EARLY STAGE OF NUCLEIC ACID ELECTROCHEMISTRY. DETECTION OF DNA DAMAGE IN X-RAY-IRRADIATED RATS

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To the memory of Professor Robert Kalvoda.

First papers on electroactivity of DNA and RNA were published more then 50 years ago. For about 8 years oscillographic polarography at controlled a.c. (OP, proposed by J. Heyrovský already in 1941) was the method of choice for DNA analysis. Since approximately 1954 Robert Kalvoda developed OP for wide application in various fields. It is shown that already before 1960 it was possible to detect damage to DNA in X-ray-irradiated rats by means of OP. DNA samples from irradiated animals produced significantly larger OP anodic guanine signal indicating changes in the DNA structure. At present, radiation-induced strand breaks and damage to bases in DNA can be electrochemically detected at high sensitivity.
Keywords: Electrochemistry of nucleic acids; Oscillographic polarography; DNA damage; Ionizing radiation; Mercury electrodes.

INTRODUCTION

Electrochemistry of nucleic acids (NAs) is now a booming field producing over 700 papers per year[1]. This enormous increase in the number of papers per year took place within two decades, starting in 1990 from ca. 10 papers per year. Various reasons for such a boom could be mentioned but perhaps the most important one was the progress in genomics and particularly in the Human Genome Project[2]. Progress in genomics required new methods for the analysis of DNA and RNA. Attempts to apply optical methods for parallel analysis of NAs were successful and at present DNA arrays with optical detection for highly parallel analysis of NAs are commonly used in large hospitals and research institutes. Electrochemical methods entered the field with some delay[3–5], but at present their outlooks are very good because these methods offer a number of advantages over optical methods.

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Electrochemical approaches appear particularly useful for decentralized DNA analysis and electrochemical sensors can be easily integrated into Lab-on-chips[6–8], because they are simple, inexpensive and easy to miniaturize. While the NA sequences, including detection of point mutations/single nucleotide polymorphisms stay in the main focus of interest (reviewed e.g. in refs[9–21]), electrochemical detection of DNA damage by various factors, including envi- ronmental, chemical and physical agents, can be also very important[22–25].

In this paper I would like to go back to the beginning of the NA electrochemistry, and (i) show that first electrochemical detection of DNA damage was done already by the end of the 1950's by means of the oscillographic polarography with controlled a.c. (OP, i.e., a.c. cyclic derivative chronopotentiometry by the present nomenclature). This result was a part of Ph.D. Thesis and remained unpublished until now and (ii) discuss the role of the OP in the early stage of the DNA electrochemistry and the pivotal role of Robert Kalvoda (RK, Fig. 1) who developed OP for its application in various fields of electrochemical analysis.

Less than 20 years after his invention of polarography Jaroslav Heyrovský (JH) proposed OP as a new method, which differed from the d.c. polarography in a number of points (see refs[26,27] for details). It took little more than 10 years from the publication of the paper introducing OP[28] to the production of the first commercially available instrument "POLAROSKOP P524". Considering that JH's paper was published in the war-time and that the first years in Czechoslovakia after the Second World War were poli-

FIG. 1
Professor Robert Kalvoda at J. Heyrovský Centennial Congress on Polarography (on the occasion of the 100th Anniversary of Jaroslav Heyrovský's birthday)

tically and economically rather difficult, the time from the scientific invention to its commercialization was relatively short. Soon in the 1950's, a group of Czechoslovak scientists was formed using OP in their research, particularly in analysis of low MW compounds. Oscillopolarographic meetings (attended by scientists from different countries) were regularly organized at Smolenice Castle (Slovakia) by RK et al., and attended usually by JH. Surprisingly, many excellent JH's pupils were skeptical about OP, stressing advantages of the classical d.c. polarography, and at the Polarographic Institute in Prague (PI, headed by JH), RK was the only main agent propagating OP.

At present time the RK's name is frequently connected with various aspects of electrochemical analysis such as polarometric titrations in pharmaceutical analysis, application of computers in laboratory and particularly in electrochemical devices, etc.[29–44] He is also well known by organizing exhibitions propagating electrochemistry over the World. For about two decades OP was his main domain; he was greatly interested in capacitive phenomena and in adsorptive stripping methods. In my mind his name was related to the OP [26,45–49] and JH already during my University studies in the 1950's.

As undergraduate biochemistry students in Brno we had access to the PI at Vlašská Street in Prague (next to the US Embassy), where JH and RK had their laboratories. Our schoolmate Osvald Manoušek closely collaborated with Petr Zuman and did a part of his diploma in the same building. As an organic chemist he was excited by the ability of some thiolated substances to form sparingly soluble compounds with the electrode mercury[50]. Main task of my diploma was analysis of horse sera fractionated on $Ca_3(PO_4)_2$ by means of d.c. polarographic Brdička's reaction. This reaction involved hydrogen evolution catalyzed by cysteine-containing proteins in presence of cobalt ions[51–53]. I showed that cobalt can be replaced by Ni^{2+} ions[54]. Using a solid copper amalgam electrode, we showed that various amino acids and other compounds could be detected directly on the filter paper[55]. Amino acids, peptides and other compounds inactive in d.c. polarography yielded interesting OP responses[56,57]. These findings later helped me in my decision to try OP for the DNA analysis.

BEGINNINGS OF DNA ELECTROCHEMISTRY

I joined the Institute of Biophysics of the Czechoslovak Academy of Sciences (IBP) in Brno in 1955 as the only chemist among medical doctors. My task was to study the damage of DNA by ionizing radiation. In the 1950's radiobiology was a “must” at the IBP due to the danger (stressed by the Director of the Institute) that the “Cold War” might turn into a real Atomic War. The equipment available in my lab included simple microscope, slow centrifuge, the U.S. Army Field colorimeter and roentgen apparatus for irradiation of small animals. Using these instruments, it was possible to irradiate rats and isolate DNA from their organs, but it was difficult to analyze the DNA damage. According to the literature among NA bases, only adenine was claimed as polarographically reducible (requiring strongly acidic medium)[58] and DNA was supposed to be polarographically inactive[59]. Considering my experience with d.c. polarography and OP during my diploma I believed that it might be reasonable to try OP for analysis of DNA, RNA and their components. Unfortunately there was no Polaroscope at the IBP. I took advantage of the kindness of the supervisor of my diploma Professor V. Morávek and his assistant Z. Pechan at the Department of Biochemistry of the Masaryk University who allowed me to use their Polaroscope. I performed my measurements after working hours, outside the IBP. After showing my first OP results (Fig. 2) to the Director of IBP Professor F. Herčík, he helped me in borrowing a Polaroscope at the Military Technical Academy to do my work at IBP. Doing OP at IBP was more convenient for me, but

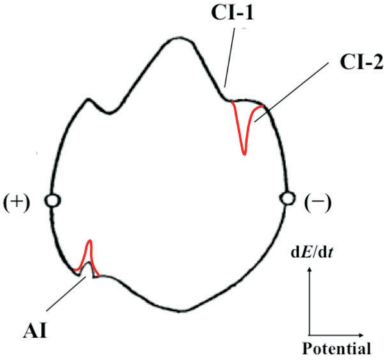

FIG. 2
Schematic representation of oscillopolarograms dE/dt vs E of native double-stranded (ds) and denatured single-stranded (ss) DNA. Indentations of ssDNA are in red. OP indicated that ssDNA is reducible (indentation CI-2) in difference to absence of the reduction signal in dsDNA in which reducible adenine and cytosine residues are hidden inside the molecule forming a part of the hydrogen bonding system. Anodic peak G was assigned to guanine residues representing the so-called “OP artifact” appearing only when the electrode was polarized to strongly negative potentials of the right marginal point

again I had to do it in the late afternoon, because during my working hours I was busy with other things.

DNA DAMAGE BY IONIZING RADIATION

My supervisor at IBP believed that in the radiation sickness, DNA is deprived of pyrimidine bases. He speculated that the sickness might be cured by supplying organisms with pyrimidine nucleoside triphosphates (not available in Czechoslovakia at that time) to improve the DNA synthesis. His conclusions were based on a paper by Berenbom and Peters[60,61] and others[62,63] showing that the base content of DNA isolated from irradiated rats did not follow the Chargaff rules[64] (i.e. that content of purines in DNA is equal to that of pyrimidines) and displayed an excess of purines over pyrimidines. I was skeptical about these results[60,61], but my experiments[65] showed a good agreement with them. Moreover, it turned out that injections of cytosine and thymine deoxyribonucleotides in rats shifted the DNA base content in direction to normal values[66]. I suspected that the Schmidt–Thannhauser method (involving precipitation of DNA with 6% $HClO_4$), used for DNA isolation[61] might catch not only highly polymerized DNA but also DNA degradation products (including large oligonucleotides insoluble in $HClO_4$). After changing the method to that by which only highly polymerized DNA was isolated, normal base DNA base content was found in a good agreement with Chargaff's rules[67].

ELECTROCHEMICAL DETECTION OF DNA DAMAGE BEFORE 1960

Highly polymerized DNA displayed significant differences between OP of DNA samples from irradiated rats and from non-irradiated controls (Fig. 3). The results were included in my Ph.D. thesis defended in 1959 [68]. Changes in the chromatographically-determined DNA base content were published[65–67] while the DNA OP studies were supposed to be published shortly afterwards. But further development of my research of the DNA OP was unexpectedly fast and my OP responses of DNA from irradiated rats were forgotten and remained unpublished. Here I want to show that already in the 1950's it was possible to electrochemically detect DNA damage induced by ionizing radiation. Figure 3 shows anodic parts of oscillopolarogram of DNA samples isolated from irradiated and control (non-irradiated) rats. DNA from irradiated animals displayed significantly deeper (22 mm) anodic indentation AI (assigned to guanine residues) as compared to that (12 mm) of non-irradiated rats. This indentation corresponds to CV peak G, explained by oxidation of the reduction product of guanine residues in DNA[69]. The large difference in the depths of AIs from irradiated and control rats were explained by relatively small changes in the DNA secondary structure induced by irradiation of the animals. The DNA samples shown in Fig. 3 were also measured in 1 M KOH, i.e. in the medium where DNA displayed only non-faradaic capacitive OP signals. In this medium almost no difference between OP signals of irradiated and non-irradiated DNA samples was observed. In 1 M KOH denaturation of DNA took place, disturbing the DNA secondary structure in both samples. Later it was shown that derivative pulse polarography (DPP) and voltammetry with HMDE were very sensitive to formation of single-strand breaks in DNA, representing one type of DNA damage by ionizing radiation[70] (with covalently closed circular DNA a single break among 2 × 10^5 intact phosphodiesteric bonds can be determined[72]). Using DPP we showed that after irradiation relatively short

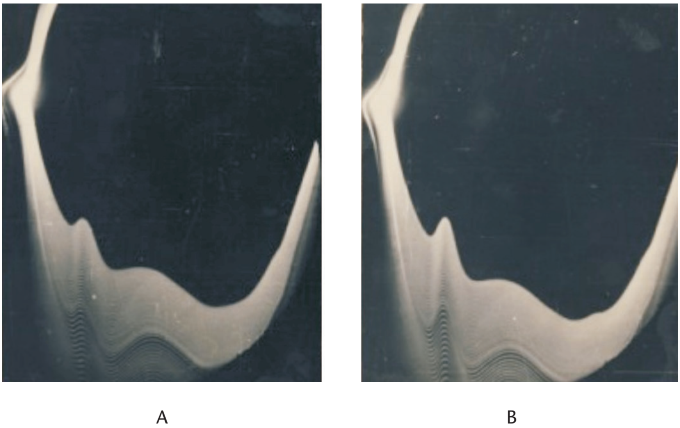

FIG. 3
Anodic parts of oscillopolarograms d*E*/d*t* vs *E* of native double-stranded DNA isolated from spleen of control (A) and X-ray irradiated (B) rats. Wistar rats were exposed to total body irradiation with a dose of 400 r, using a Super Sanax apparatus as described[65]. Highly polymerized DNA was isolated the 5th day after irradiation by Zamenhof's method[71]. Oscillopolarographic measurements were performed at a DNA concentration of 30 µg/ml in 1 M $HCOONH_4$, with a dropping mercury electrode, using 0.4 mA polarizing a.c., open to air. 1 M ammonium formate (pH 5.6) was used as a background electrolyte, in which cathodic indentations CI-1 and CI-2 and anodic indentation AI were displayed (Fig. 2). Cathodic part of the oscillopolarogram is not shown because for obtaining indentation CI-2 (Fig. 2) (specific for single-stranded DNA) the first curve technique was necessary. In 1959 this technique was available at PI[47] but not at IBP in Brno. The reduction of adenine and cytosine residues in single-stranded DNA was completely irreversible and the indentation, which appeared on the first curve, disappeared already at the second curve. This behavior of DNA was later documented by means of CV[69]

pieces of DNA could be split off from the DNA duplex producing pulse polarographic peak III, specific for ssDNA [70]. Of course, *in vivo* DNA damage was caused not only by ionizing radiation and radiation-induced free radicals but also by enzymes acting in the irradiated organism. In addition to strand breaks, ionizing radiation induces also damage to DNA bases but until recently electrochemical determination of base damage was much less sensitive than determination of strand breaks. Recently we showed that using DNA repair enzymes, the damaged bases can be transformed to strand breaks[73] and determined at very high sensitivity[74]. Using this method it was possible to detect UV radiation-induced DNA damage not only *in vitro* but also in cells. To my knowledge this is the most sensitive electrochemical method of the detection of damage to DNA bases. Such electrochemical methods are based on the decrease of the reduction or oxidation signals of bases in DNA (e.g. refs[75–77]) or on appearance of a signal of a modified base, such as 8-oxoguanine[78,79], being by their nature by several orders of magnitude less sensitive than methods based on formation of DNA strand breaks.

REDUCIBILITY OF SINGLE-STRANDED DNA AT MERCURY ELECTRODES

It took some time to collect samples of DNA, RNA and their components. In 1957 I knew that all NA bases are electroactive and that in addition to adenine also cytosine is reducible. Moreover, guanine produced an anodic peak, which was yielded also by DNA and RNA, suggesting that the anodic NA signal is due to a single base type. In the same year the paper was published claiming DNA and RNA as electroinactive species[59]. This discrepancy was recently discussed and clarified[1]. Having my data I visited RK and showed him the oscillograms. He considered my results as interesting (stating: "we have to show it to our prof") and concluded that I should inform JH about my work and immediately arranged my visit to the Director of the PI. JH showed great interest in my work and asked me about publication of my results. I told him that I plan a communication for *Naturwissenschaften*[80] followed by a full paper on NA components in *Collection of Czechoslovak Chemical Communications*[81]. JH made me quite happy by encouraging me in continuing my work, because in that time most of electrochemists were telling me to stop working with DNA which is too long, too slowly diffusing, poorly defined, similar to sheep milk, etc.

I believe for several years I was the only one in the World involved in DNA and RNA electrochemical analysis and for about a decade the only one who claimed that DNA undergoes faradaic processes at electrodes yielding reduction and oxidation signals[82]. In a relatively short time I assigned DNA

OP signals to DNA bases[83,84] and showed that DNA denaturation and its reversal – DNA renaturation/hybridization – can be followed electrochemically[85,86]. Moreover, I demonstrated that traces of single-stranded DNA could be detected in native double-stranded DNA samples by OP [87] and by derivative pulse polarography[88]. The exceptional sensitivity of the electrochemical signals at Hg electrodes for changes in DNA structure[86] resulted in early detection of DNA premelting and polymorphy of the DNA double helix[89].

ROBERT KALVODA AND BEGINNINGS OF NUCLEIC ACID ELECTROCHEMISTRY

In many steps of my electrochemical research, I was in a close contact with the PI and particularly with RK. He, together with Michael Heyrovský and other colleagues, were always ready to help me with advice and to encourage me in continuing my work. Doing my DNA experiments at the PI at Vlašská Street and particularly in RK's laboratory became quite common. For example, I was allowed to measure directly in the JH's laboratory at his multifunctional oscillographic polarograph (some of the oscillograms are in my Ph.D. Thesis[68]) and my first DNA a.c. polarographic measurements showing differences in adsorption/desorption of native and denatured DNAs were done in RK's laboratory[90]. There is no doubt that the scientific atmosphere at the PI strongly contributed to the fact that DNA electrochemistry started already by the end of the 1950's. In that time it was difficult to imagine that electrochemistry of DNA could begin with other but mercury electrodes. On the other hand, many electrochemists believed that d.c. polarography and not OP was the method of choice. In reality d.c. polarography was perhaps the worst way of electrochemical analysis of long chromosomal DNA (MW between 10^7–10^8). Polarographic analysis of such giant molecules (at concentrations used at that time by biochemists using UV absorbance measurements) required at least short accumulation at the electrode. With short oligonucleotides, such as 10 to 20-mers, available at present, d.c. polarography could produce interesting results, but now nobody wants to work with the dropping mercury electrode. On the other hand, mercury-containing solid amalgam electrodes[91,92] (with potential windows similar to those of liquid mercury electrodes) appear more and more useful in voltammetric and chronopotentiometric analysis of NAs [93], proteins[94,95] and carbohydrates[96].

CONCLUSION

In the recent decade, development of electrochemical DNA sensors and sensing assays has made a great progress. Determination of any nucleotide sequence, including detection of single nucleotide polymorphisms/point mutations, in PCR-amplified DNA represents no problem. Recently new methods have been proposed, based on combination of biochemical and electrochemical principles, which allow determination of specific nucleotide sequences in some DNAs and RNAs without PCR amplification. These methods must be usually applied to biological materials, such as blood, urine, saliva and cell cultures. Such analysis can be hardly done with simple probe-modified electrodes or e.g., with gold electrodes using binary layers to shield the electrode surface[97–99]. For analysis of biological materials special approaches are necessary: (a) application of the so-called double surface technique[100–104] in which the DNA capture/hybridization is performed not at an electrode but on a special surface, such as on magnetic beads, at which the non-specific adsorption is minimized; (b) recently proposed ternary layers at gold electrodes[105] allowing analysis in undiluted blood and urine, shows extremely high signal-to-noise ratio and may even surpass in this respect DST with magnetic beads. There is no doubt that electrochemical DNA sensors are coming of age. I would like to remind here that the first important steps towards the present state, such as findings of the DNA structure-sensitive electroactivity[83], detection of DNA denaturation and renaturation[85], covalent electroactive labeling of DNA [106,107], application of solid electrodes in NA analysis[108] and invention of DNA-modified electrodes[109] were done before 1990, i.e. before the year when the boom of DNA electrochemistry began.

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