Electrochemical Reduction Using Glassy Carbon Electrode in Aqueous Medium of a Potential Anti-Chagas Drug: NFOH
Chagas’ disease affects about one-quarter of the population in Latin America. Moreover, according to the World Health Organization, approximately 100 million people live under the risk of contracting this parasitosis and 16–18 million people might be infected. In Brazil, where nearly 6 million people are infected, the main problem concerning treatment is the Trypanosoma cruzi resistance to nifurtimox. As an alternative to this, benznidazole (a 2-nitroimidazole) is the only drug used in Brazil to combat Chagas’ disease. Unfortunately, both drugs are effective only in the acute phase of the disease. Even though, few new compounds have been assayed clinically.

Some new nitroheterocyclic compounds have been proposed as candidate drugs for Chagas’ disease chemotherapy, some of which have a trypanocidal action due to their ability to be reduced by flavoproteins generating the nitro radical anion or forming the superoxide and hydrogen peroxide. Consequently, the electron transfer from the radical to the molecular oxygen causes direct or indirect cellular damage, the former by reaction with various biological macromolecules and the latter by generation of the highly reactive hydroxyl radical. The biological action of nitrofurazone (NF) has been reported on T. cruzi through trypanothione reductase inhibition, an enzyme found in the parasite rather than in the host.

It is generally accepted that the nitro radical anion and hydroxylamine derivative are the main species responsible for some nitroheterocyclic compounds’ cytotoxic action. As a result, electrochemical studies can be relevant for the comprehension of the redox cycles involved in these biological processes. In this sense, the differences in the biological activities of the nitroheterocyclic as anti-microbial agents were explained by electrochemical studies, because significant differences among the reduction potential values of these compounds were registered. Considering Chagas’ disease, the voltammetric peak potential values obtained for several nitrofurans analogs do not unequivocally indicate a relationship with their trypanocidal activities, which demonstrates that other factors such as stereochemistry, lipophilicity, diffusion, and kinetics can determine the anti-Chagas action of these compounds. This indicates that for different biological targets, different physicochemical parameters can set the action of biologically active compounds.

Classically, the kinetic stability of nitro radical anion can be observed by cyclic voltammetry through the one-electron reversible couple due to the redox system R–NO2/R–NO2. For this, a convenient experimental condition (aprotic medium, mixed solvents, alkaline-pH medium), in which the low disponibility of protons favors the R–NO2 kinetic stability, is necessary. Under such conditions, the electrochemical behavior of NF has been extensively studied by using working electrodes such as mercury, gold, glassy carbon, and boron-doped diamond. The molecular modification has been the most promising approach to introduce drugs in therapeutics and to obtain better drugs. Hydroxymethyl derivatives are generally prodrugs which are more hydrophilic than the parent compound. Generally, NH-acidic drugs such as amides, imides, or ureides are potential targets to N-hydroxymethylation.

Hydroxymethylnitrofurazone (NFOH), synthesized from NF and formaldehyde (Mannich reaction), has proven to be a potential drug against Chagas’ disease. NFOH proved to be about four times more effective against T. cruzi than NF, besides being less mutagenic. However, in a previous work, the NF and NFOH electrochemical studies have not indicated significant differences in the ease of their reduction. Despite this, we must keep on studying NFOH due to its importance in establishing the best electrochemical condition which can contribute to a full understanding of its mechanism of action. The existence of a possible additional mechanism of NFOH action, besides the nitro moiety reduction, was already suggested.

We report, in this work, a thorough study of the voltammetric behavior of NFOH (Fig. 1) at a glassy carbon electrode (GCE) in an aqueous medium, with emphasis on the R–NO2/R–NO2 redox couple. The second-order rate constant of the decay of the nitro radical anion and its half-time life have been determined. A brief comparison between benznidazole (MTZ) and chloramphenicol (CFN) voltammetric behaviors by using a carbon-fiber microelectrode is also presented.
Experimental

Reagents and solutions.—Stock solutions (0.01 M) of NF (Avo-
cado Co.) and NFOH were prepared through direct dissolution in
deonized water and ethanol (1:1) using an ultrasonic bath; MTZ
(Rhodia Farma Ltd.) and CFN (Sigma Chemical Co.) were prepared
by dissolution only in purified water. pH study was accomplished
with a universal buffer (Britton–Robinson) starting from the mixture
of phosphoric, acetic, and boric acids with NaOH. All solutions
were prepared using analytical-grade reagents from Merck and pu-
rified water from a Gehaka UV system.

As NFOH is not commercially available, it was synthesized fol-
lowing procedures previously described.5,20 The obtained product
was monitored using thin-layer chromatography (mobile phase:
chloroform–methanol–acetic acid, 85:10:5, v/v/v) and the spec-
tronic data corresponding to the one previously reported.25

Apparatus and methods.—Cyclic and linear-sweep voltam-
mograms were recorded using an Autolab PGSTAT 30 potentiostat/gal-
vanostat from Eco-Chimie, Utrecht, Netherlands, coupled to a 15
mL cell with a three-electrode system: A GCE and carbon-fiber mi-
electrode as working electrodes, Ag/AgCl as a reference elec-
trode, and Pt as an auxiliary electrode. The data acquisition and
treatment were performed using the GPES 4.9 program (Eco-
Chimie). Dissolved air was removed from the solutions by 10 min
bubbling with nitrogen. pH control was carried out by using
Metrohm 654 pH meter and the combined-glass electrode at room
temperature.

The GCE (Ø = 2 mm Analion, Brazil) was manually and
intensely polished with 1 µm diamond suspension in spray on Supra
metallurgical synthetic velvet (granulometry 1/4 µm). The carbon-
fiber microelectrode (Ø = 11 ± 2 µm, BAS) was also polished us-
ing the same procedure. These treatments were made by using prod-
ucts from Arotec S/A, Brazil. After polishing, both electrodes were
rinsed with purified water.

Using the theoretical model,26 the \(I_{\text{NO}}/I_{\text{NO}}\) values were mea-
ured at each scan rate and inserted in a work curve to determine the
\(\omega\) parameter, which incorporates the effects of rate constant, drug
centration, and scan rate. Considering \(\sigma \tau = 4\), the \(\omega\) vs \(\tau\) plot
results in a linear relationship that can be described by the equation
\(\omega = k_2C_D\tau\), where the \(k_2\) value (the rate constant of the second-order
reaction for the nitro radical anion decay) is obtained from this plot
slope. \(C_D\) is the drug concentration, and \(\tau = (E_{1/2} - E_A)/v\). The nitro
radical anion stability was calculated by the half-life equation
\(t_{1/2} = 1/[\text{drug concentration}]\), assuming that \([\text{drug concentration}] = \text{drug concentration}\).

Results and Discussion

As already mentioned, NFOH was obtained by reaction with
formaldehyde via a Mannich reaction. Consequently, it could be
converted back to NF at physiological pH.19 However, the conver-
sion of NFOH to NF is extremely slow at pH 7.4 (half-life of 134 h
at 37°C) but quite fast in acidic medium (half-life of 1.5 h at pH
1.2).27 Therefore, we hypothesize that there was no conversion of
NFOH to NF during the electrochemical experiments at pH values
higher than 8 because all solutions tested were freshly prepared
before each voltammetric measurement.

The cyclic voltammogram of NFOH in aqueous media showed
two reductive peaks corresponding to hydroxylamine (RNHOH) and
amine (RNH₂) formation, usual for nitrofuran compounds.27-29 At
pH 7.4 and a scan rate of 0.1 V s⁻¹, the −0.469 and −0.951 V
potential peak values were, respectively, registered. The number of
electrons transferred during the reduction process was estimated by
comparing the NFOH limiting currents with those obtained for MTZ
and CFN at the same concentration.27-29 The electrochemical reduc-
tion for both latter drugs involves four electrons at pH 7.4.12,13,16,17
As these nitro compounds and NFOH have similar diffusion coeffi-
cient values, the comparison of the limiting currents \(I_L\) obtained
by linear-sweep voltammetry at microelectrodes29 is a good approxi-
mation of the number of electrons involved. Figure 2 shows the
linear-sweep voltammograms of the studied nitro compounds using
a carbon-fiber microelectrode. The \(I_L\) values are closely related \(I_L\)
NFOH/\(I_L\) MTZ and \(I_L\) NFOH/\(I_L\) CFN are close to 1, indicating that
these compounds have similar reduction processes, involving the
same number of electrons, which confirms the previously published
results.16,17 Therefore, it can be assumed that the hydroxylamine
derivative is the main product of NFOH voltammetric reduction.

As observed in Fig. 3, the NFOH reduction peak obtained in an
acidic medium is split at pH 7.20. At this pH, a shoulder appears
around −0.5 V and becomes a peak at pH > 9.2. A corresponding
oxidation peak becomes visible at pH > 7.58. A similar voltammet-
ric behavior was observed for 5-nitromidazole under the same ex-
perimental conditions (results not shown). This reduction peak is
due to the reversible one-electron reduction to the radical anion and the other peak is due to the further three-electron reduction of the radical anion to the hydroxylamine:

$$\text{R–NO}_2^+ + 3e^- + 4\text{H}^+ \rightarrow \text{R–NHOH} + \text{H}_2\text{O}$$

Figure 4 clearly shows that the NFOH voltammetric reduction is pH dependent. The $E_{p1}$ values are linearly ($\Delta E_{p1}/\Delta \text{pH} = -49.7$ mV/pH) shifted to negative potential values in an acidic medium. However, it is evident that a distinct process occurs on the working electrode at pH values higher than 7.58. The reduction peak ($E_{pnc}$) due to the one-electron reduction to the radical anion appears at more positive potentials. $E_{p1}$, due to the three-electron reduction to the hydroxylamine, is shifted to more negative potentials with increasing pH but not linearly. In contrast, $E_{pnc}$ does not change with pH. Therefore, the potential difference between $E_{p1}$ and $E_{pnc}$ becomes larger as the proton availability decreases. As already proposed, the three-electron reduction step to the hydroxylamine most probably involves first the protonation of the radical anion, which is followed by further reduction of the radical. The shift of $E_{p1}$ to more negative potentials in the case of the GCE might be due to competitive adsorption, preventing the nitro radical anion surface protonation. The performed comparisons with mercury and gold electrodes confirm this behavior. Moreover, the voltammetric study of nitrofurans has demonstrated that the nitro radical anion was not sufficiently and kinetically stable to produce a couple using a mercury electrode in protic medium, even at alkaline pH. The behavior herein described shows that the GCE polishing using diamond powder produced a similar effect to that observed in an aqueous medium after the alumina removal from the electrode surface by sonication in ethanol.

Figure 5 shows the first reduction step cyclic voltammogram of NFOH at pH 10.2, corresponding to the R–NO$_2$/R–NO$_2^-$ couple, with $E_{pnc} = -0.512$ V and the reverse anodic peak with $E_{pna} = -0.391$ V. While the $E_{pnc}$ values did not change with scan rate, the cathodic peak current ($I_{pnc}$) increased linearly with $v^{1/2}$ (0.3 ≤ $v$ ≤ 2 V), indicating that the process is controlled by diffusion according to $I_{pnc}(\mu \text{A}) = 11.7v^{1/2}/(\text{s}^{1/2})$; $r = 0.998$. Although the $\Delta E$ values are large, by applying the Randles–Ševčík equation ($I_p = -2.69 \times 10^8 n^{1/2}A^{1/2}/C_{\text{NFOH}}^{1/2}$) and using the NFOH diffusion coefficient predicted ($6.72 \times 10^{-6}$ cm$^2$ s$^{-1}$), the one-electron participation can be estimated for the first reduction step, confirming the nitro radical anion formation.

The radical kinetic stability was evaluated through the current

Figure 3. Cyclic voltammograms of 0.5 mmol L$^{-1}$ NFOH registered in several pH values in aqueous medium, using GCE with scan rate = 1.0 V s$^{-1}$, pH: (A) 1.99, (B) 5.10, (C) 7.20, (D) 7.58, (E) 9.20, and (F) 12.0.

Figure 4. Change of peak potential with pH in aqueous medium using GCE and scan rate = 1.0 V s$^{-1}$. [NFOH] = 0.5 mmol L$^{-1}$. $E_{p1}$ corresponds to hydroxylamine derivative formation and $E_{pnc}$ is the cathodic peak of the R–NO$_2$/R–NO$_2^-$ couple.

Figure 5. Cyclic voltammogram corresponding to R–NO$_2$/R–NO$_2^-$ couple at pH 10.2 and [NFOH] = 0.5 mmol L$^{-1}$ using GCE, scan rate = 2.0 V s$^{-1}$, and switching potential = -0.57 V.
ratio ($I_{pNa}/I_{pNc}$) analysis corresponding to the one-electron reversible couple due to the R–NO$_2$/$R$–NO$_2$− redox system. Figure 6 shows that the kinetic stability increases between pH 8 and 9 and remains constant at higher pH values. Due to the overlap of the current of the first peak with that of the second peak (formation of the hydroxylamine), the average value of $I_{pNc}$ corresponds to about 97% of the real value taking into account the switching potential and the baseline correction for $I_{pNa}/I_{pNc}$.

The effects of the scan rate and drug concentration on the $I_{pNa}/I_{pNc}$ ratio are presented in Fig. 7 and 8, respectively. While the former shows that, as the scan rate is increased, the current ratio tends toward unity, yet the latter shows that the current ratio decreases as the NFOH concentration increases. These diagnostic criteria fulfill the requirements for a coupled homogeneous electrode process, in which an irreversible chemical reaction follows a reversible charge-transfer step. Furthermore, this chemical irreversible reaction is considered second order, as the current ratio is not reagent-concentration dependent on the first-order reaction.$^{11,27}$ The behavior described herein leads to a conclusion that NFOH undergoes an irreversible reaction of disproportionation after the nitro radical anion generation, as depicted in the overall mechanism below$^{9}$

$$2R–NO_2^- + 2H^+ \rightarrow R–NO_2 + R–NO + H_2O$$

As it is considered a second-order reaction, its rate constant ($k_2$) can be determined by the Olmstead–Nicholson method.$^{26}$ Recently, this mechanism was very well described by Squella et al.$^{11}$ for several nitroheterocyclic compounds. Figure 9 shows the plots, based on this theory, where a linear relationship between the kinetic parameters, $\omega$ vs $\tau$, is obtained at different pH values. Table I presents...
three examples of $k_2$, showing that the nitro radical anion stabilization improves in alkaline medium, which confirms that the R–NO$_2^-$ disproportionation is slower at higher pH values, as stated by the calculated half-time life ($t_{1/2}$). Compared with other nitrofuran compounds, the $k_2$ values obtained for NFOH in this work are around 1 order of magnitude larger than those registered in an aprotic or mixed medium. From these aspects, it is possible to infer that the nitro radical anion decay is facilitated in a protic medium. As there are no indications that the R–NO$_2^-$ itself reacts in water ($k \approx 0$), the protic medium can favor the nitro radical anion decay providing enough protons for the R–NO$_2$H formation, facilitating the fast protonation reaction between the conjugate base R–NO$_2^-$ and the neutral radical formed, as depicted below 9,32.

$$R–NO_2H^+ + R–NO_2^+ (H^+) \rightarrow R–NO_2 + R–NO + (H^+)$$

Based on the discussion above, even though the nitro radical anion is less kinetically stable in an aqueous/protic medium than in an aprotic/mixed medium (for instance, 60% dimethylformamide and 40% H$_2$O), it is stable enough to carry out quantitative studies on its interactions with biological targets under experimental conditions as close as possible to the biological system. 11

**Conclusions**

NFOH was reduced in an acidic medium at a GCE, producing only one reduction peak involving four electrons due to the hydroxylamine derivative formation. Furthermore, the generation and observation of the nitro radical anion in an alkaline aqueous medium were possible because of its slow protonation. This work also clearly indicates that the GCE surface activation through polishing with diamond powder contributed to the kinetic stability of the nitro radical anion due to the hydrophobic properties of the interface, hindering or even suppressing the radical anion protonation in the diffusion layer. This behavior corroborates the previous hypothesis 10 that the acid–base properties of the alumina on the GCE surface does facilitate deprotonation of R–NO$_2^-$.

Therefore, the study of the nitro radical anion generation in an aqueous medium may allow us to increase our knowledge about biological targets, leading to a better comprehension of the nitroheterocyclic compounds’ mechanism and its charge-transfer process in experimental conditions close to the biological system, which will certainly contribute to the development of potential antichagasic derivatives.

### Table 1. The $k_2$ and $t_{1/2}$ values for the nitro radical anion disproportion at different pH values obtained from [NFOH] = 0.5 mmol L$^{-1}$.

<table>
<thead>
<tr>
<th>pH</th>
<th>$k_2 \times 10^{-3}$ (L mol$^{-1}$ s$^{-1}$)</th>
<th>$t_{1/2}$ (s)</th>
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<tr>
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