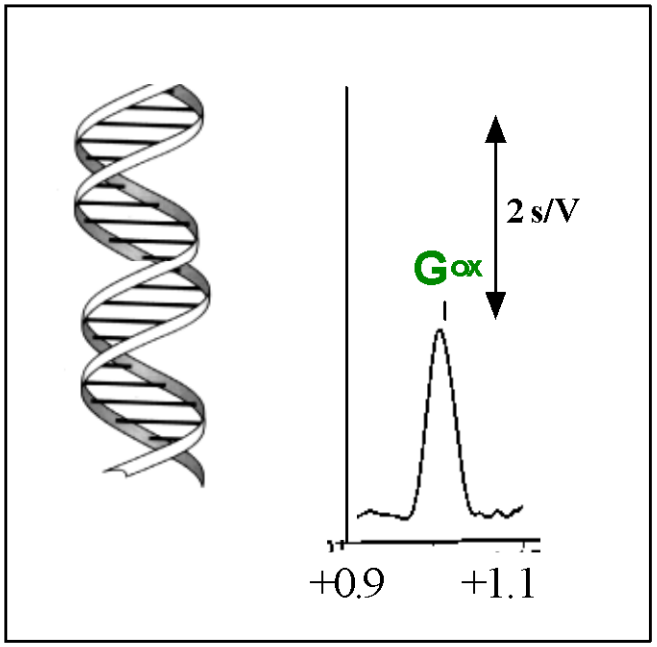
DNA oxidation at carbon electrodes is less influenced by DNA structure

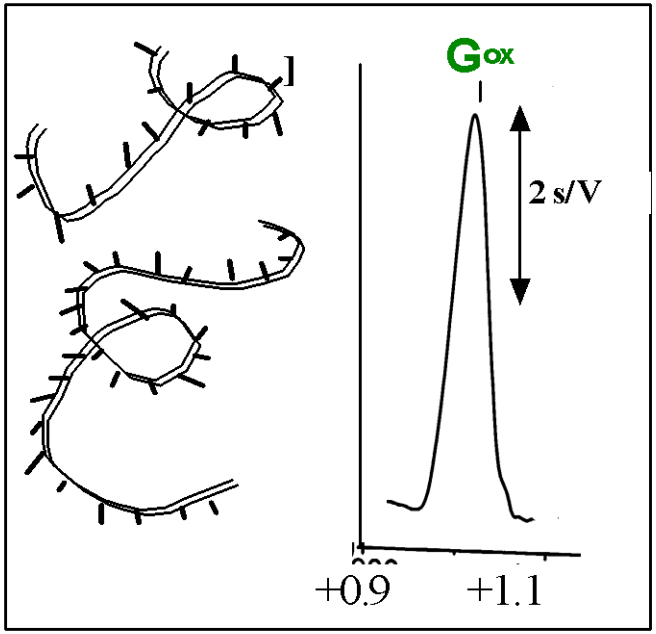
- oxidation sites of guanine and adenine in dsDNA are located closer to the double helix surface and are accessible via the double helix grooves





DNA oxidation at carbon electrodes is less influenced by DNA structure



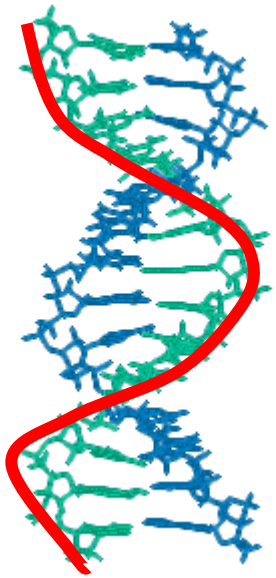


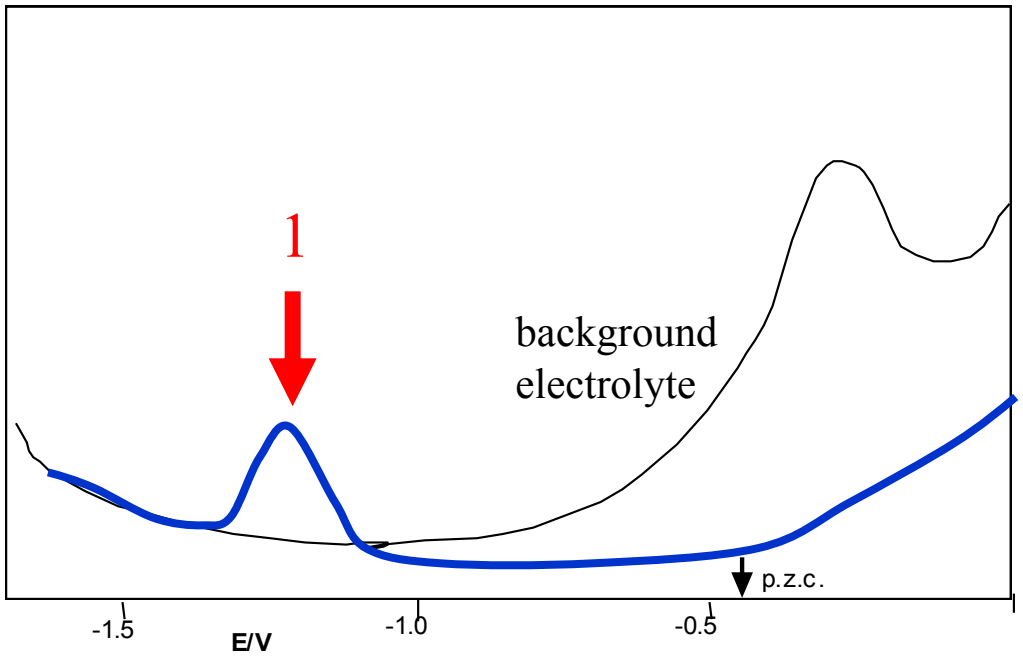
chronopotentiometry at CPE

At mercury electrodes in weakly alkaline media,
adsorption-desorption (tensammetric) signals
of nucleic acids can be detected
(e.g., using AC polarography, voltammetry, AC Z)

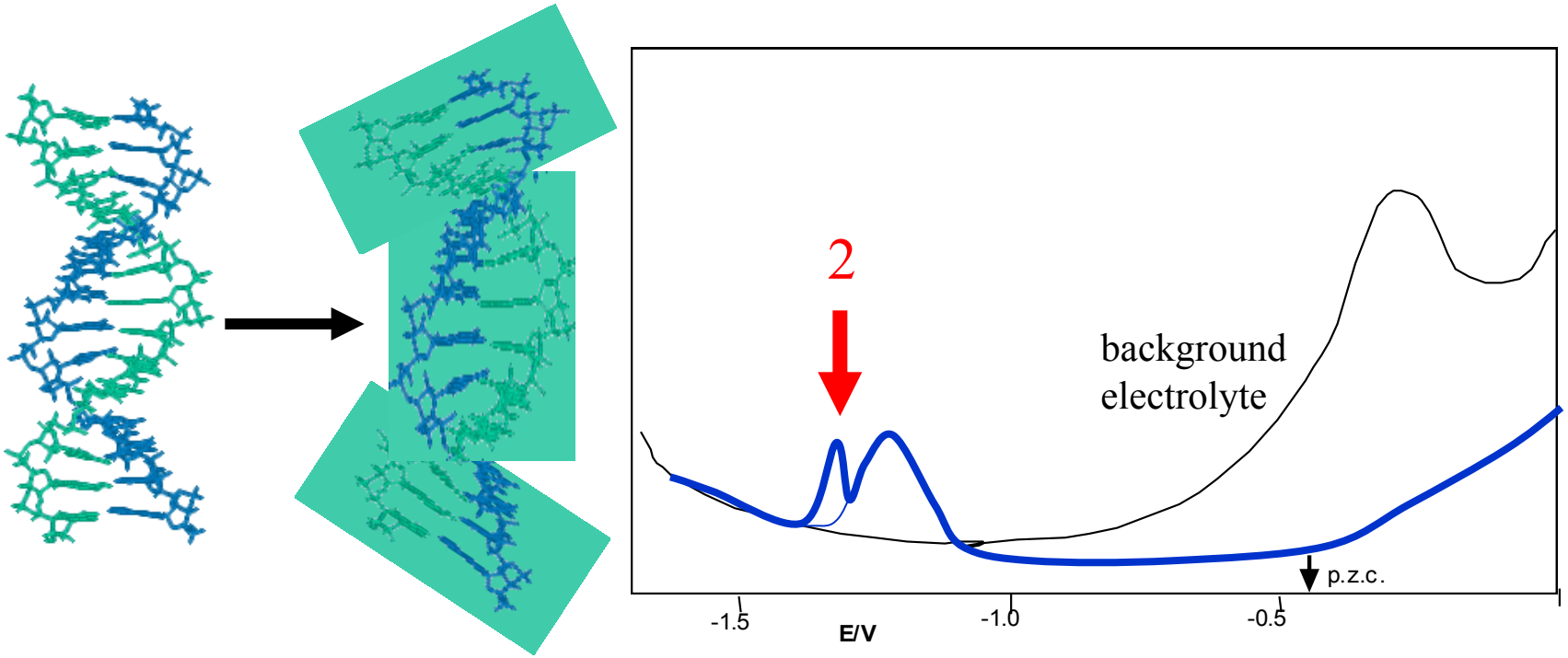
- depending on the conditions and on DNA structure, individual components of the polynucleotide chains may be involved in adsorption/desorption processes

-at moderate ionic strenght, **double-stranded DNA** yields peak 1 due to desorption/reorientation of DNA segments adsorbed via the sugar-phosphate backbone

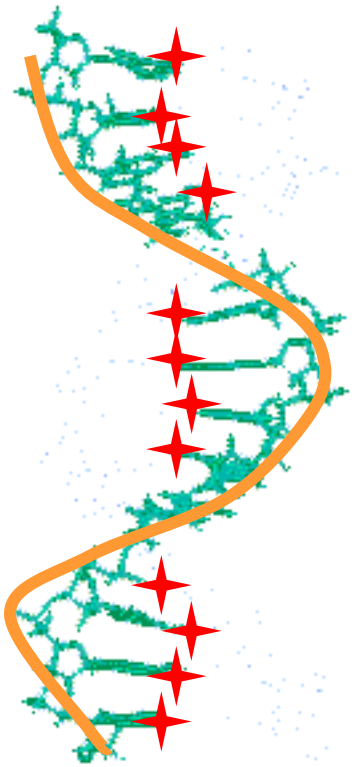


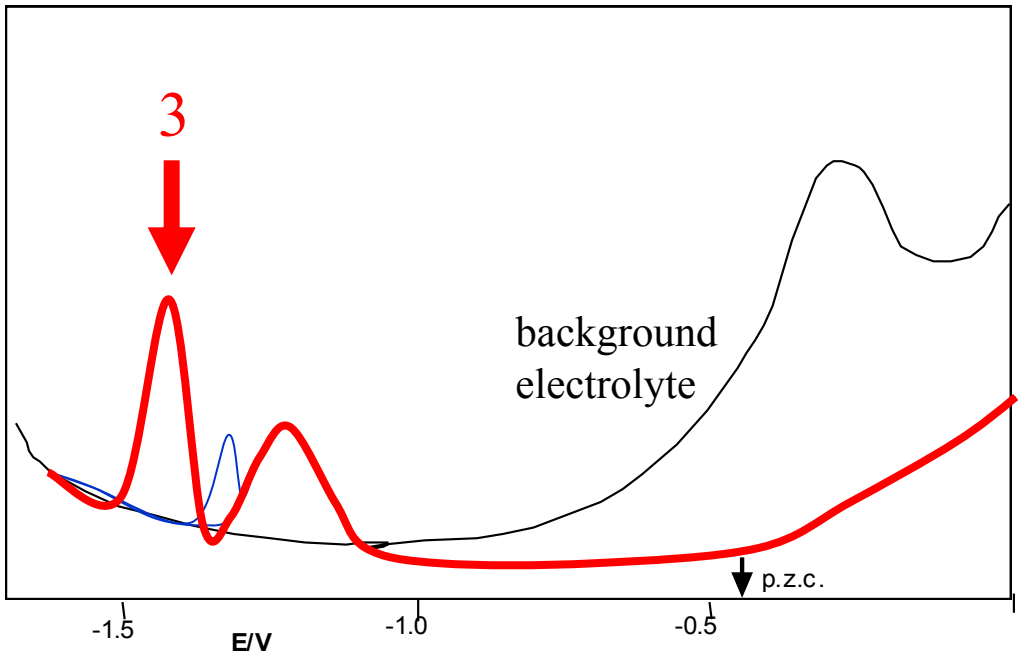


-distorted or regions of double-stranded DNA yield **peak 2**

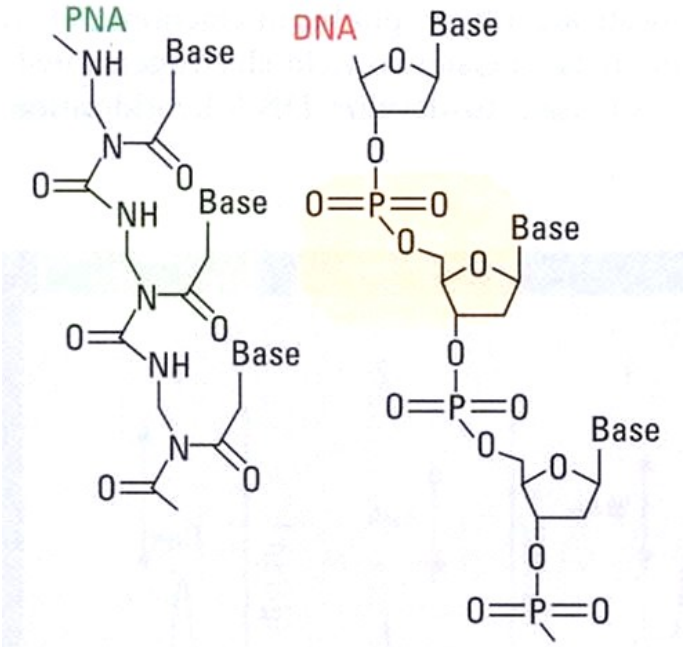


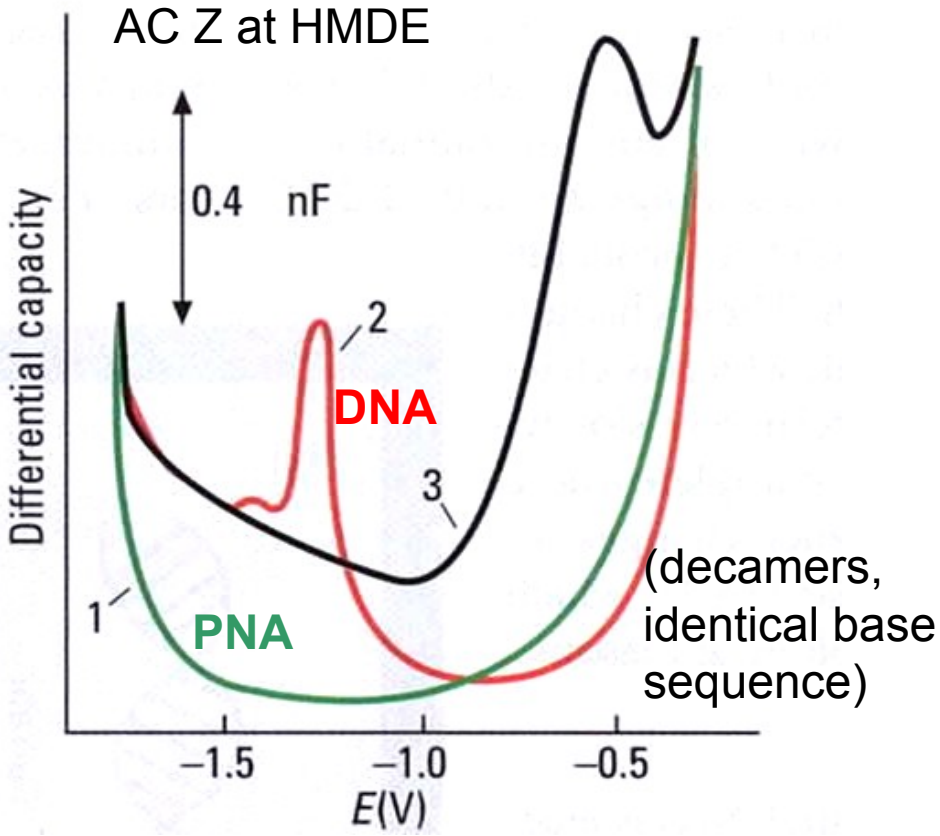
single-stranded (denatured) DNA yields peak 1 (due to the sugar-phosphate backbone) and **peak 3** due to desorption/reorientation of DNA segments adsorbed via **freely accessible bases**





adsorption/desorption behavior of DNA at electrodes is strongly related to negative charge of its sugar-phosphate backbone
(together with a strong adsorption of nucleobases via hydrophobic forces)

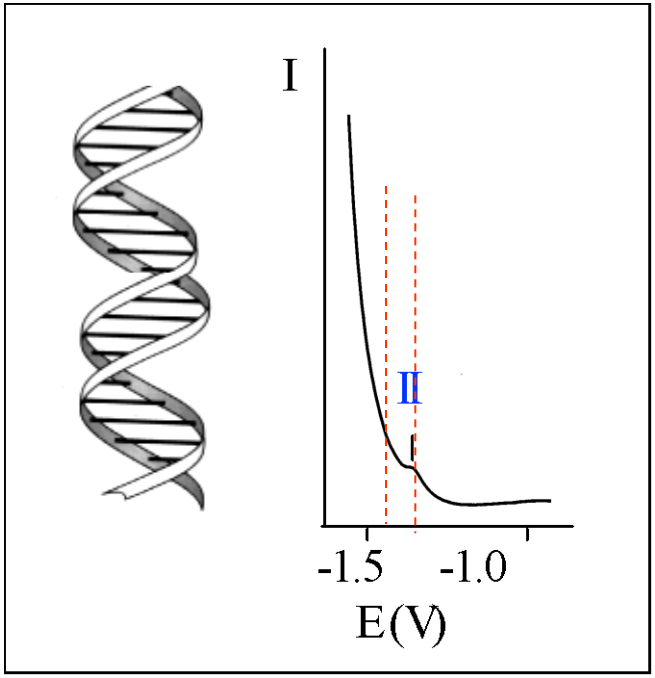


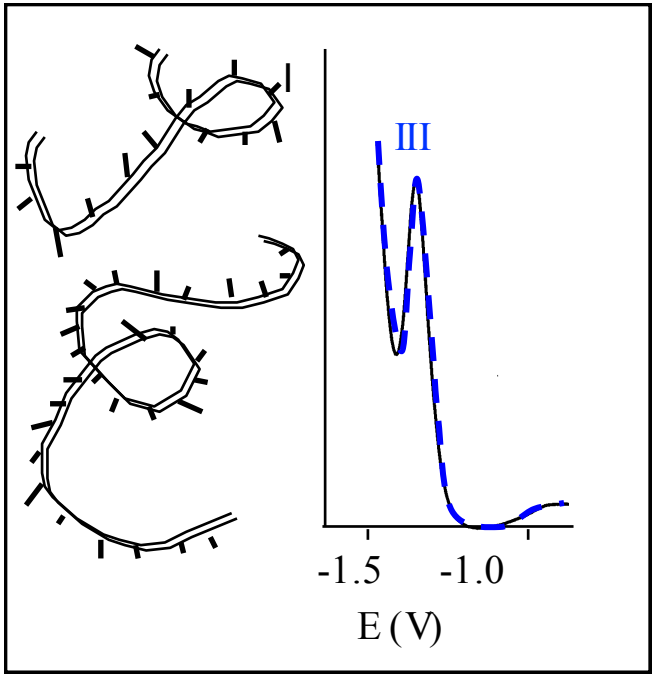


peptide nucleic acid: DNA analogue with neutral backbone

differential pulse polarography

- used in nucleic acid studies in the 60-70´s
- discrimination between ss and dsDNA

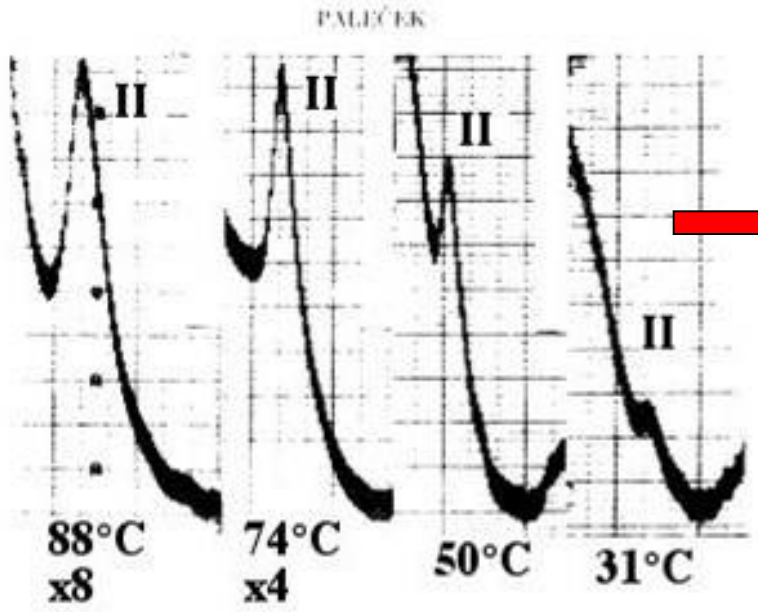




differential pulse polarography

- peak II: high sensitivity to subtle changes of dsDNA structure (&dynamics)

- DNA premelting



DPP peak II

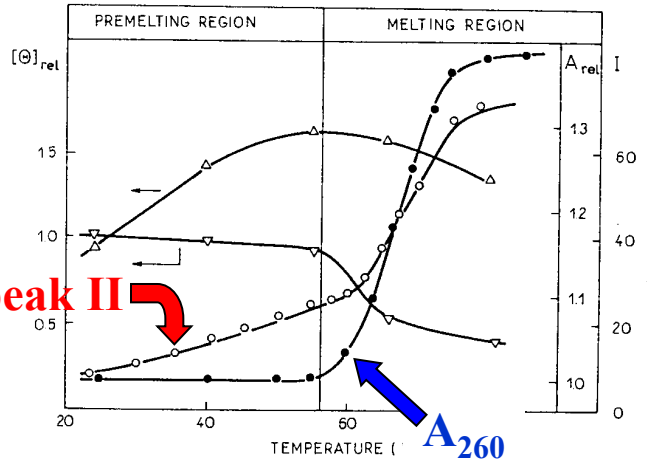


FIG. 2. Thermal transition of calf thymus DNA followed by circular dichroism (CD), derivative pulse polarography and spectrophotometry. CD: △——△, positive band (275 nm); ▽——▽, negative band (245 nm); ○——○, polarography; ●——●, absorbance at 269 nm. θ_{rel}, the ratio between the ellipticity at the given temperature and at 25°C. A_{rel}, the ratio between the absorbance (260 nm) at the given temperature and the absorbance at 25°C. I, the height of the pulse-polarographic peak in divisions. Adapted from Paleček and Frič (*21*).

[1] Nonstandard abbreviations: CD, circular dichroism; ORD, optical rotatory dispersion; ds, double-stranded; ss, single-stranded.

differential pulse polarography

- **strand breaks**

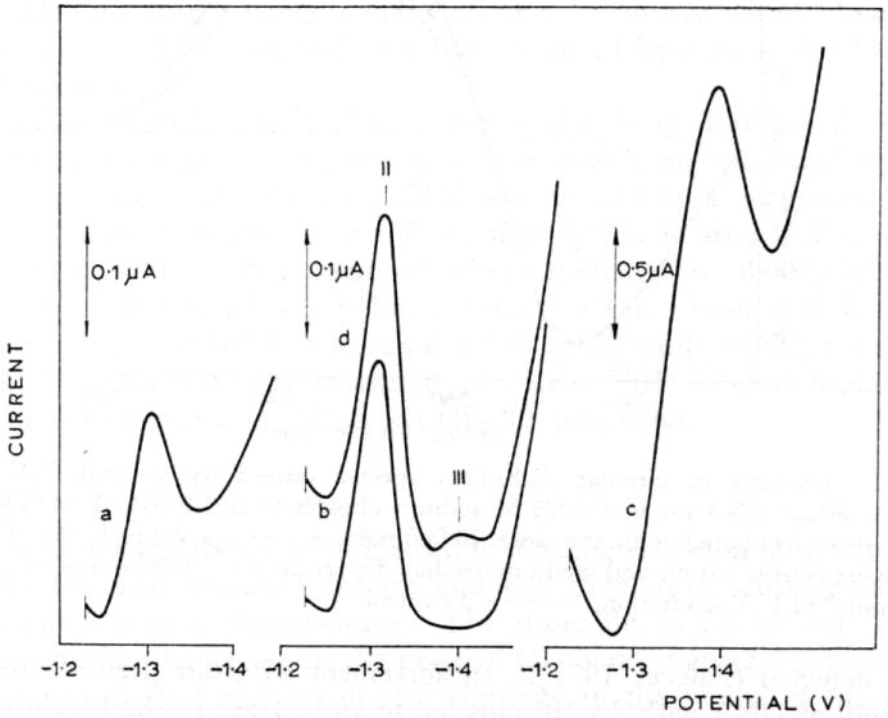


Figure 3. Differential pulse polarograms of DNA irradiated with ionizing radiation. DNA was irradiated in the concentration 460 μg/ml in the medium given in figure 1. (*a*) a control; (*b*) 10^4 rads; (*c*) 6×10^5 rads; (*d*) the sample (*b*) heated at 50°C for 6 min and quickly cooled. The differential pulse polarograms were measured in 0·3 M ammonium formate, 0·1 M sodium phosphate, pH 6·9 at DNA concentration 400 μg/ml. Sensitivity of the apparatus was 1 μA in parts (*a*), (*b*) and (*d*), and 5 μA in part (*c*).

differential pulse polarography

- **chemical modification of DNA: platinum adducts**

distinction of the kind of structural change caused by modification with different Pt complexes

peak II: conformation distortion, base pairing preserved

peak III: base unpairing

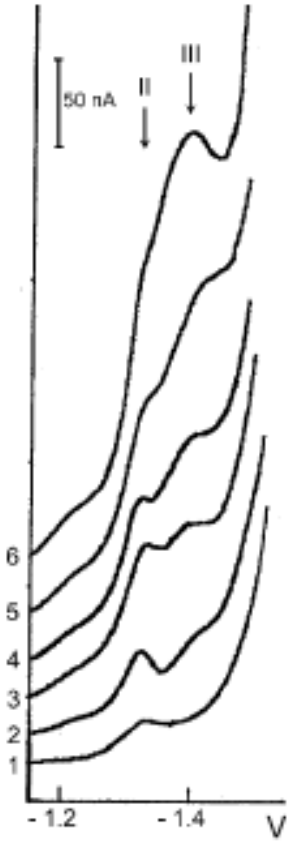


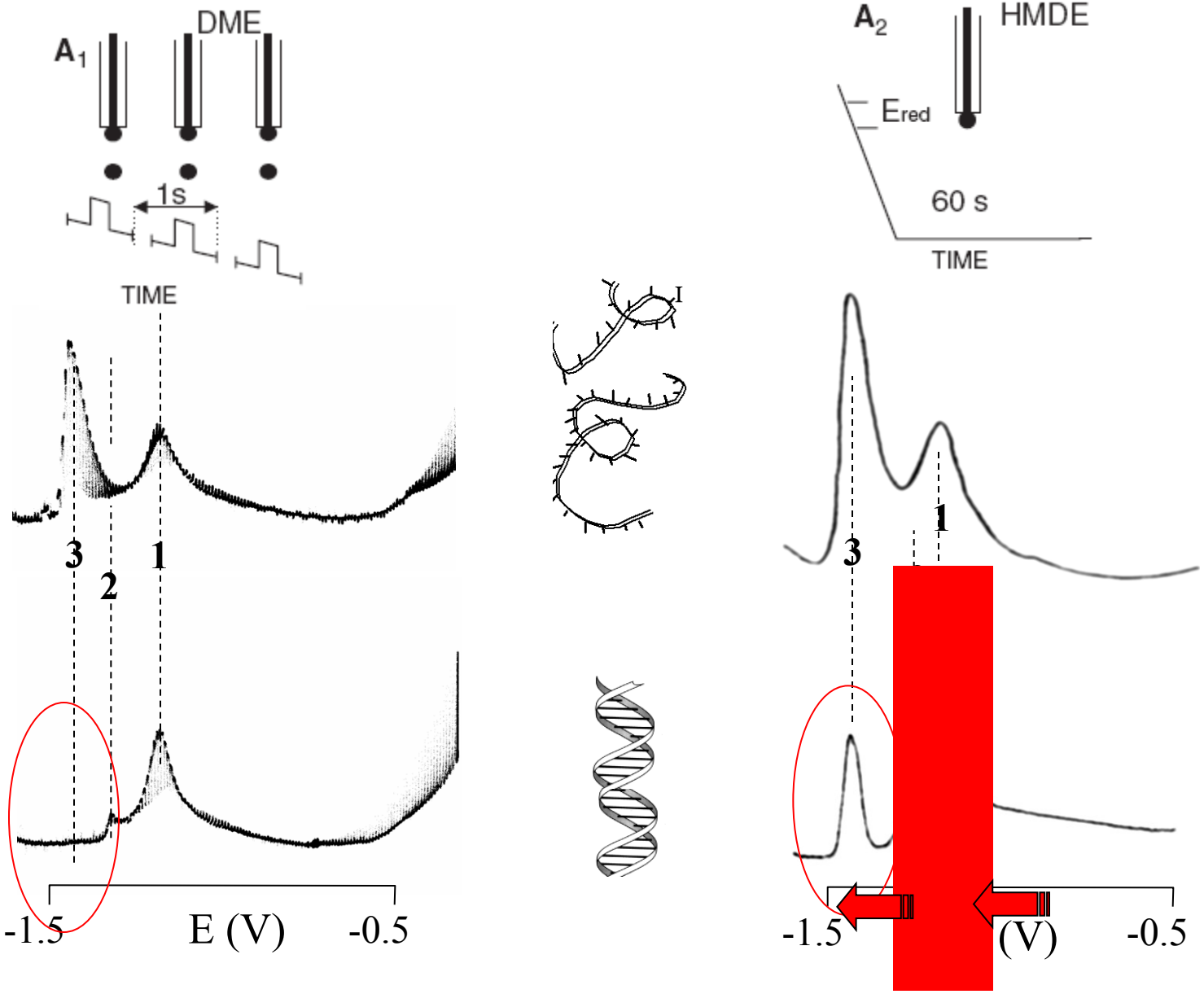
Fig. 7 Differential pulse polarographic analysis of CT-DNA modified by ORGANObisPt. DNA at a concentration of 0.4 mg/mL in 0.3 M ammonium formate with 0.01 M phosphate buffer, pH 6.8. *Curve 1*: control, unmodified DNA; *curves 2–6*: DNA modified by ORGANObisPt at $r_b = 0.001, 0.003, 0.005, 0.007, 0.01$, respectively; the *arrows* marked *II* and *III* indicate potentials *E* (against saturated calomel electrode) at which native or denatured DNA samples yielded DPP peaks II or III, respectively (see text)

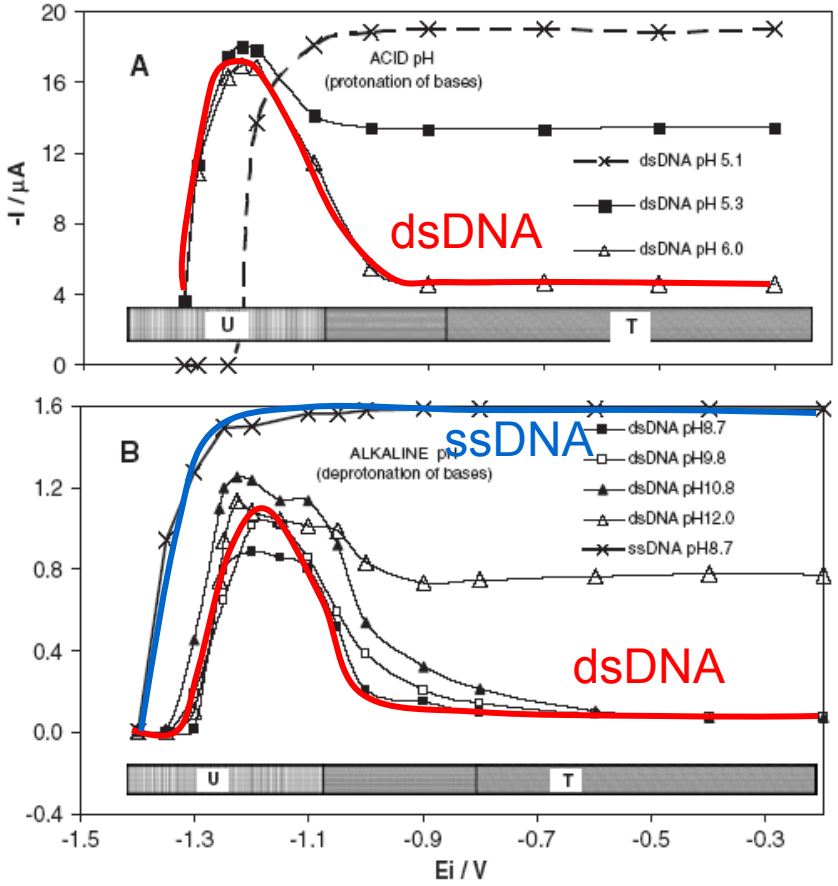
(Brabec et al.)

Changes of DNA structure at electrically charged surface

DME (SMDE)

HMDE





intensities of ssDNA-specific signals

prolonged exposure to
(accumulation at)
potential given on x-axis

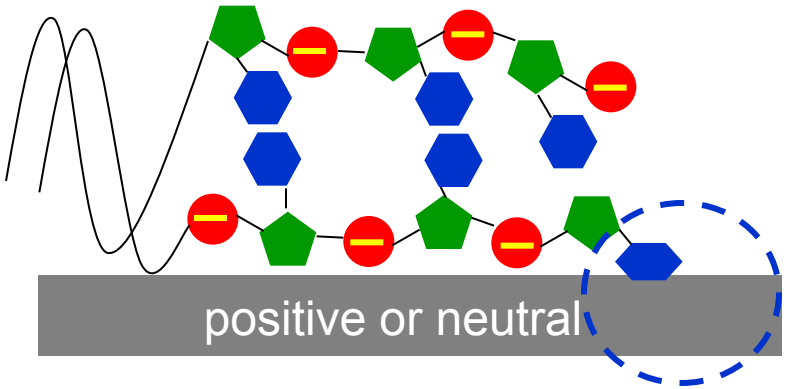
pH close to neutral (bases not ionized):

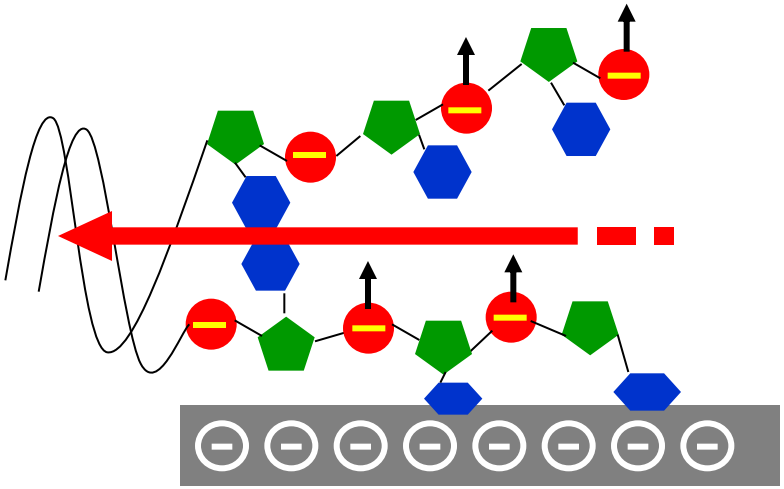
region T: – negligible structural changes due to adsorption

region U: surface denaturation

- close to the duplex ends (or single-strand breaks), some bases can be unpaired and make contact with the mercury surface

- phosphates repelled from negatively charged surface
- randomly adsorbed bases represent relatively firm anchor sites
- constraints in the double helix cause its (slow) unwinding
- more (unpaired) bases are coming into contact with the electrode



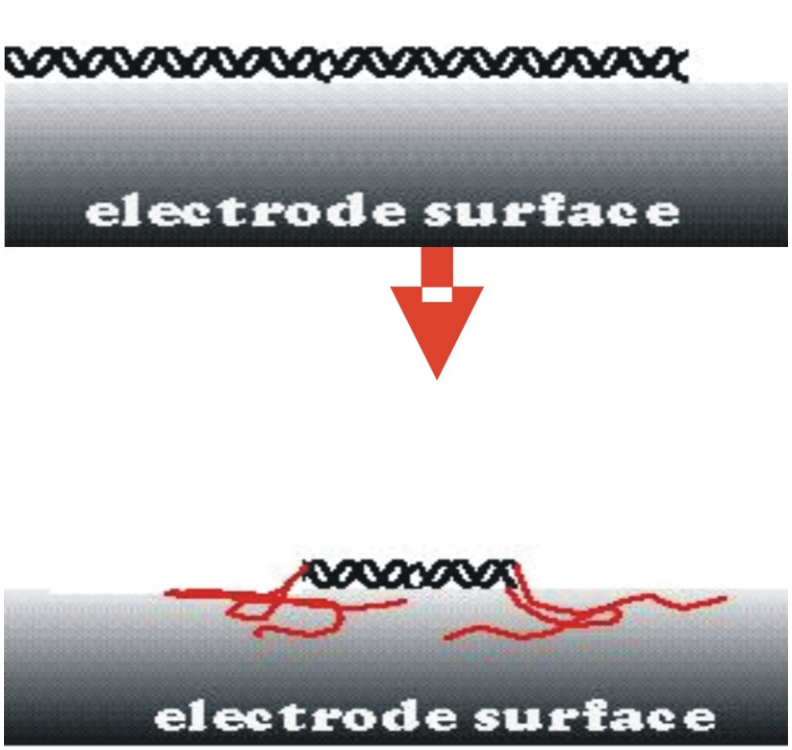


base

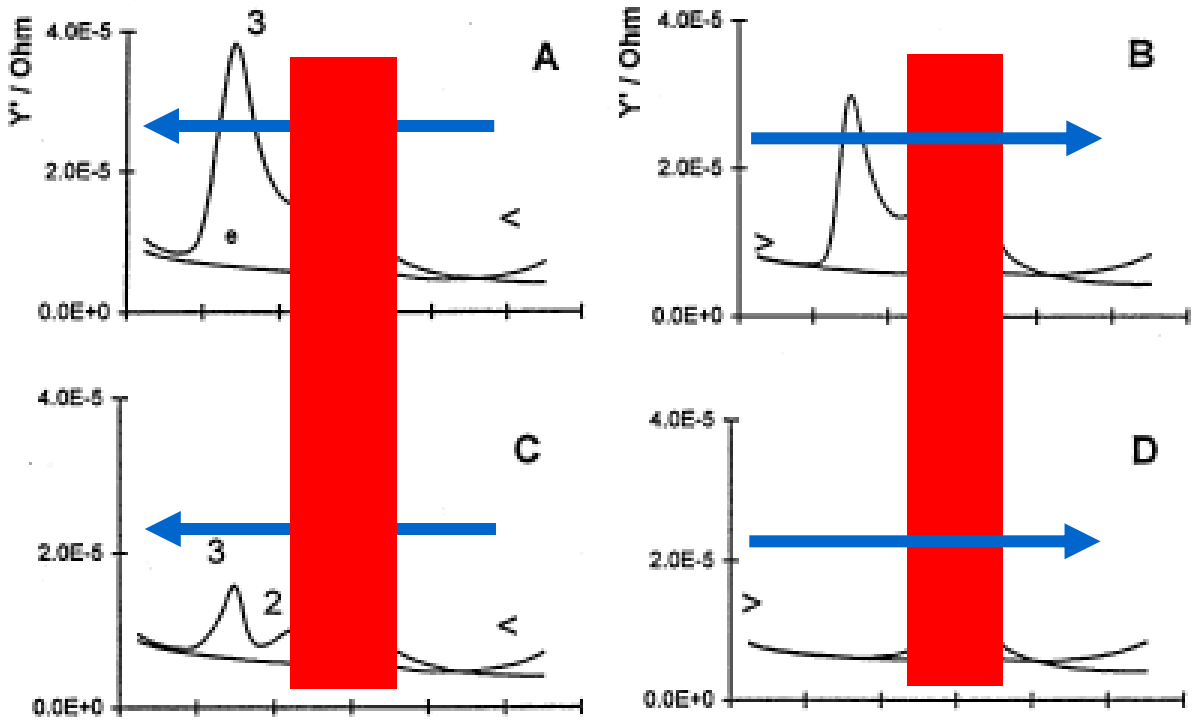
sugar

phosphate

(in real situation the strands must rotate around one another; the process requires repeated adsorption/desorption events)


electrode surface
electrode surface

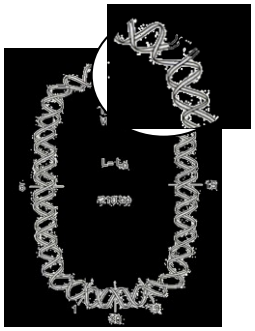
effects of initial potential and scan direction



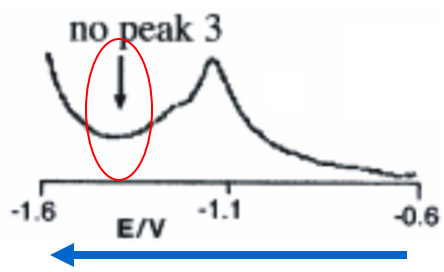
DNA with or without ends

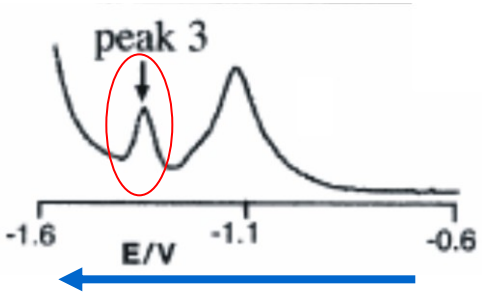
supercoiled

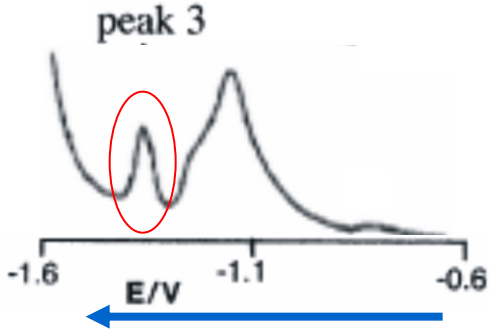
open (nicked) circular



linear

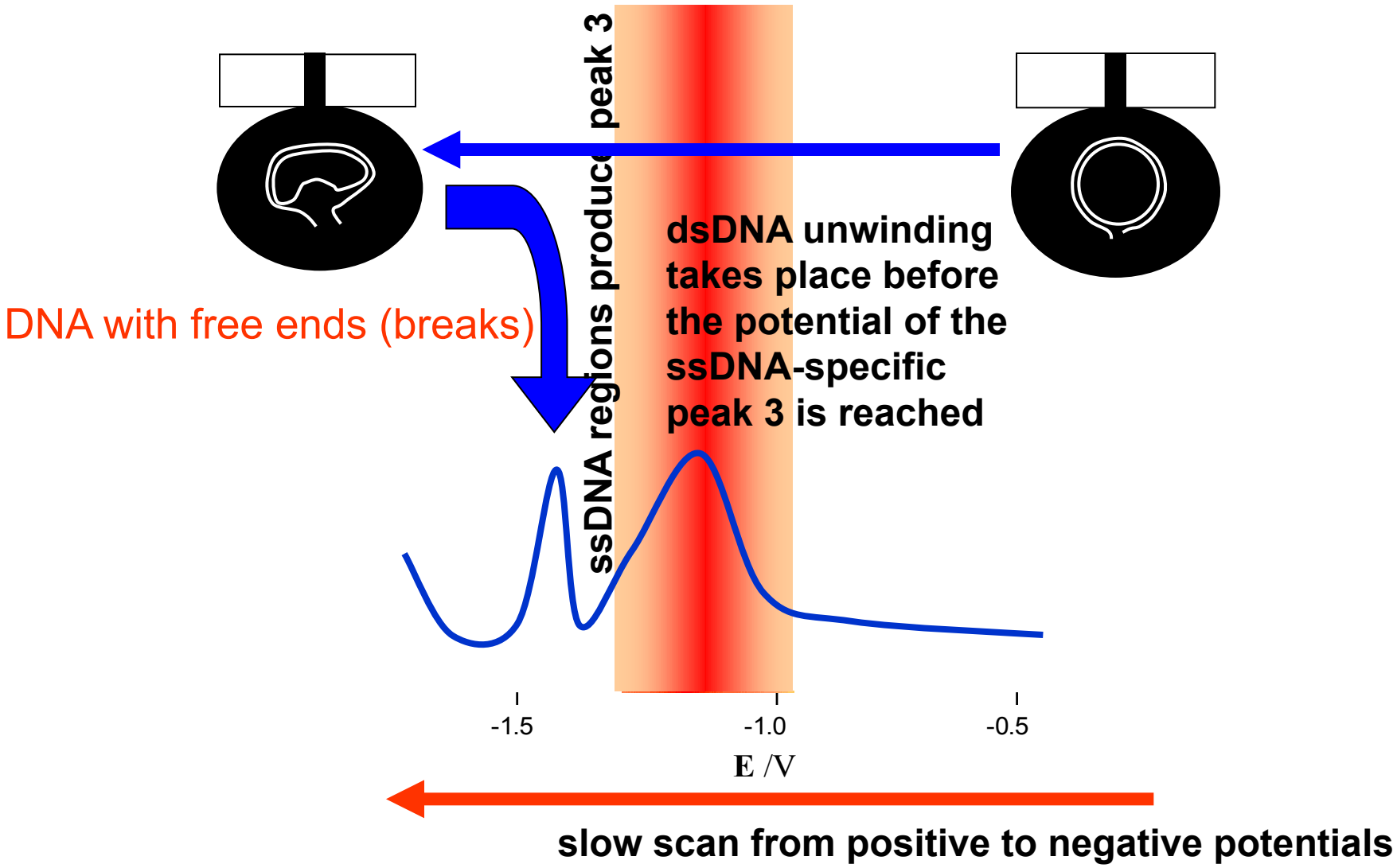




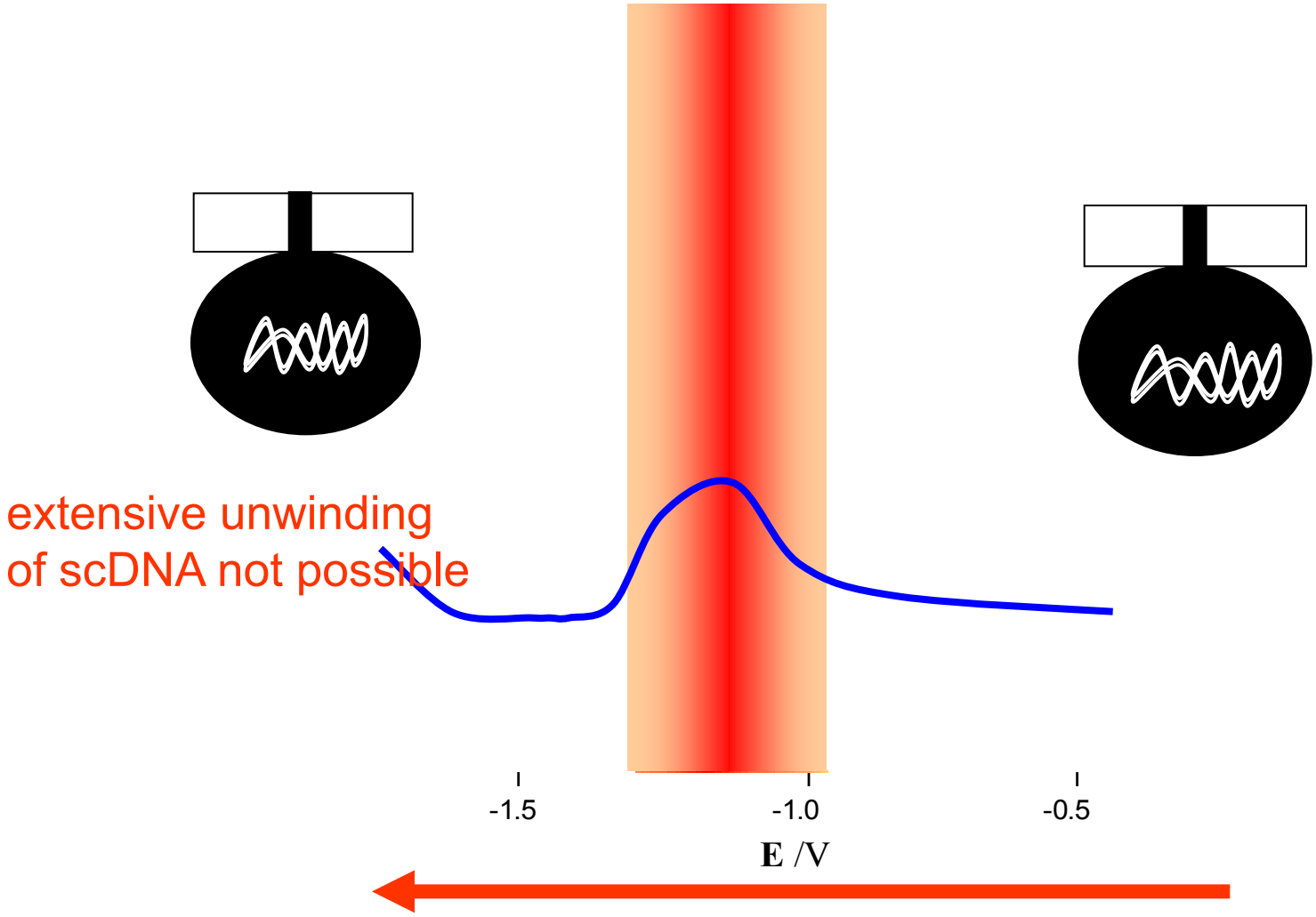


detection of DNA strand breaks using supercoiled DNA and mercury electrodes

surface denaturation of dsDNA at the HMDE within the „**region U**“



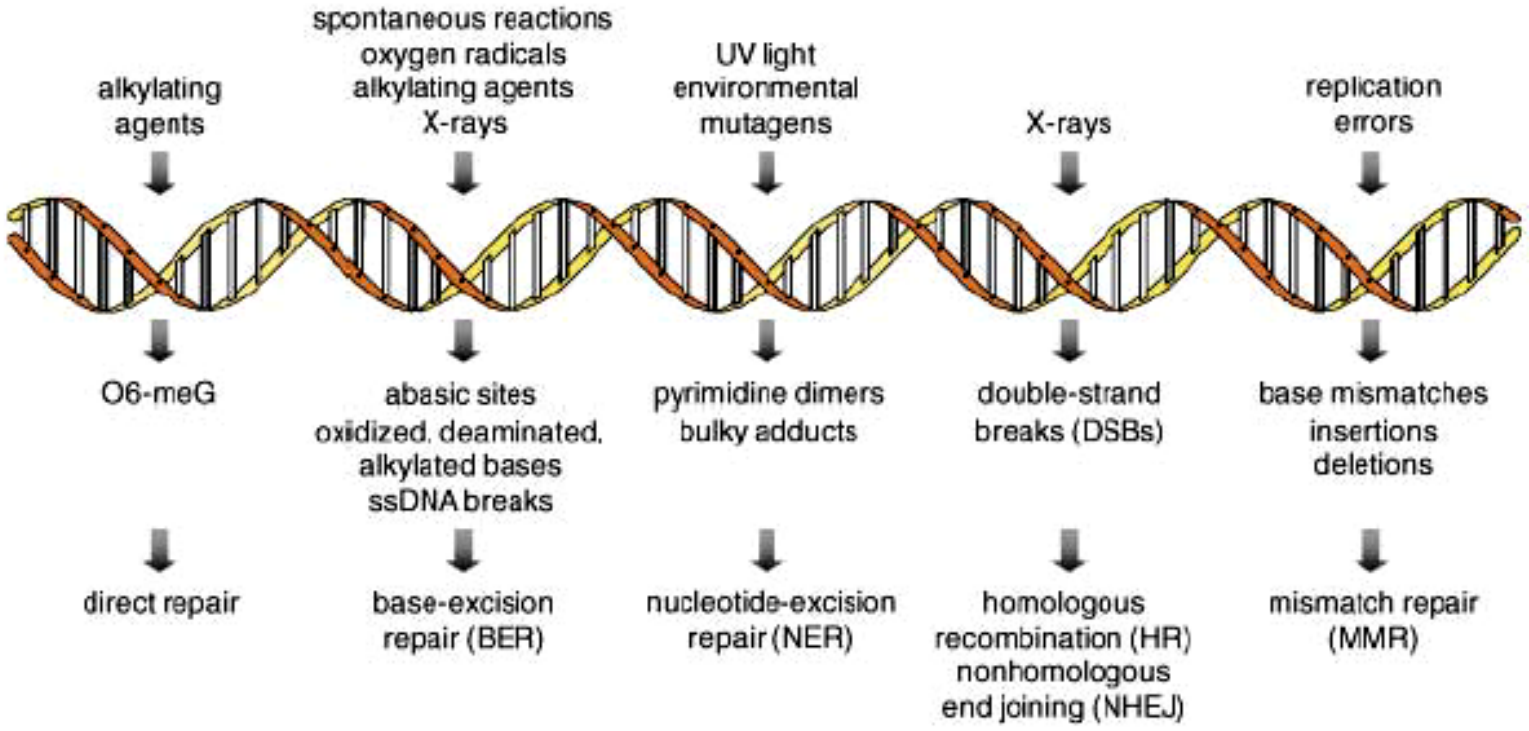
surface denaturation of dsDNA at the HMDE within the „**region U**“



detecting DNA damage

DNA in the cells is permanently exposed to various chemical or physical agents

- endogenous - products and intermediates of metabolism
- exogenous - environmental (radiation, pollutants)



Scharer, O. D. (2003) Chemistry and biology of DNA repair, *Angew. Chem. Int. Ed. 42*, 2946-74.

Most frequent products of DNA damage („lesions“)

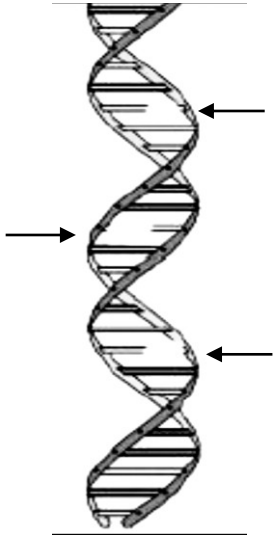
interruptions of DNA sugar-phosphate backbone

single-strand break

double-strand break

- reactive oxygen species
- action of nucleases
- consequence of base damage

interruption of the N-glykosidic linkage



abasic sites

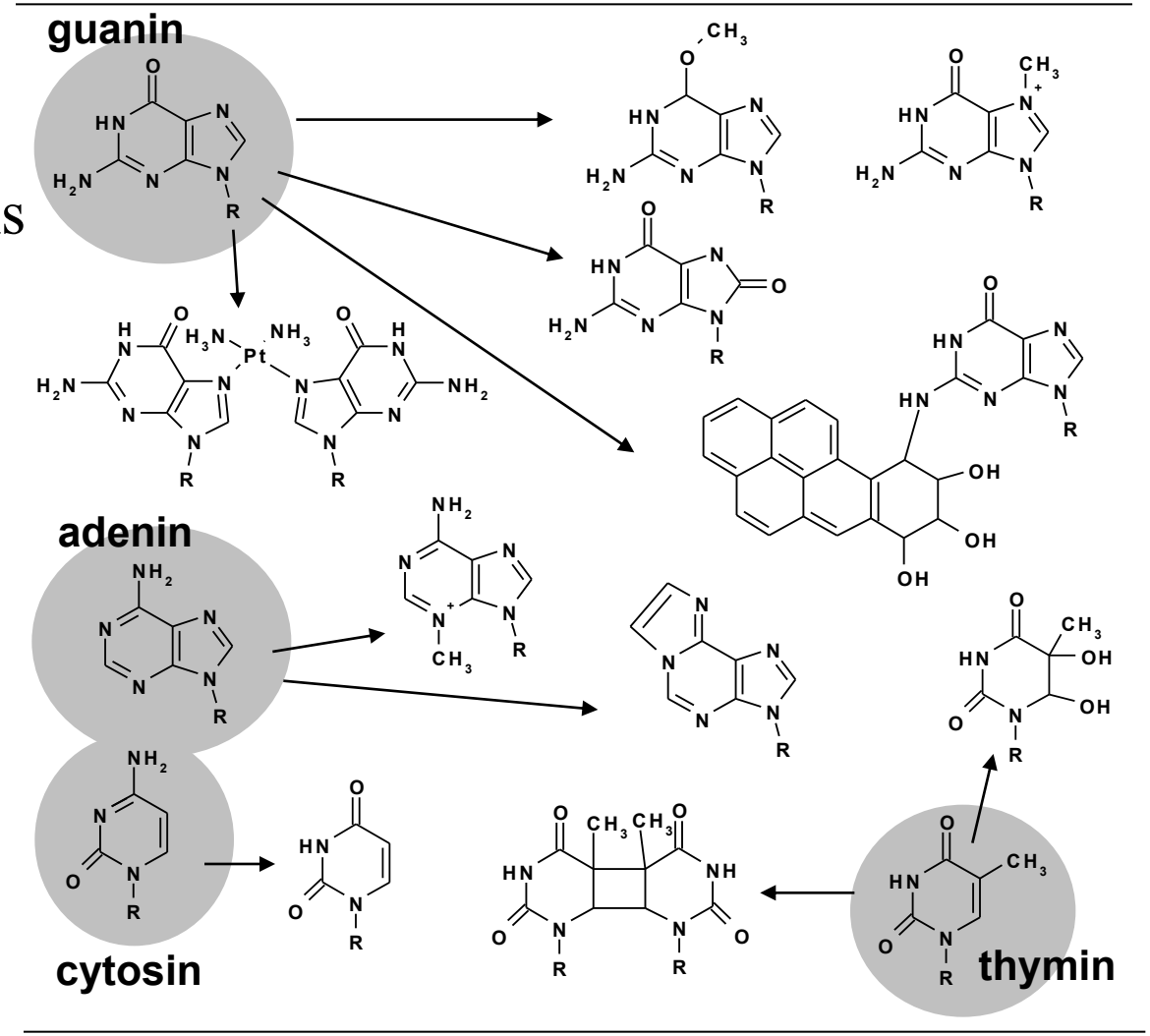
- spontaneous hydrolysis (depurination)
- consequence of base damage

Most frequent products of DNA damage („lesions“)

base damage:

chemical modifications

- **alkylation**
- **oxidative damage**
- **deamination**
- **damage by UV radiation (sunlight)**
- **metabilically activated carcinogens**
- **anticancer drugs**



How to detect DNA damage?

1. Techniques involving complete DNA hydrolysis followed by determination of damaged entities by chromatography or mass spectrometry

2. Monitoring of changes in whole (unhydrolyzed) DNA molecules: electrophoretic and immunochemical techniques

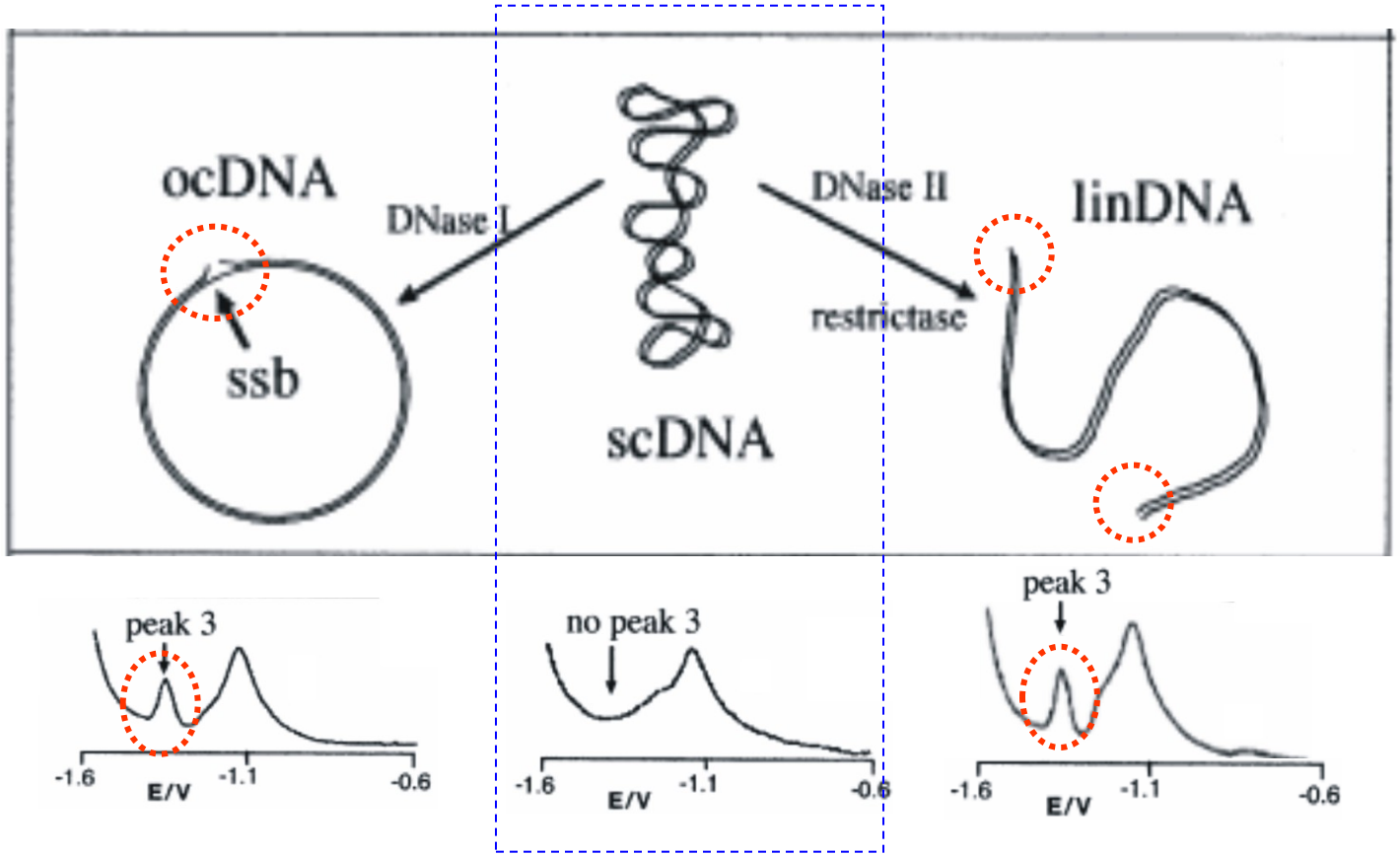
Can electrochemistry help?

chemical modification of DNA can:

- cause strand breakage detectable primarily with mercury (amalgam) electrodes
- cause distotions of the double helix detectable primarily with mercury (amalgam) electrodes
- hit electroactive sites of nucleobases thus affecting their electrochemical activity (mercury or carbon electrodes)
- result in introducing new electroactive moieties (principially any electrode - depending on the electroactive group introduced)

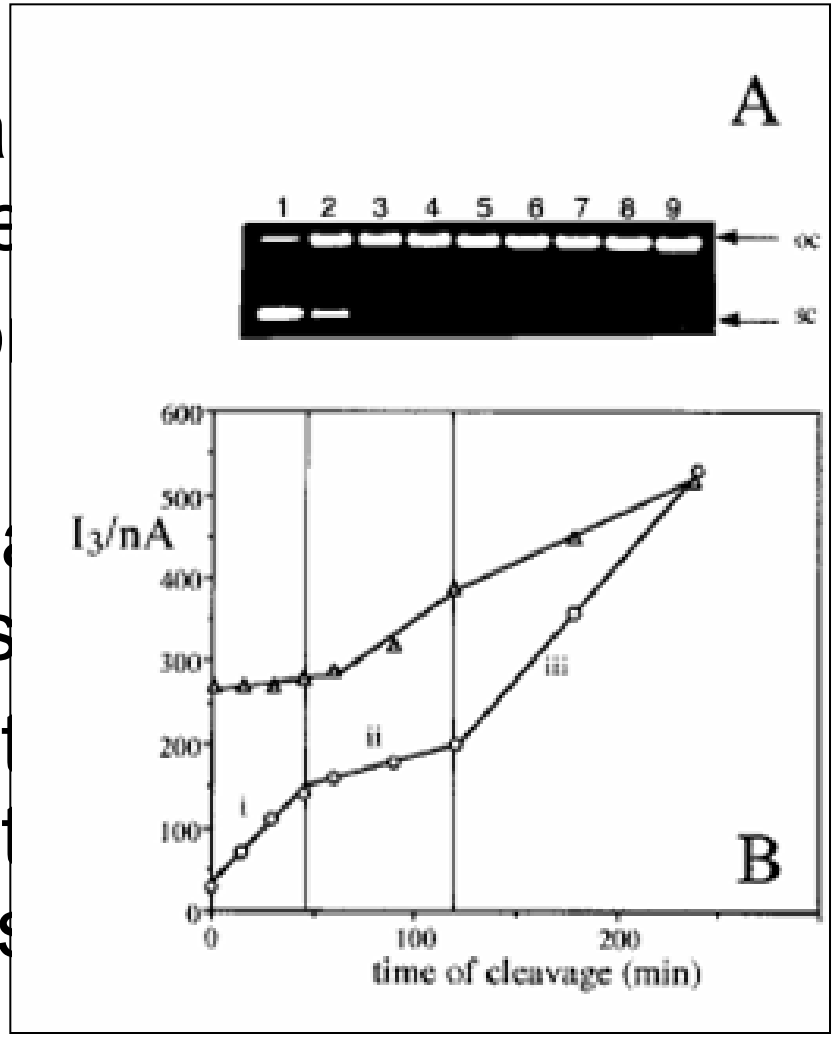
Detecting strand breaks with mercury-based electrodes

difference in behavior of covalently closed circular and nicked or linear DNAs at a mercury electrode

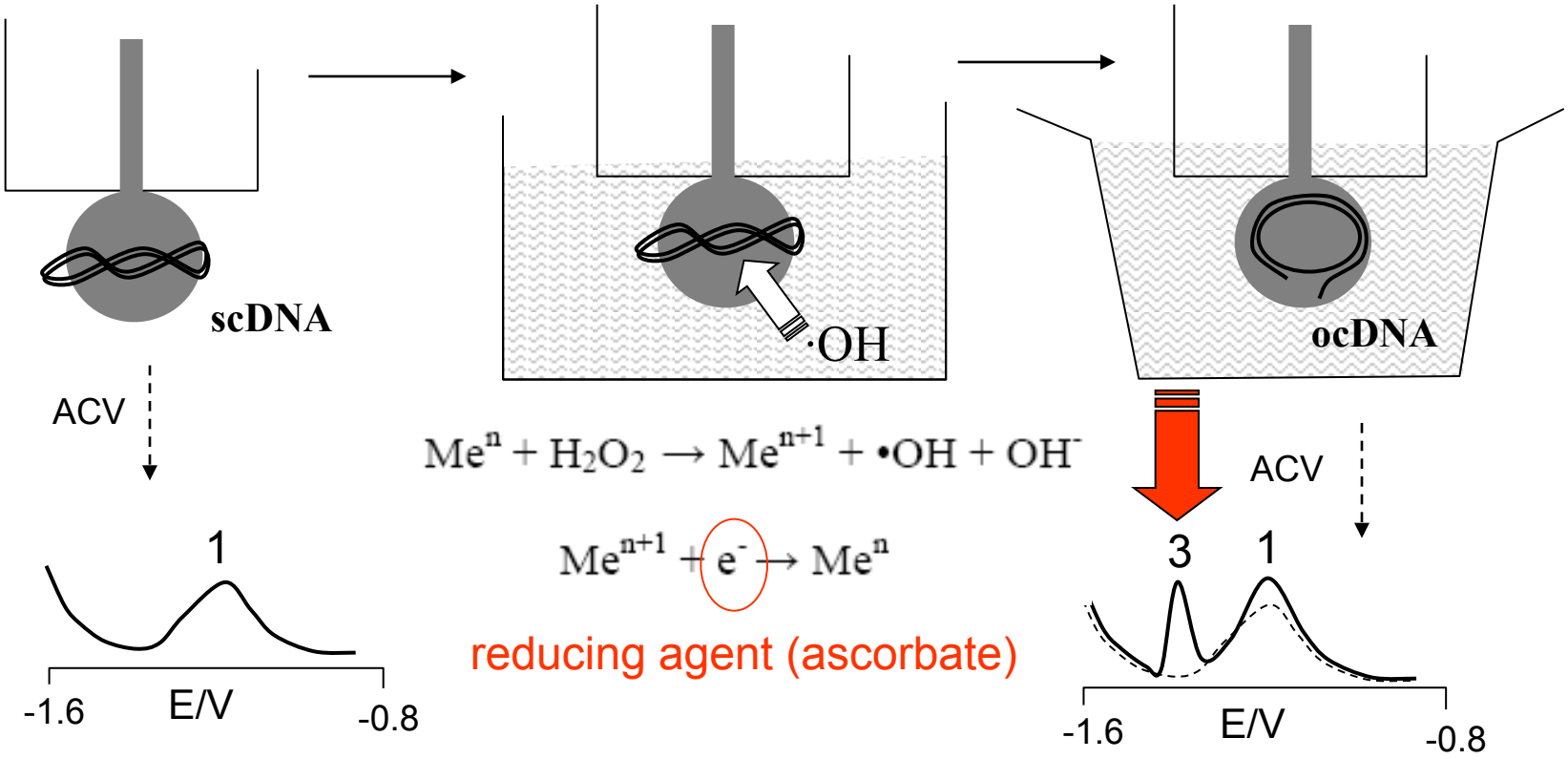


High sensitivity of ssb detection with mercury electrodes

- one break in ~1% of a
  molecules can be dete
- that is one lesion amo
  nucleotides
- 200 ng of DNA per ana
  sensitivity than agaros
- detection of multiple st
  molecule possible (not
  of native electrophores



Mercury electrode modified with scDNA: sensor for DNA damaging agents



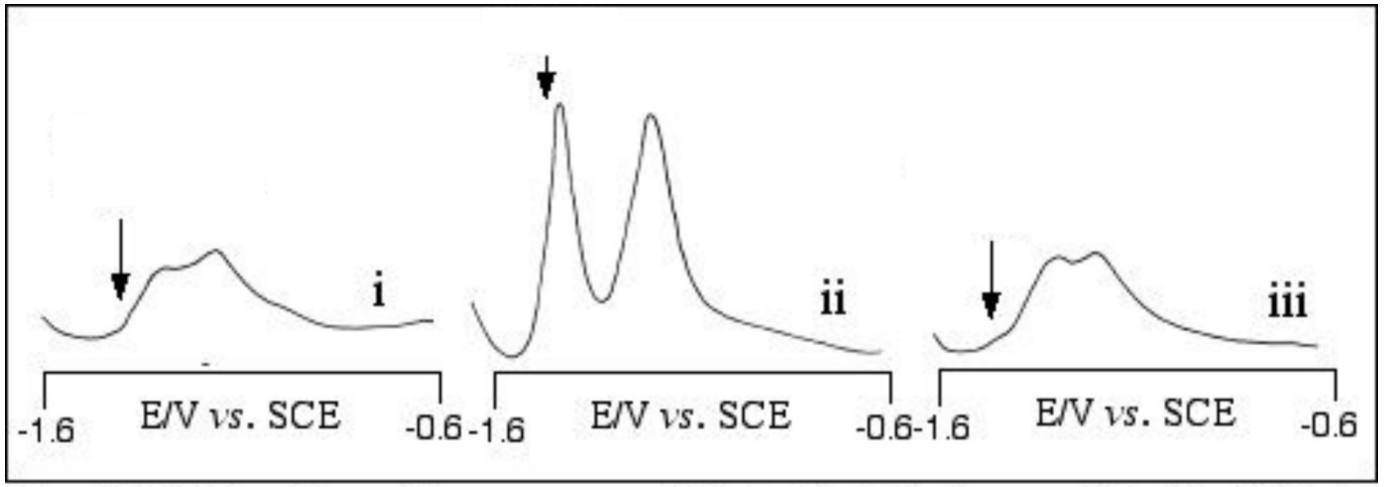
example of the sensor application: detection DNA damaging agents in waste (industrial) waters

(uranium mines, Dolní Rožínka)

blank

mine water – input of purification plant

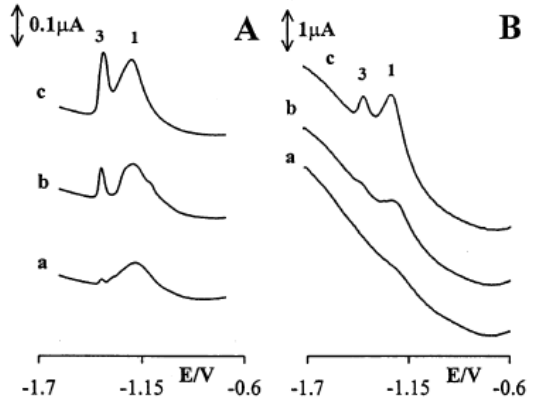
output of the water purification plant



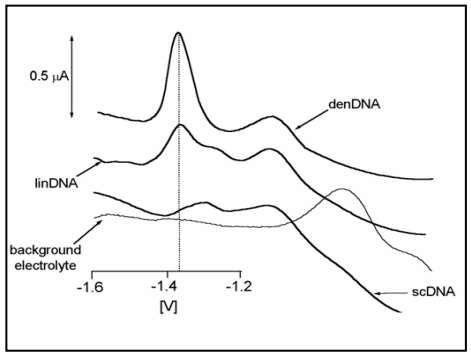
(containing considerable amounts of transition metals like Fe, Mn)

similar responses to DNA damage like with the HMDE can be obtained

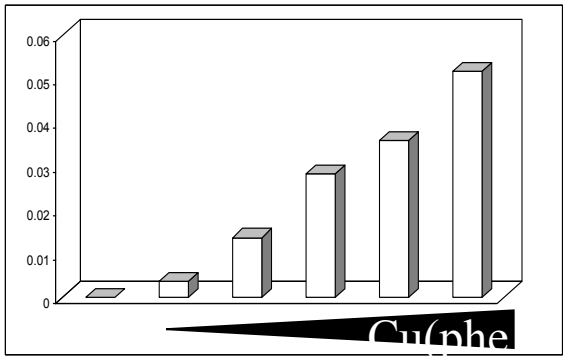
- **with mercury film electrodes (Kubičárová 2000)**



- **with amalgam electrodes (Cahová-Kuchaříková, Fadrná, Yosypchuk, Novotný 2004)**



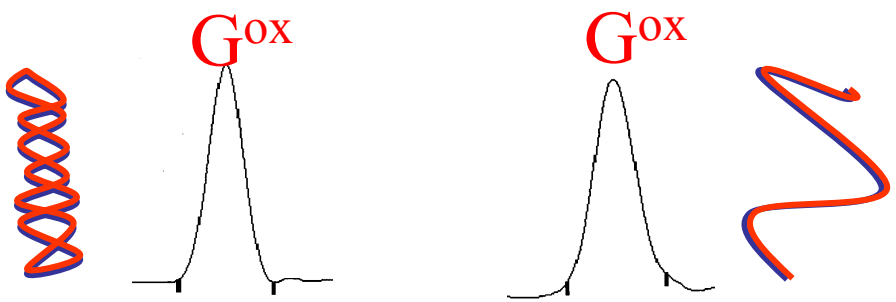
AC voltammograms of sc, linear ds and denatured DNA at m-AgSAE



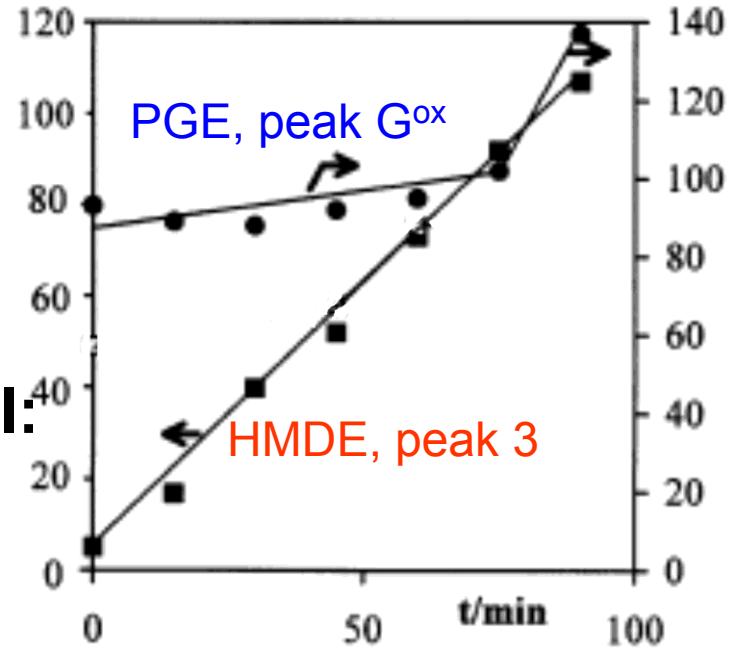
changes in the peak 3 height (at m-AgSAE) due to scDNA exposure to a chemical nuclease $Cu(phen)_2$

guanine oxidation signal **at carbon electrodes** is not sensitive to formation of individual strand breaks

- practically indistinguishable responses of sc, oc and linear DNAs
- small sensitivity to DNA structure: intact dsDNA yields a large signal
- absence of (extensive) surface denaturation of dsDNA at carbon

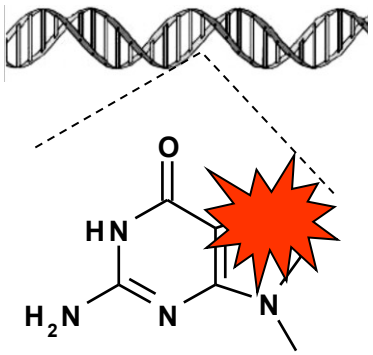


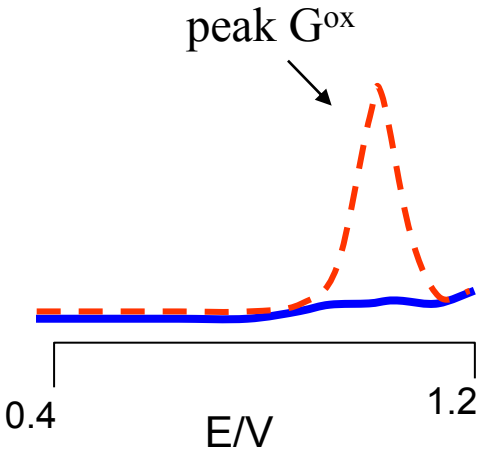
cleavage of scDNA by DNase I:



Damage to DNA bases

- **techniques based on a loss of electrochemical activity of chemically modified bases**
- **usually guanine**





- guanine signals at carbon or mercury electrodes
- alkylating agents, hydrazines, PCBs, cytostatics, acridines, arsenic oxide…

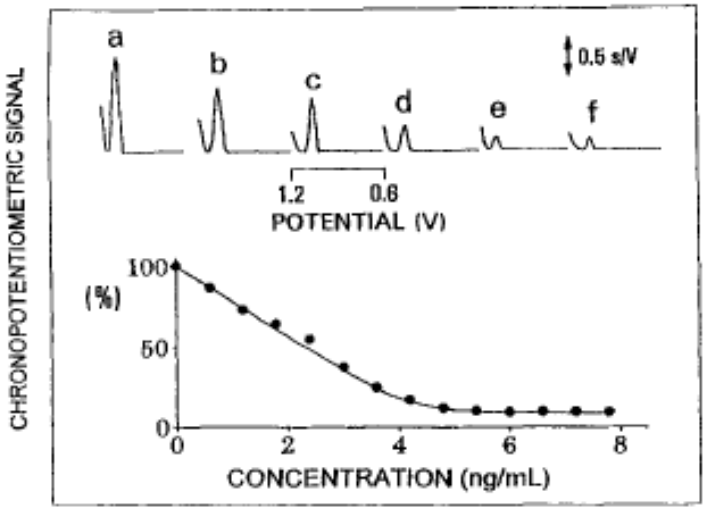


Fig. 6. Chronopotentiometric response of the DNA carbon paste biosensor for increasing levels of dimethylhydrazine in 1.2 μg l^{-1} steps (b)–(f), along with the resulting calibration plot. Also shown (a) is the response of the sensor prior to the hydrazine addition. Interaction time, 10 min. (See [21] for details.)

- **some base adducts yield electrochemical signals distict from those corresponding to the unaffected bases**
- **e.g., 8-oxoguanine**

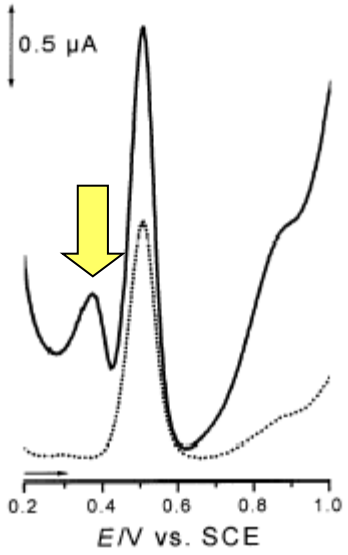


Fig. 8. Differential pulse voltammograms in pH 4.5 0.1 M acetate buffer obtained with a thin layer dsDNA-modified GCE after being immersed in a 5 μM adriamycin solution during 3 min and rinsed with water before the experiment in buffer: (···) without applied potential; (—) after applying a potential of −0.6 V during 60 s. Pulse amplitude 50 mV, pulse width 70 ms, scan rate 5 mV s^{-1}. First scans.

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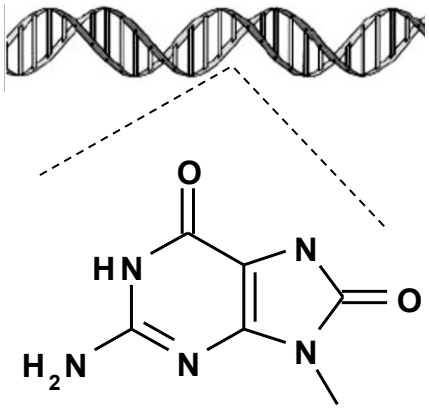
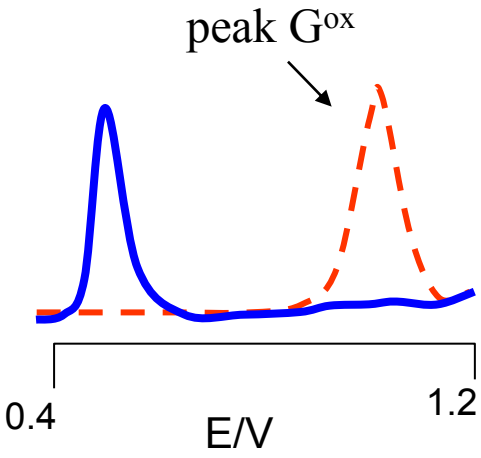
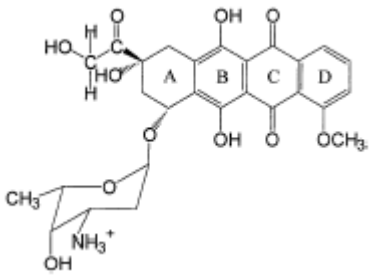




8-oxoG elecrochemically generated in DNA at GCE in the presence of adriamycin (A.M. Oliveira-Brett)



What to do when the DNA damage product of interest:

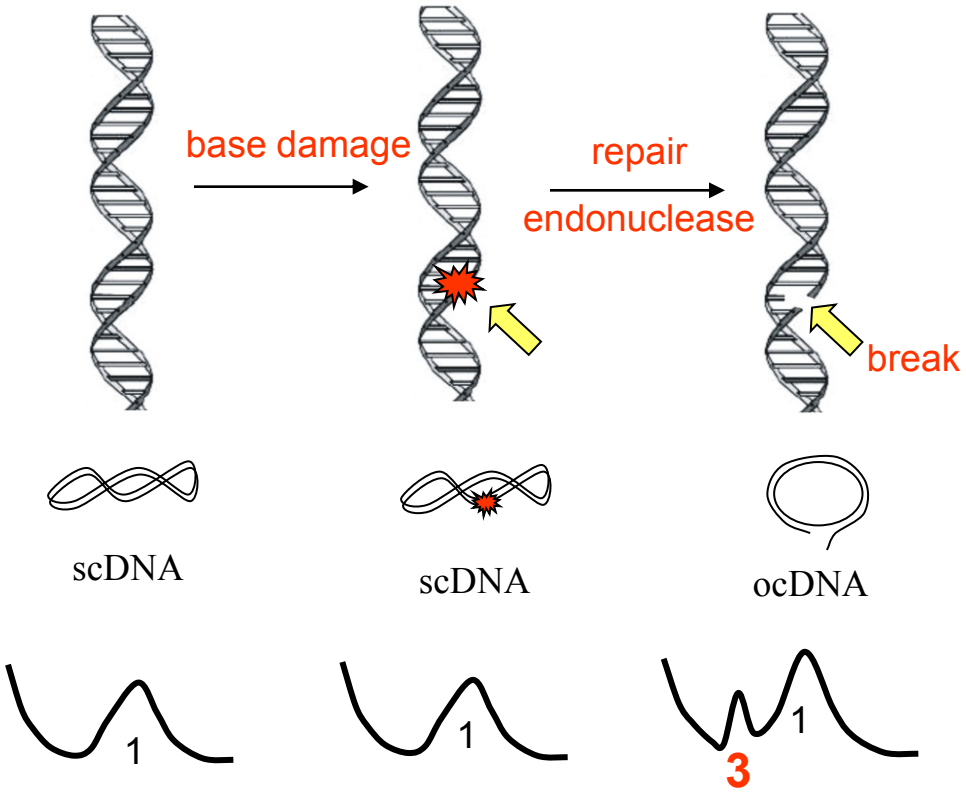
- is not electroactive
- does not affect intrinsic electroactive sites of DNA
- is too rare to be detectable via e.g. degrease of the guanine signal?

Anal. Chem. **2005**, *77*, 2920–2927

Use of DNA Repair Enzymes in Electrochemical Detection of Damage to DNA Bases in Vitro and in Cells

Kateřina Cahová-Kuchaříková, Miroslav Fojta,* Tomáš Mozga, and Emil Paleček

base damage converted to strand breaks
→ sensitive detection at mercury (amalgam) electrodes



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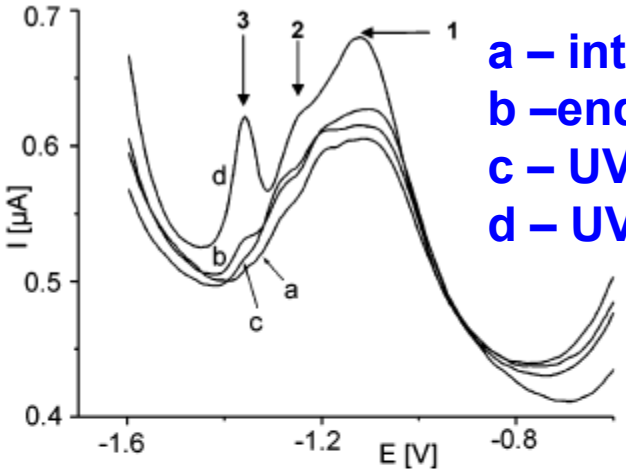
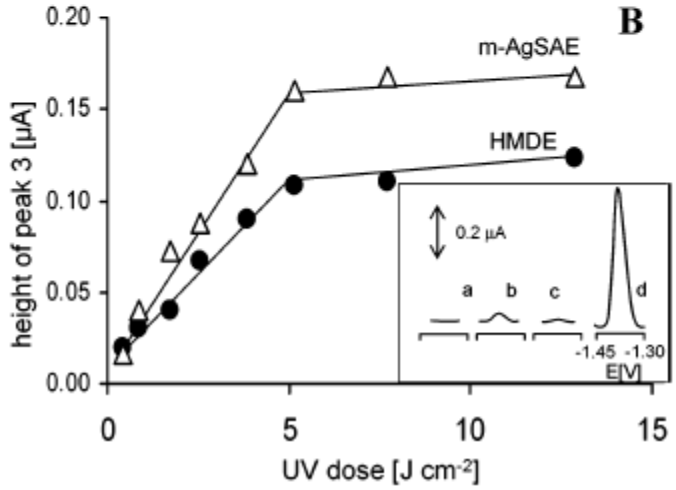
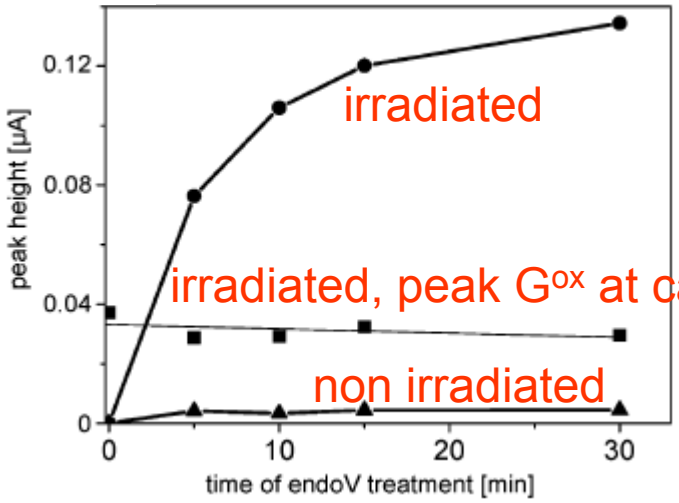
Py dimers detected by endonuclease V



a – intact scDNA
b –endoV treated scDNA
c – UV irradiated scDNA
d – UV+endoV



dependence on UV dose



dependence on enzymatic cleavage time

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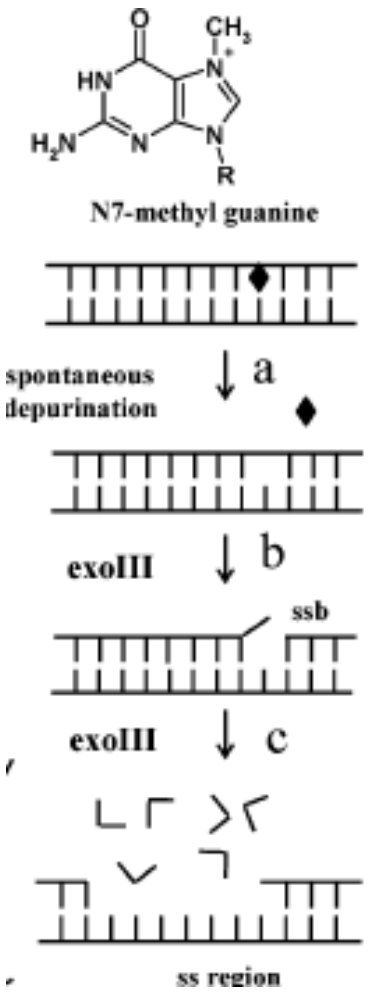
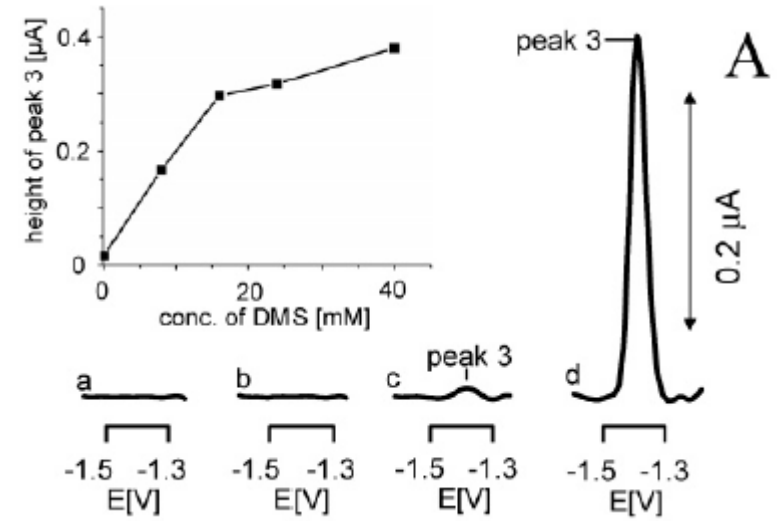
Kateřina Cahová-Kuchaříková, Miroslav Fojta,* Tomáš Mozga, and Emil Paleček

apurinic sites detected by exonuclease III





scDNA | **scDNA +ExoIII** | **scDNA +DMS** | **scDNA +DMS +exoIII**

(peak 3 details)

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Use of DNA Repair Enzymes in Electrochemical Detection of Damage to DNA Bases in Vitro and in Cells

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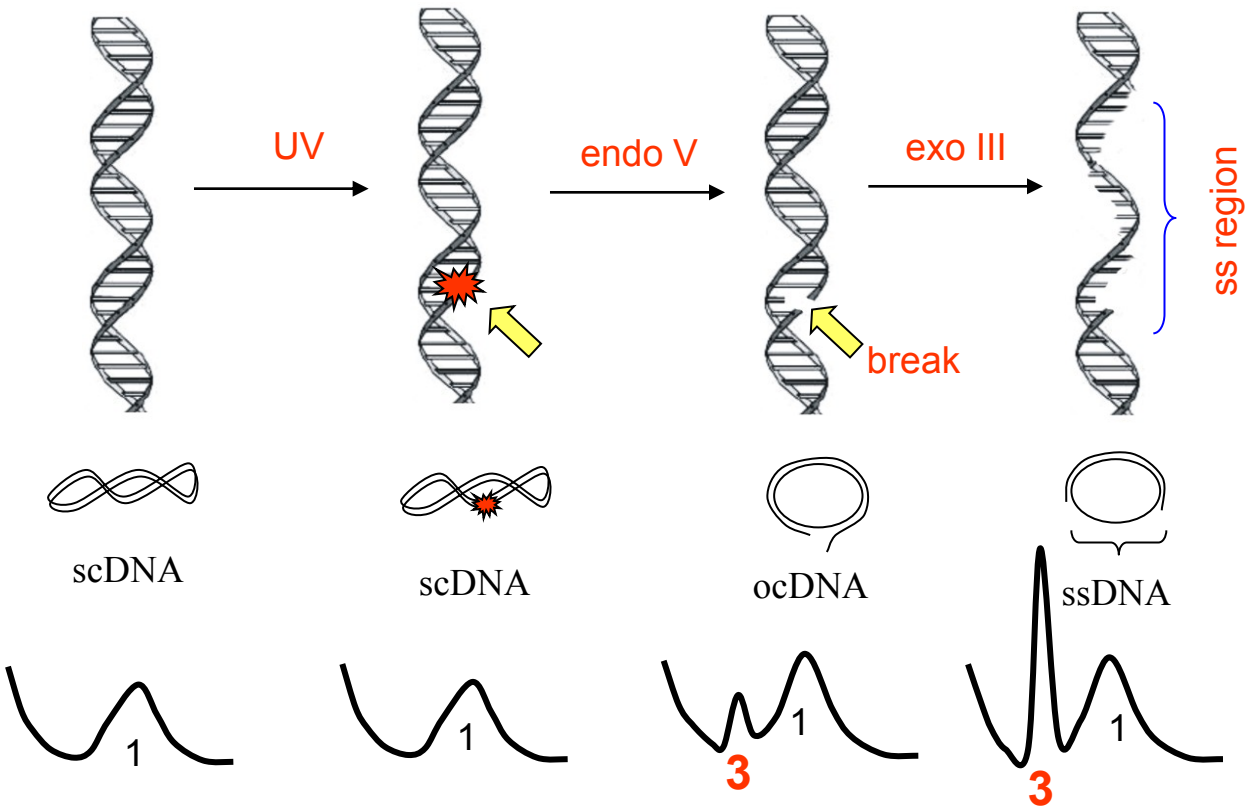
enhancement of the ssb signal using exonuclease III cleavage



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Use of DNA Repair Enzymes in Electrochemical Detection of Damage to DNA Bases in Vitro and in Cells

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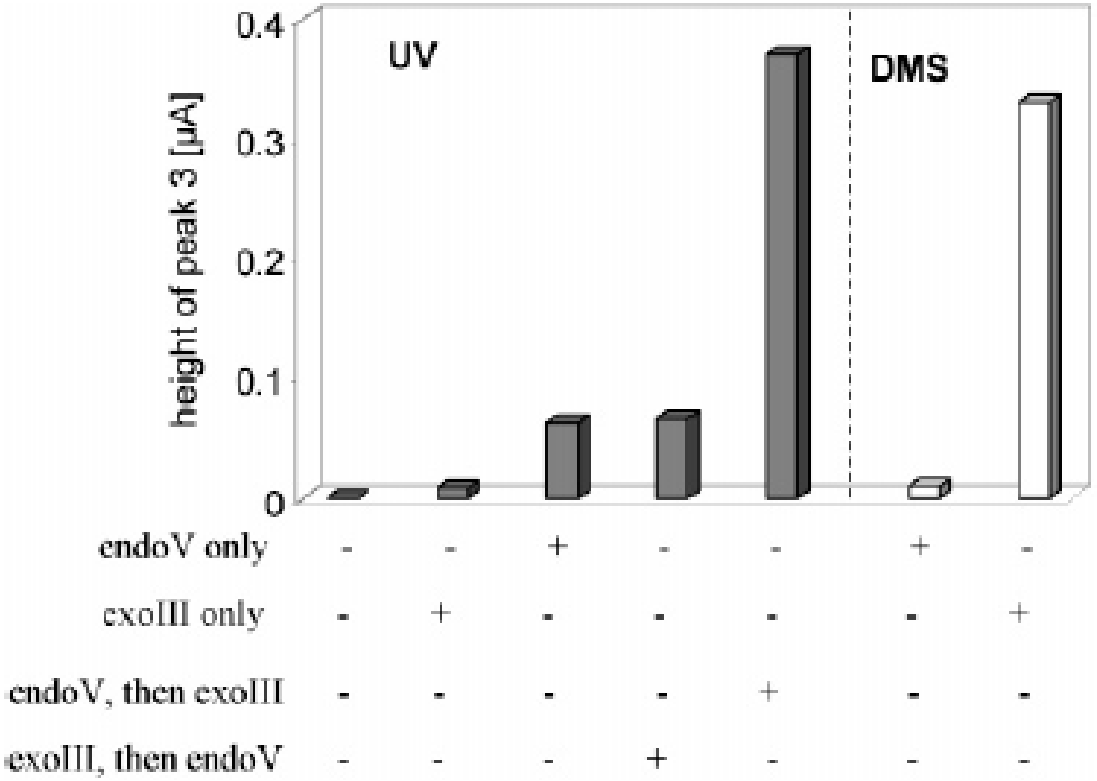
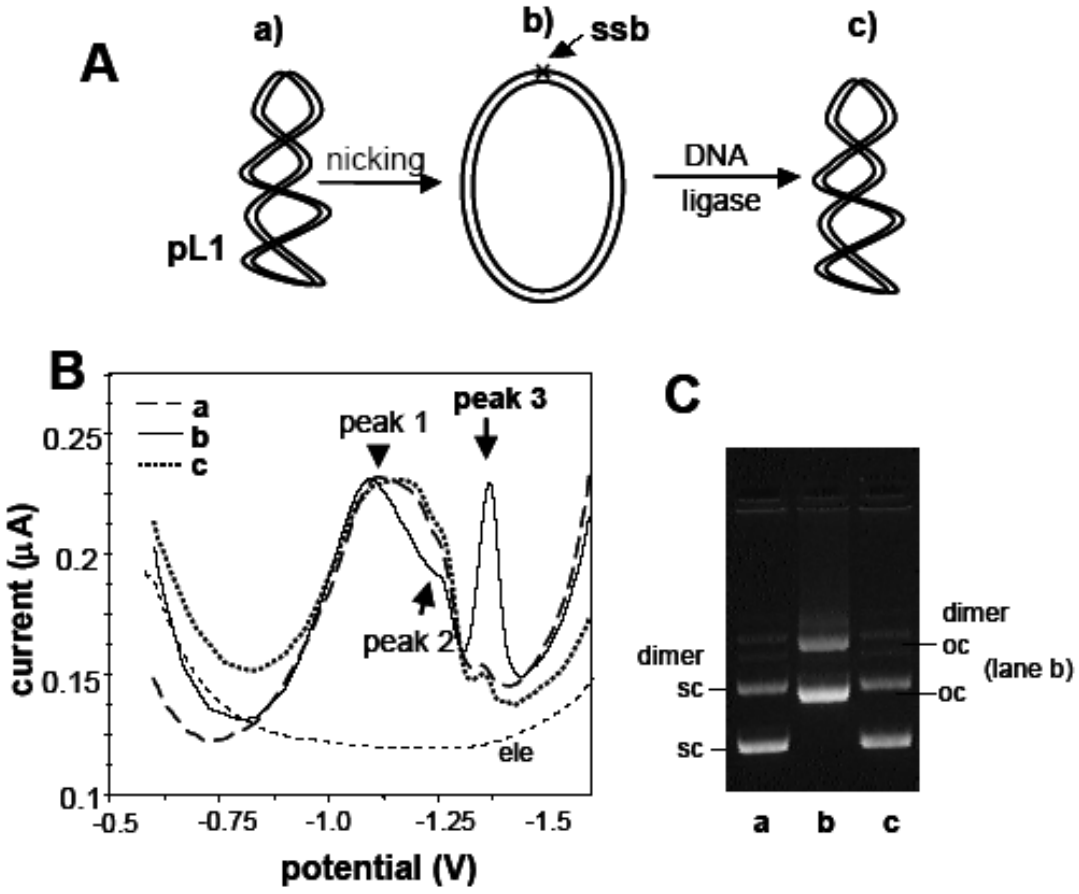
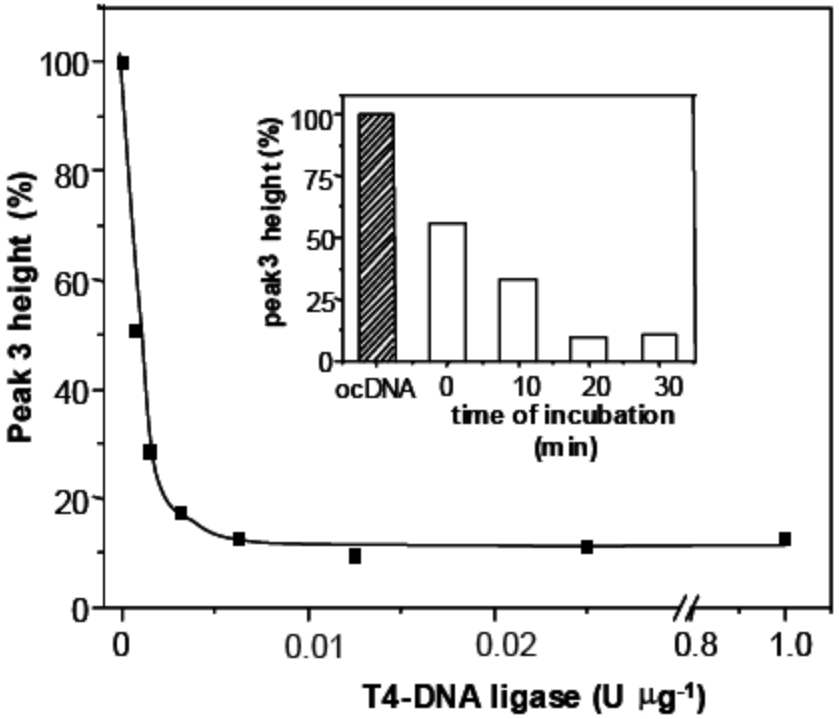


substrate specificity of the enzymes → specificity of adduct detection

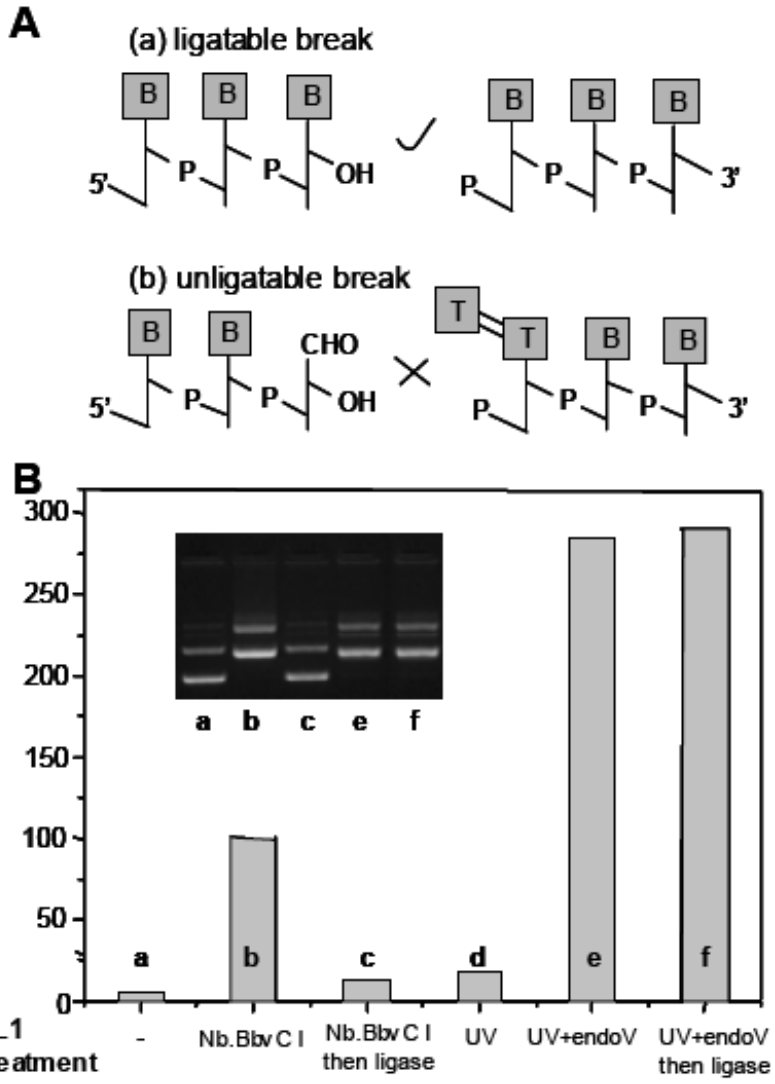
Ligation (repair) of strand breaks





Ligatable and unligatable strand breaks

only 3‘-OH, 5‘-phospho junctions can be sealed directly by ligases



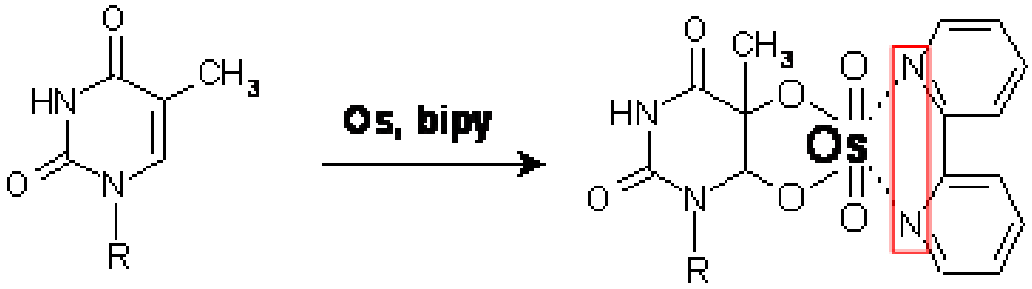
Sensitive detection of strand breaks

(DNA damage convertible to strand breaks)

with carbon electrodes:

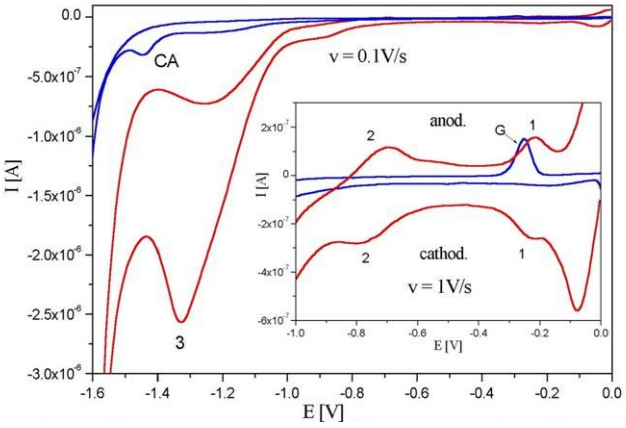
Utilization of an DNA structure-selective electroactive marker

should using „bad dangerous“ mercury be avoided?



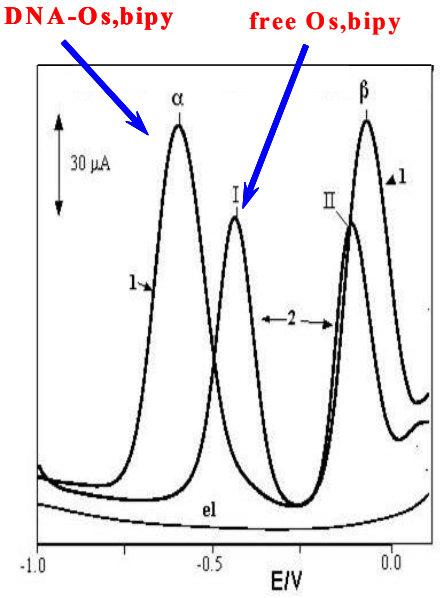
thymine

voltammetric responses of the Os labels



HMDE

- fast reaction with thymine, slow with cytosine, practically no with purines
- DNA structure selectivity: thymines in duplex DNA are protected from modification



PGE

Anal Bioanal Chem
DOI 10.1007/s00216-008-1850-1

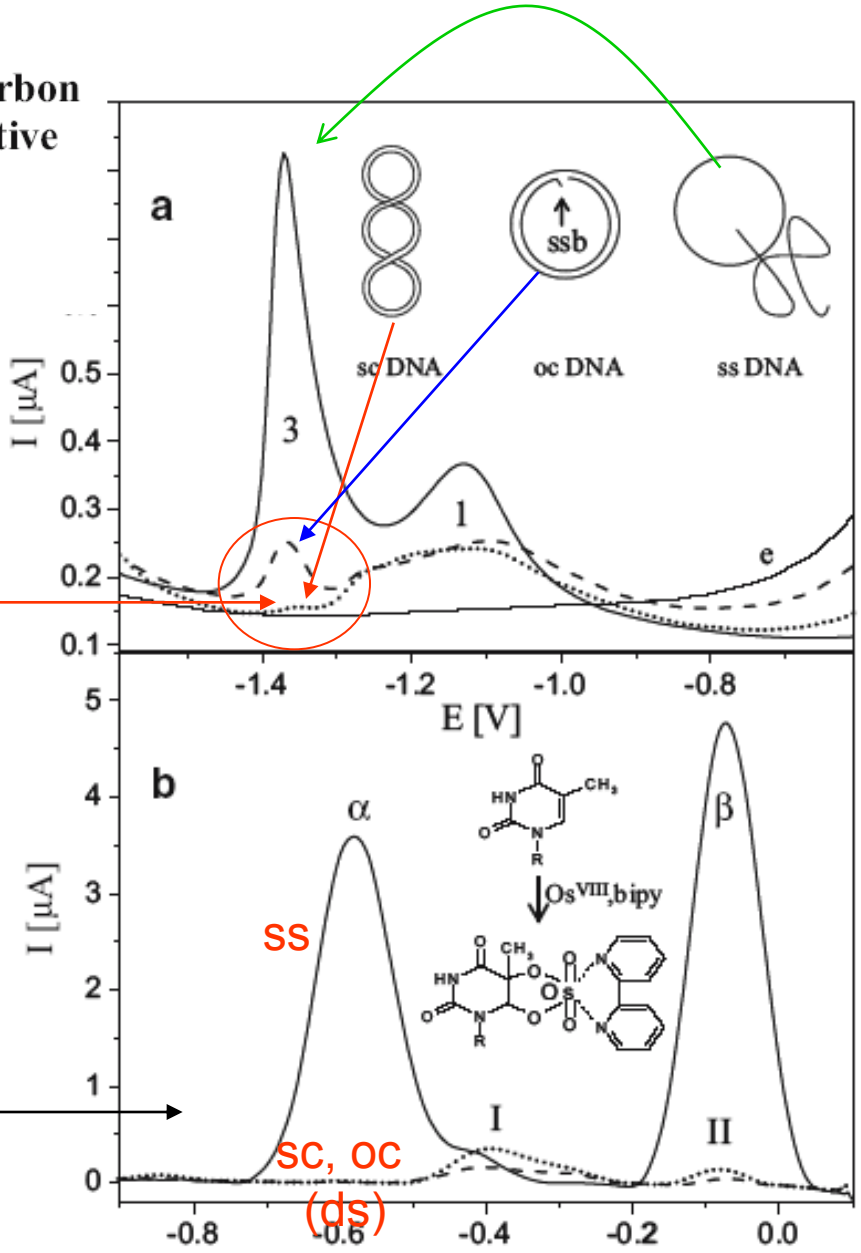
ORIGINAL PAPER

Sensitive voltammetric detection of DNA damage at carbon electrodes using DNA repair enzymes and an electroactive osmium marker

Luděk Havran · Jan Vacek · Kateřina Cahová · Miroslav Fojta

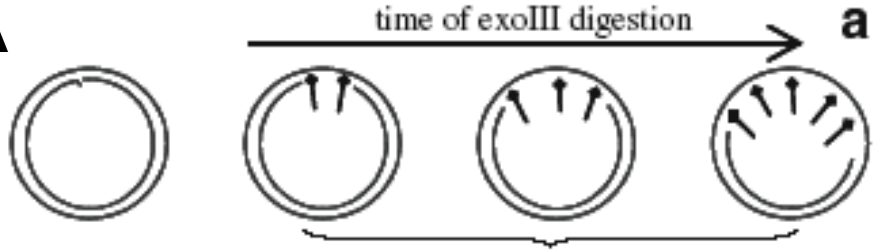
Label-free AC voltammetry at mercury electrode: discrimination between dsDNA without breaks and with breaks

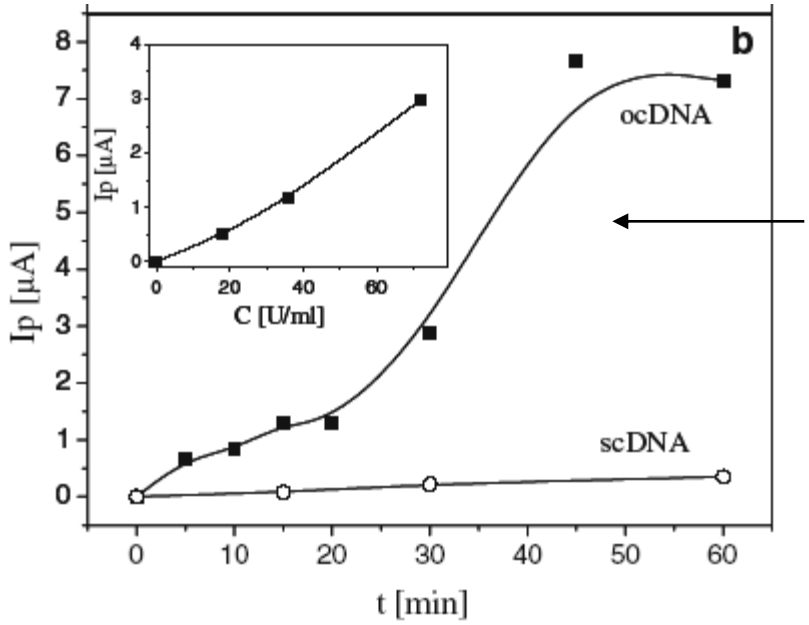
Os,bipy: highly selective for ssDNA – strong voltammetric signal due to ssDNA modification



Creation of ssDNA stretches in dsDNA possessing free ends (=breaks) using exonuclease III

(no cleavage of intact DNA)





exo III, then Os,bipy; SWV measurement

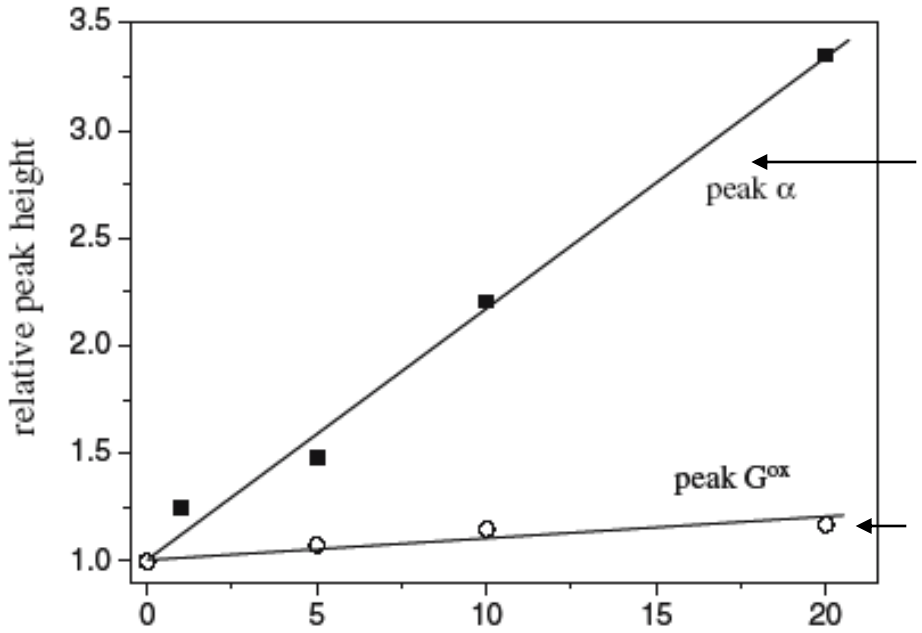
time of enzymatic treatment

- **highly selective for DNA containing breaks or abasic sites (=damaged)**
- **in combination with other DNA repair enzymes, also for other types of nucleobase damage**

- it is the combination of the DNA repair enzyme combined with the chemical probe Os,bipy, not the carbon electrode, what renders the assay highly sensitive!

relative increase of signals:

(mixtures intact/single-nicked plasmid DNA, exo III treatment)



osmium peak
negligible signal for intact DNA, remarkably increasing with number of breaks
→more selective

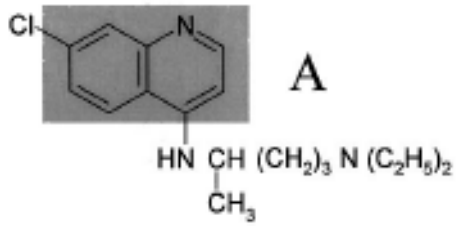
guanine peak (no Os,bipy treatment)
considerable signal for intact DNA, slightly increasing

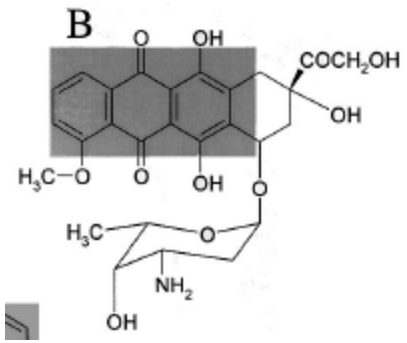
(1 % corresponds to one strand break per 3.10^5 nucleotides)

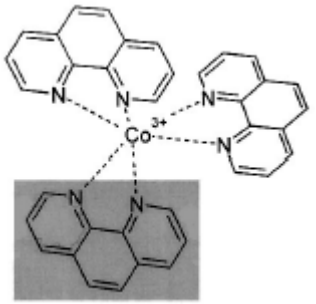
DNA structural changes due to intercalation

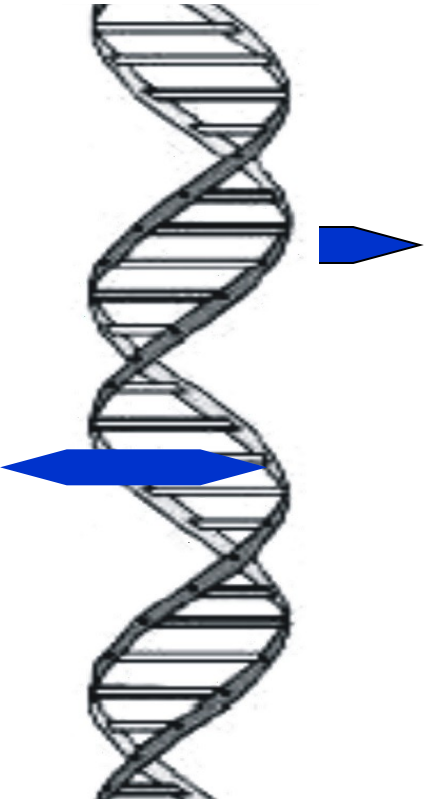
Adsorptive Transfer Stripping AC Voltammetry of DNA Complexes with Intercalators

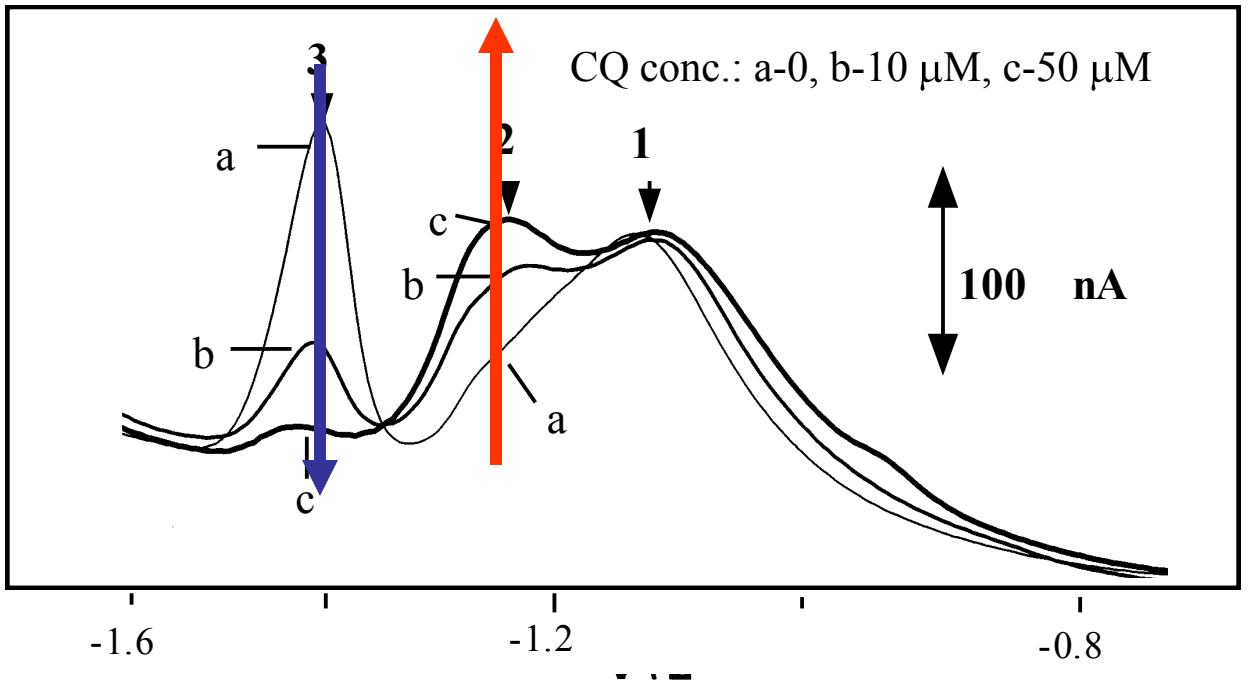
Miroslav Fojta, Luděk Havran, Jana Fulnečková,* and *Tatiana Kubičárová*











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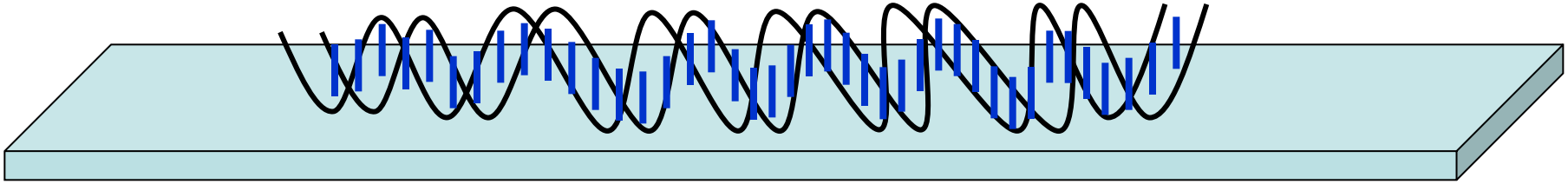
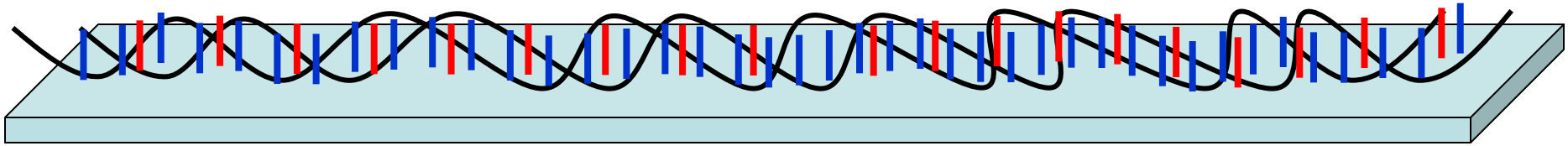
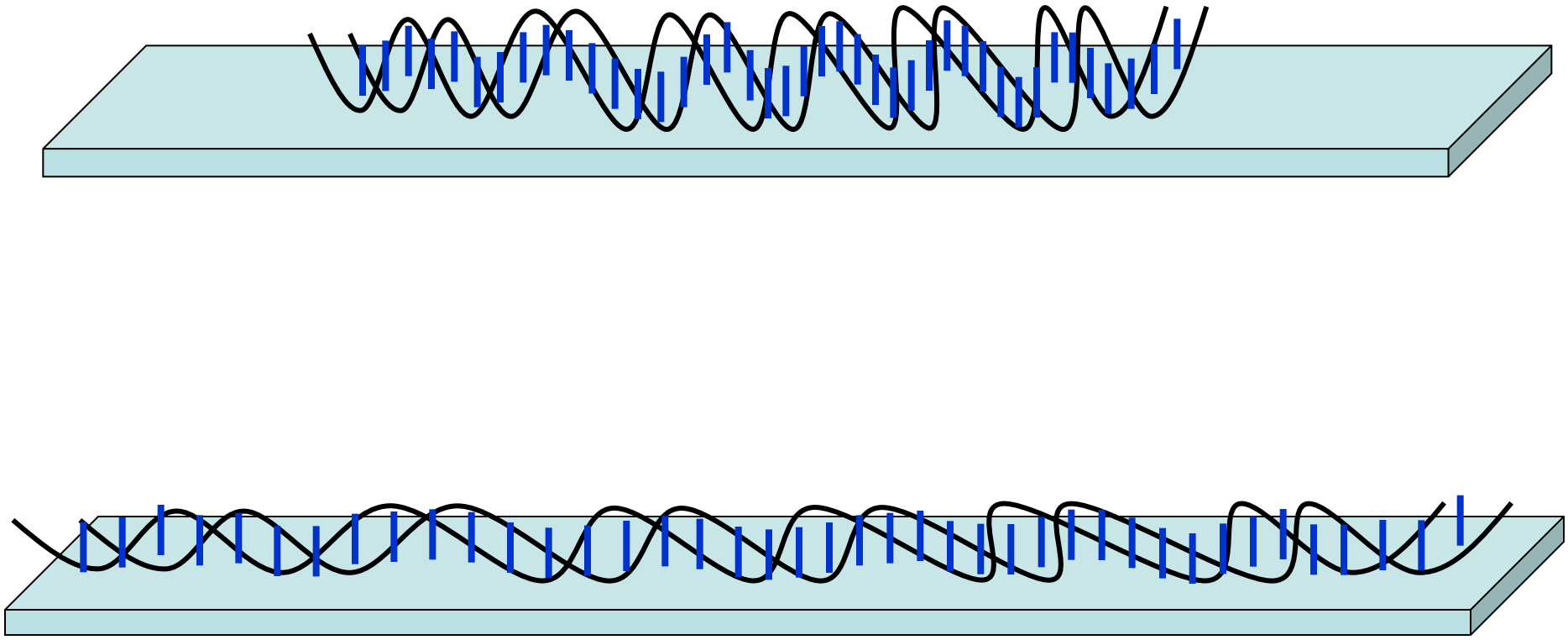


DNA in the absence of intercalator: B-form, bases hidden in the double-helix interior, relatively far from the electrode surface



DNA saturated with the intecalator (**intDNA**): untwisted and lengthened double helix, less deep grooves – bases closer to the surface, contacts between the surface and the base pair edges



after removal of the intercalator, the intDNA conformation is preserved

the adsorbed untwisted regions of intDNA yield the AV voltammetric peak 2