Reduction of Different Nitro-Compounds on Electroenzymatic System: Cytochrome c Reductase-NAD(P)H Modified Carbon Paste Electrode

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Abstract

A flavoenzyme cytochrome c reductase NAD(P)H mixture has been used to modify a carbon paste electrode. The modified electrode was characterized by cyclic voltammetry varying the scan rate and temperature. Cathodic and anodic peaks were observed at −0.45 and −0.39 V, respectively, for a scan rate of 100 mV/s. The enzyme-electrode is found to reduce electrocatalytically nitroimidazole and nitrofurane compounds. However, this effect was not observed on the reduction of nitrobenzene derivatives such as nitrobenzene substituted 1,4-dihydropyridines. Conditions such as pH and percentage of DMF have been optimized for the determination of these nitro-compounds. The best electrocatalytic reactions were obtained at pH 8.5, coincident with the optimal activity of the enzyme.

Keywords: Electrocatalysis, Cytochrome c reductase, Modified carbon paste electrode, Nitroimidazoles, Nitrofuranes, Dihydropyridines

1. Introduction

The ingenious concept to combine the recognition properties of macromolecular biological molecules to the sensitivity of electrochemical devices, has led to the emergence of biosensors as valuable analytical tools for monitoring of target analytes in different technological areas [1]. Biosensor research has attracted the endeavors of the world's scientific community in the last few decades and is a very active area at present as evident by the number of scientific publications, monographs, symposia, and reviews [2–8]. Modified electrodes based on the incorporation of enzymes within carbon paste are gaining considerable attention. Short response times accrue from the absence of supporting membranes and the close proximity of the biocatalytic and graphite sites. The bulk of the paste serves as a source of the biocatalytic activity, and fresh biosurfaces can easily be obtained by renewing the surface. The fact that many enzymes retain their activity within the hydrophobic paste environment is not surprising considering the known activity of the enzymes in organic media. This avenue is not limited to enzyme electrodes [8]. The reduction of aromatic nitro-compounds catalyzed by nitroreductase enzymes has been studied in aqueous and mixed media [9–14], but it has not been studied in a heterogenuous system like an enzyme modified carbon paste electrode. This type of modified electrode may have a direct action in situ on the reduction of these nitro-compounds.

There are manyfold studies described in biological systems at modified carbon paste electrodes using different enzymes as modifier. However, the principal interest of the present study is focused to plasma and urine samples. In this scope, potential interferents in the range from 0 to −0.7 V (versus Ag/AgCl) should be consider metabolites like ethanol, glucose-6-phosphate, glycerol and L-lactate at −0.15 V, pH 8.5 [15]; inosine, thymidine, cytidine, adenosine, uridine, deoxycytosine and deoxyadenosine at −0.1 V [16]; phenol, catechol, 4-cresol, hydroquinone, pyrogallol, L-tyrosine, tyramine, 3,4-dihydroxyphenylalanine, chlorogenic acid at −0.1 V [17]; hydrogen peroxide, methanol, 1-propanol, 2-propanol, 1-butanol at −0.05 V [18–22]; bilirubin at −0.2 V [23]; dopamine, epinephrine at −0.2 V [24]; D-alanine, D-valine, D-leucine, D-isoleucine, D-serine, D-aspartic acid, D-glutamic acid, D-lysine, D-histidine, D-arginine, D-phenylalanine, D-tryptophen, D-methionine, D-proline at −0.05 V [25, 26]; and all 20 common L-amino acids at −0.05 V [18, 20, 26].

Flavoenzymes mediating electron transfers processes are involved in the regulation of neurotransmitters and the detoxification of xenobiotics [27]. Many of these enzymes react directly on substrates, while others act intermediate in the respiratory chain by bridging the electron-transport gap between the pyridine nucleotides [28]. Mammalian cytochrome c reductase (CCR) has a flavin group as active site [27, 29, 30]. Its formal potential is −460 mV (vs. Ag/AgCl). It presents good redox properties making this enzyme immobilized on carbon paste electrode a potential electrocatalyzer for the reduction of different compounds. Therefore, electrocatalysis of nitro-compounds reduction such as nitro-imidazoles, nitrofuranes and nitro-dihydropyridines has been proposed. In this way, the reduction potential of each compound would be shifted in a positive sense, decreasing the overpotential of these reactions.

The interest to study the reduction of these nitro-compounds is due to their application as therapeutic drugs to treat infection produced by anaerobic bacteria and protozoarium. The mechanism of the biological action of these drugs depends on the reduction of the nitro-group which leads to intermediate species which interact with DNA, oxidizing it and liberating thymidine phosphate, causing a lesion characterized by destabilization and damage of the double helix [31]. For this reason, it is important to study the labile intermediate species formed during reduction of nitro-compound derivatives. Previous studies [32] have shown the formation of the anion radical $R\text{-}NO_2^{\cdot -}$ is perhaps responsable for the damage of DNA, as an intermediate in the mechanism.

In this article, considerable efforts were made to obtain an electroenzymatic system (cytochrome c reductase-NAD(P)H-CP electrode) that to catalyze nitro-compounds reduction thus obtaining an important tool for biodegradation studies.

2. Experimental

Cytochrome c reductase of porcine heart (EC 1.6.99.3) and NADPH were obtained from Sigma Chemical Co. and NADH from Merck. Metronidazole, tinidazole, nitrofurantoin, nifuroxazide, nitrofurazone, nifedipine, nitrendipine, and nisoldipine were supplied by Laboratorio Chile S.A. Phosphate buffer solutions 150 mM were used in all experiments with pH ranged between 6.7 and 11.0. The nitrofurantoin reduction also has been studied in mixed media at different DMF percentages (20–40 %).

The enzyme was purified as described in the literature [16]. Electrophoresis of the enzyme after purification was homogeneous and showed one band corresponding to 78 000 according to the literature. Protein was determined by the Bradford method.

The working electrode (geometric area 0.04 cm^2) was carbon paste modified with cytochrome c reductase (CCR) and its cofactor (NAD(P)H), the counter electrode was a Pt wire, and the reference electrode was Ag/AgCl, all contained in a two-compartement cell. The CCR-NAD(P)H modified carbon paste electrode was prepared by mixing 4 mg of the enzyme, 4 mg of cofactor and 16 mg of carbon paste (Metrohm 6.2801.000). The resulting paste was packed tightly into a Teflon sleeve body. Electrical contact was established with a copper wire. The surface was polished to a smooth finish before use. Then, the stable response of the modified electrodes were obtained through of continuous cycling of potentials (20 sweep) at 0.5 V/s. Before each electrochemical measurement, the surface of the modified working electrode was renewed and the application of the continuous cycling of potential was realized.

Before all experiments, nitrogen was bubbled through the solution for 10 min and was passed above the solution during experiments.

The voltammograms were recorded using a CV-50 W BAS potentiostat connected to a GATEWAY 2000 computer.

3. Results and Discussion

3.1. Characterization of the Modified Electrode

The cyclic voltammogram of the enzyme modified carbon paste electrode (CCR-NADH-CP) with the enzyme without purification is shown in Figure 1a. The cyclic voltammogram of the pure enzyme modified carbon paste electrode (CCR-NADH-CP) (solid line) is shown in Figure 1b. In this figure, the cyclic voltammogram of a CP-albumin electrode (dotted line) has been included as a current background of the modified protein carbon paste electrode. The cyclic voltammogram of CCR-NADH-CP electrode obtained by subtraction of these curves is shown in Figure 1c. As can be seen from Figures 1a and c the peak potential values remain practically inaltered by the purification procedure. A cathodic peak potential (E_{pc}) of −0.45 and an anodic peak potential (E_{pa}) of −0.39 V (vs. Ag/AgCl) were obtained after of continuous cycling, as described in Section 2. The scan rate variation studies showed that both peaks shift towards negative and positive potential values, respectively. There was also an increase in the peak current as function of the scan rate. The ΔE_p values show a quasireversible one-electron transfer.

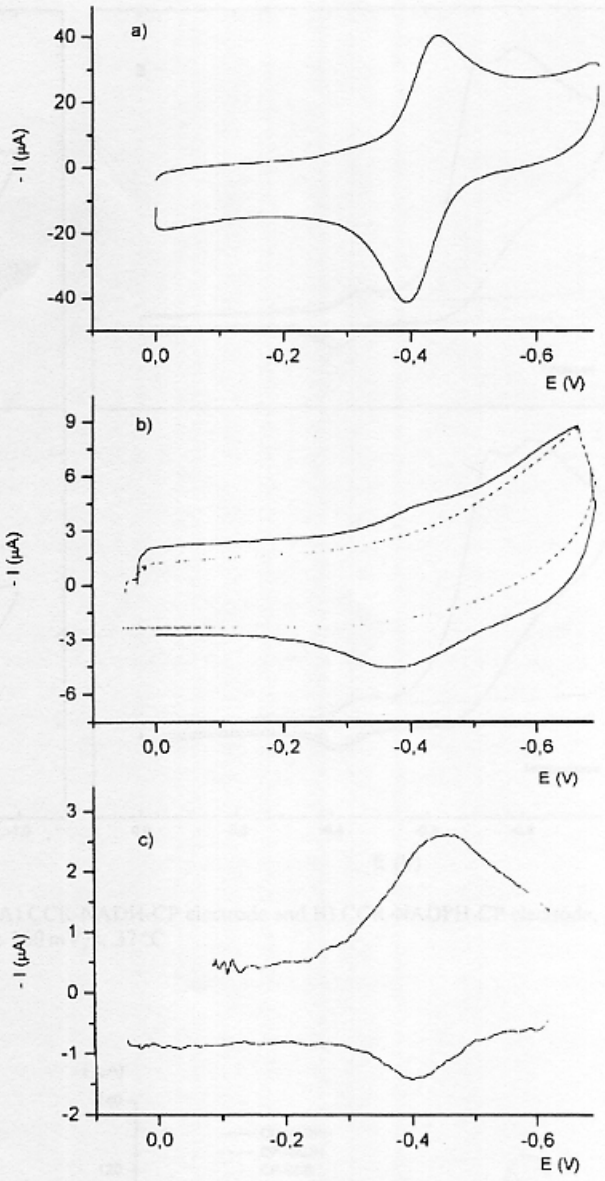


Fig. 1. CV of CCR-NADH-CP modified electrode in 0.1 M phosphate buffer pH 7.4 . Scan rate: 100 mV/s, 37 °C. a) commercial enzyme, b) enzyme after purification and c) enzyme pure with subtraction of the background current.

3.2. Electrocatalytical Reduction of Nitro-Compounds

3.2.1. Nitroimidazoles

The reduction of metronidazole and tinidazole were studied at CP and CCR-NAD(P)H-CP electrodes. Figure 2 shows the cyclic voltammograms of the enzyme-electrodes in the absence and presence of the drugs. With the drugs, a large cathodic current was observed, implying electrical contact between the immobilized enzyme and the electrode surface leading to the electrocatalyzed reduction of metronidazole and tinidazole at both types of enzymatic electrodes (CCR-NADH and CCR-NADPH carbon paste electrodes).

The catalytic reactions of the analytes used in this research are shown in Scheme 1. The first signal on the voltammograms of Figure 2a and 2b corresponds to the enzyme reduction and the second irreversible peak corresponds to the nitro-compound

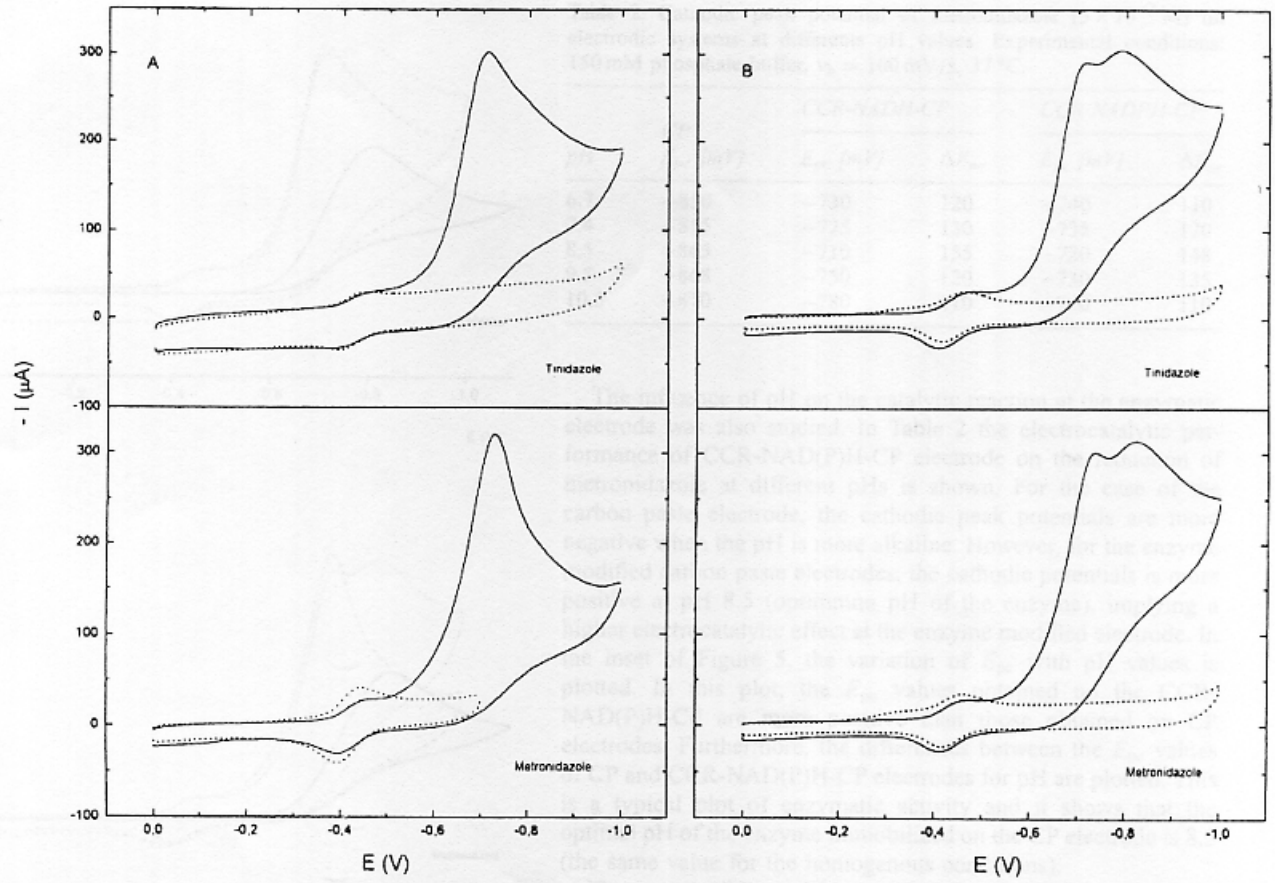
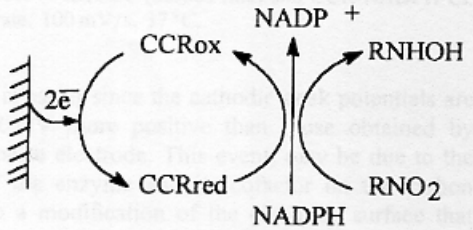


Fig. 2. CV of nitroimidazoles in 0.1 M phosphate buffer pH 6.7 on A) CCR-NADH-CP electrode and B) CCR-NADPH-CP electrode, in presence of drug (continuous line) and absence of drug (dotted line). Scan rate: 100 mV/s, 37 °C.



Scheme 1. Catalytic reaction of the nitro-compounds reduction by the CCR-NADPH-CP electrode.

reduction catalyzed by the enzymatic system according to Scheme 1.

Control experiments revealed that the enzyme-electrode without cofactors, CP-NADH and CP-NADPH electrodes (Fig. 3), produce a minor shifting when compared to the enzyme-electrode with either NADH or NADPH. This indicates that either cofactor could be used to catalyze nitro-compounds reduction.

Comparison of cyclic voltammograms for the reduction of tinidazole and metronidazole under the same experimental conditions at the enzyme modified carbon paste electrode and at carbon paste electrode, are shown in Figure 4. The response is much better using the enzyme modified carbon paste electrodes: the peak current is nearly twice that at a carbon paste electrode

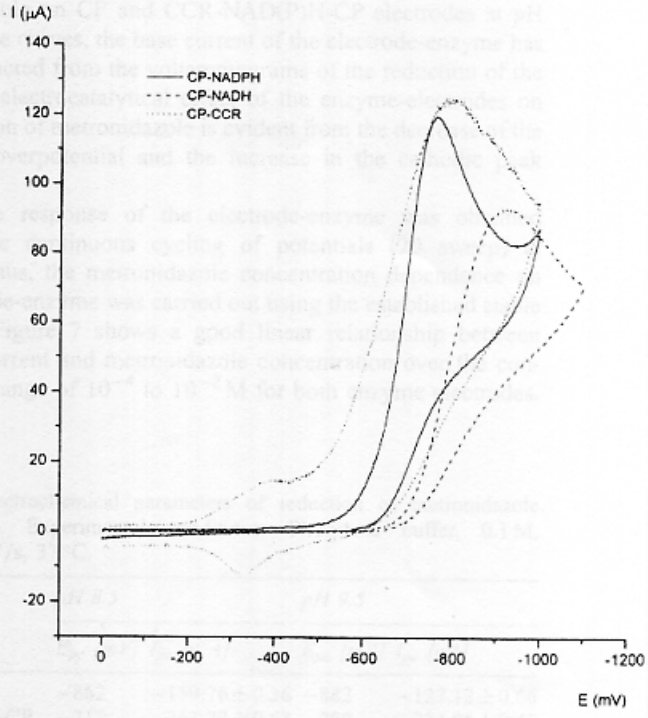


Fig. 3. CV of 5×10^{-3} M metronidazole on CP-NADPH, CP-NADH and CP-CCR modified electrodes. Scan rate: 100 mV/s, 37 °C.

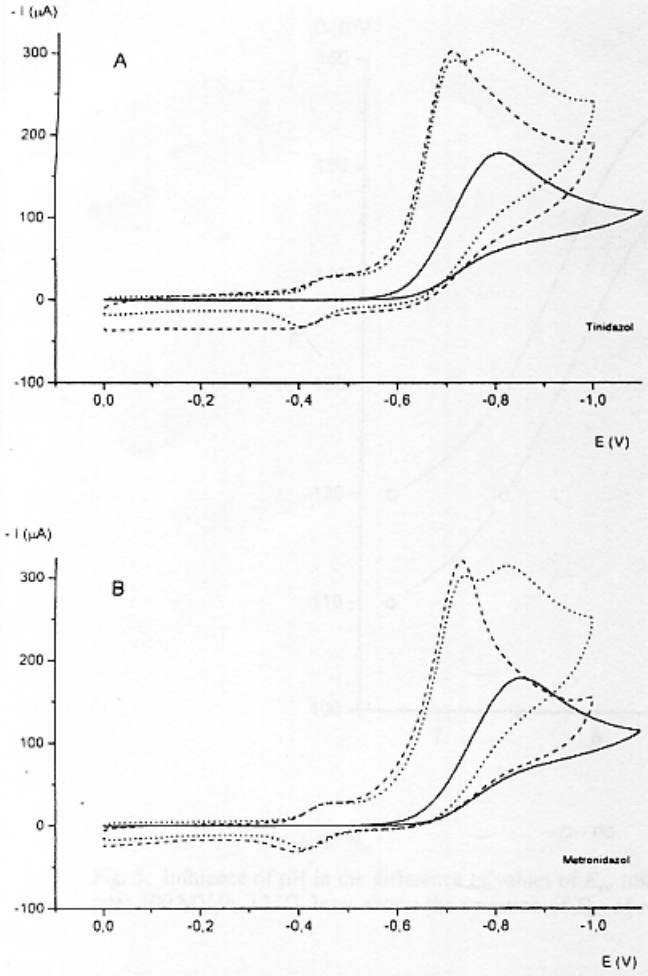


Fig. 4. Comparison of reduction of A) tinidazole and B) metronidazole in 0.1 phosphate buffer pH 6.7 on different electrodic systems. PC (continuous line), CCR-NADH-CP (dashed line) and CCR-NADPH-CP (dotted line). Scan rate: 100 mV/s, 37 °C.

and the reduction is easier since the cathodic peak potentials are approximately 100 mV more positive than those obtained by using the carbon paste electrode. This event, may be due to the immobilization of the enzyme and its cofactor on the carbon paste that leads to a modification of the electrode surface that facilitates the electron transfer. In Table 1 the values of cathodic peak potentials E_{pc} of the reduction of the differents imidazoles on carbon paste and CCR-NAD(P)H modified carbon paste electrodes are given.

Table 1. Cathodic peak potential of different nitro-compounds on bare and modified carbon paste electrodes. Experimental conditions: 150 mM phosphate buffer, pH 7.4, $\nu_b = 100$ mV/s, 37 °C.

Drug	*CP*	*CCR-NADH-CP*	*CCR-NADPH-CP*
Tinidazole	−0.81 V	−0.71 V	−0.71V/−0.80V
Metronidazole	−0.85 V	−0.72 V	−0.72 V/−0.83 V
Nitrofurantoin	−0.55 V	−0.46 V	−0.44 V
Nifuroxazide	−0.53 V	−0.49 V	−0.47 V
Nitrofurazone	−0.63 V	−0.55 V	−0.53 V
Nitrendipine	−0.79 V	−0.82 V	−0.82 V
Nisoldipine	−0.91 V	−0.92 V	−0.93 V
Nifedipine	−1.00 V	−1.00 V	−1.00 V

Table 2. Cathodic peak potential of metronidazole (5×10^{-3} M) on electrodic systems at differents pH values. Experimental conditions: 150 mM phosphate buffer, $\nu_b = 100$ mV/s, 37 °C.

		CCR-NADH-CP		*CCR-NADPH-CP*	
pH	*CP* E_{pc} *[mV]*	E_{pc} *[mV]*	ΔE_{pc}	E_{pc} *[mV]*	ΔE_{pc}
6.7	−850	−730	120	−740	110
7.4	−855	−725	130	−735	120
8.5	−865	−710	155	−720	148
9.5	−868	−750	120	−730	135
10.5	−870	−780	110	−760	110

The influence of pH on the catalytic reaction at the enzymatic electrode was also studied. In Table 2 the electrocatalytic performance of CCR-NAD(P)H-CP electrode on the reduction of metronidazole at different pHs is shown. For the case of the carbon paste electrode, the cathodic peak potentials are more negative when the pH is more alkaline. However, for the enzyme modified carbon paste electrodes, the cathodic potentials is more positive at pH 8.5 (optimmun pH of the enzyme), implying a higher electrocatalytic effect at the enzyme modified electrode. In the inset of Figure 5, the variation of E_{pc} with pH values is plotted. In this plot, the E_{pc} values obtained on the CCR-NAD(P)H-CP are more positive than those obtained on CP electrodes. Furthermore, the differences between the E_{pc} values of CP and CCR-NAD(P)H-CP electrodes for pH are plotted. This is a typical plot of enzymatic activity and it shows that the optimal pH of the enzyme immobilized on the CP electrode is 8.5 (the same value for the homogenous conditions).

The reproducibility of the modified electrodes is shown by five independent measurements for each of the different electrodes. The mean of the peak current and the standard deviation are shown in Table 3. These results supported the high reproducibility of the modified electrodes.

Figure 6 shows the voltammograms of the reduction of metronidazole on CP and CCR-NAD(P)H-CP electrodes at pH 8.5. In these curves, the base current of the electrode-enzyme has been subtracted from the voltammograms of the reduction of the drug. The electrocatalytical effect of the enzyme-electrodes on the reduction of metronidazole is evident from the decrease of the reduction overpotential and the increase in the cathodic peak current.

A stable response of the electrode-enzyme was obtained through the continuous cycling of potentials (20 sweep) at 0.5 V/s. Thus, the metronidazole concentration dependence on the electrode-enzyme was carried out using the established stable response. Figure 7 shows a good linear relationship between cathodic current and metronidazole concentration over the concentration range of 10^{-4} to 10^{-2} M for both enzyme electrodes.

Table 3. Electrochemical parameters of reduction of metronidazole (5×10^{-3} M). Experimental conditions: Phosphate buffer, 0.1 M, $\nu_b = 100$ mV/s, 37 °C.

	pH 8.5		*pH 9.5*	
	E_{pc} *[mV]*	I_{pc} *[μ A]*	E_{pc} *[mV]*	I_{pc} *[μA]*
CP	−862	−159.76 ± 0.36	−882	−127.12 ± 0.66
CCR-NADH-CP	−715	−261.72 ± 0.63	−780	−234.06 ± 0.40
CCR-NADPH-CP	−730	−289.72 ± 0.66	−790	−169.72 ± 0.88

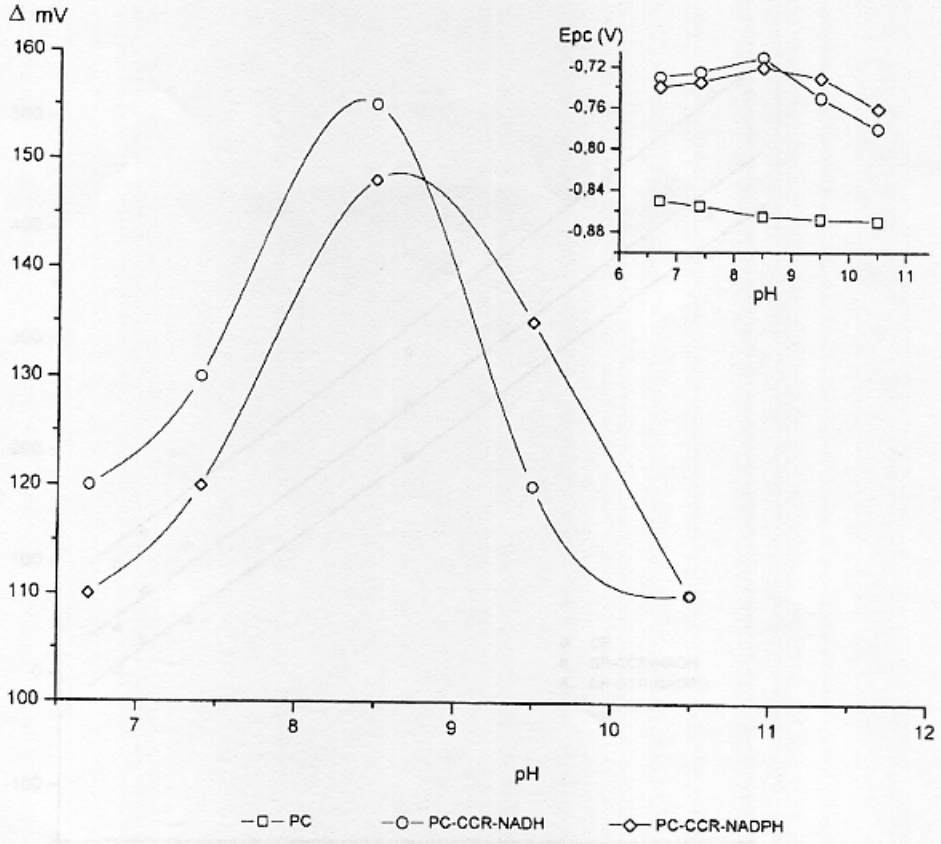


Fig. 5. Influence of pH in the difference of values of E_{pc} (mV) of reduction of 5×10^{-3} M metronidazole between CP and CCR-NAD(P)H-CP. Scan rate: 100 MV/s, 37 °C. Inset shows the variation of E_{pc} of reduction of metronidazole with the pH on the electrodic systems.

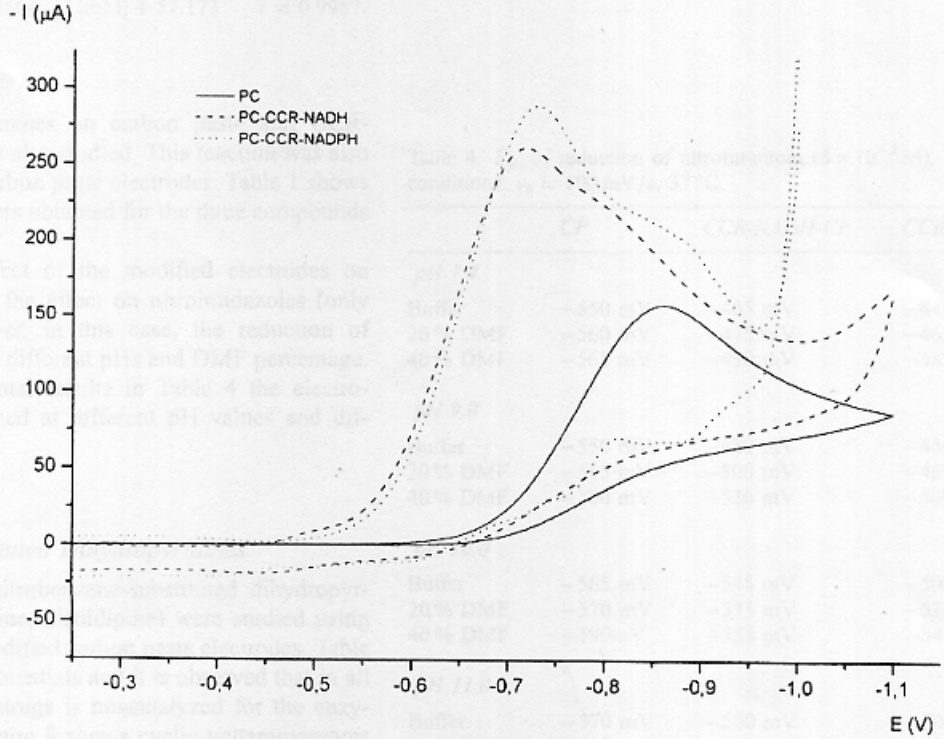


Fig. 6. CV of 5×10^{-3} M metronidazole in 0.1 M phosphate buffer pH 8.5 on CP and enzyme modified CP electrode. Scan rate: 100 mV/s, 37 °C. The background current of the enzyme-electrode has been subtracted from the voltammograms of the drug.

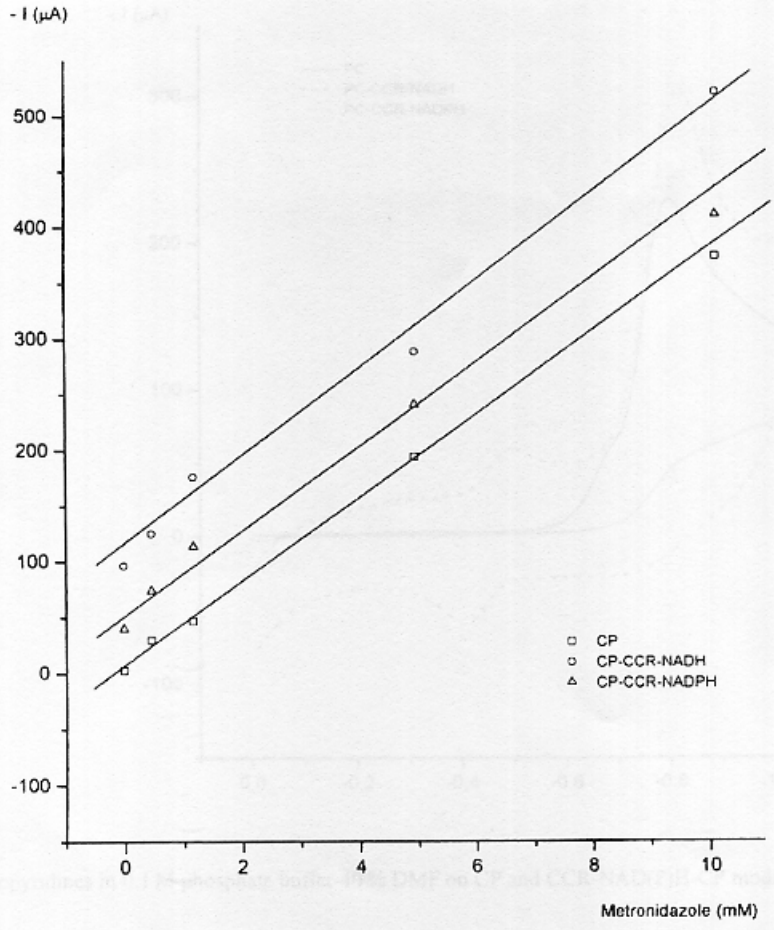


Fig. 7. Calibration plot obtained for reduction of metronidazole on the different electrodic systems at pH 8.5:
CP $I\ [\mu A] = 36480 \cdot C\ [mM] + 7.557 \quad r = 0.9995$
CCR-NADH-CP $I\ [\mu A] = 40406 \cdot C\ [mM] + 106.716 \quad r = 0.9962$
CCR-NADPH-CP $I\ [\mu A] = 35564 \cdot C\ [mM] + 57.173 \quad r = 0.9967.$

3.2.2. *Nitrofuranes*

The reduction of nitrofuranes on carbon paste and CCR-NAD(P)H-CP electrodes was also studied. This reaction was also catalyzed by the modified carbon paste electrodes. Table 1 shows the electrochemical parameters obtained for the three compounds of this type.

The electrocatalytical effect of the modified electrodes on nitrofuranes is smaller than the effect on nitroimidazoles (only 60 mV in the last). However, in this case, the reduction of nitrofurantoin was studied at different pHs and DMF percentage. According to the experimental results in Table 4 the electrocatalytical effect is maintained at different pH values and different DMF contents.

Table 4. E_{pc} of reduction of nitrofurantoin (5×10^{-3} M). Experimental conditions: $\nu_b = 100$ mV/s, 37 °C.

	CP	*CCR-NADH-CP*	*CCR-NADPH-CP*
pH 7.4			
Buffer	−550 mV	−465 mV	−445 mV
20% DMF	−560 mV	−475 mV	−465 mV
40% DMF	−565 mV	−490 mV	−485 mV
pH 9.0			
Buffer	−550 mV	−480 mV	−450 mV
20% DMF	−565 mV	−500 mV	−480 mV
40% DMF	−580 mV	−520 mV	−500 mV
pH 10.0			
Buffer	−565 mV	−545 mV	−500 mV
20% DMF	−570 mV	−535 mV	−530 mV
40% DMF	−590mV	−555 mV	−545 mV
pH 11.0			
Buffer	−570 mV	−500 mV	−500 mV
20% DMF	−580 mV	−545 mV	−535 mV
40% DMF	−595 mV	−555 mV	−560 mV

3.2.3. *Nitrobenzene-Substituted Dihydropyridines*

The reduction of three nitrobenzene-substituted dihydropyridines (nitrendipine, nifedipine, nisoldipine) were studied using carbon paste and enzyme modified carbon paste electrodes. Table 1 shows the cathodic peak potentials and it is observed that in all cases, the reduction of the drugs is noncatalyzed for the enzymatic-electrode systems. Figure 8 shows cyclic voltammograms of nitrendipine on carbon paste and CCR-NAD(P)H carbon paste electrodes.

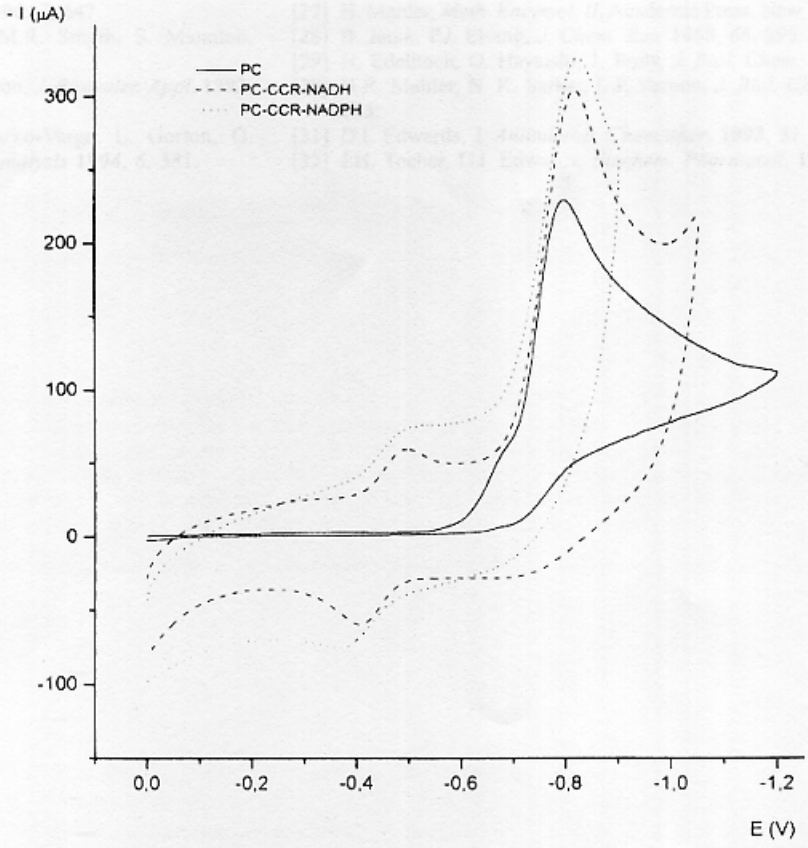


Fig. 8. CV of dihydropyridines in 0.1 M phosphate buffer-40 % DMF on CP and CCR-NAD(P)H-CP modified electrode. Scan rate: 100 mV/s, 37 °C.

4. Conclusions

The results demonstrate that the enzyme cytochrome c reductase immobilized on carbon paste electrode together with its cofactor produce an important catalytic effect on the reduction of nitroimidazoles and nitrofuranes. However, this effect did not occur on the reduction of nitrobenzene-substituted dihydropyridines. The study of the variation of E_{pc} of metronidazole reduction with pH shows that the better electrocatalytic effects were obtained at pH 8.5, coincidental with the optimal pH for the enzyme activity. The addition of different percentages of DMF produce a shifting of the E_{pc} in the negative sense. However, percentages higher than 40 % are not possible to use, because the inactivation of the enzyme can occur.

Immobilizing cytochrome c reductase enzyme on the carbon paste electrode inaugurates a new tool to study the biodegradation mechanisms of nitro-compounds of biological significance.

5. Acknowledgements

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6. References

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