

Polarography of Cytochrome c in Ammoniacal Buffers Containing Cobalt Ions. The Effect of the Protein Conformation

V. BRABEC

*Institute of Biophysics, Czechoslovak Academy of Sciences,
Královopolská 135, 612 65 Brno, Czechoslovakia*

Abstract. Catalytic currents yielded by cytochrome c in ammoniacal buffers containing cobalt ions at a dropping mercury electrode (Brdička's catalytic currents) were investigated by means of direct current, differential pulse, normal pulse (NP) and phase-selective alternating current polarography. It was found that Brdička's catalytic current of cytochrome c, (the more negative part of Brdička's double wave, wave B) is influenced by the presence of cytochrome c denaturants in the background solution. The wave B rose with the increasing concentrations of urea and sodium perchlorate, and increased in parallel with absorbance changes at 409 and 695 nm measured for identical cytochrome c solutions. The latter absorbance changes reflect unfolding of cytochrome c molecules in the bulk of solution by these denaturants. The results of NP polarography (a technique working with large potential excursion during the drop lifetime) indicate that in Brdička's solution cytochrome c could extensively be unfolded due to its adsorption at the mercury electrode, polarized to potentials around that of zero charge.

Key words: Cytochrome c — Polarography — Absorption and fluorescence spectroscopy — Brdička's catalytic currents — Unfolding of cytochrome c

Introduction

It has been shown that methods of electrochemical analysis are useful tools in analysis of biologically significant compounds including biomacromolecules (for a review, see e.g. Dryhurst 1977; Paleček 1983). In electrochemical analysis of proteins, polarography is one of the most used methods. It allows reduction/oxidation properties of disulphide groups or electroactive prosthetic groups (in conjugated proteins) to be observed. Nevertheless, polarographic analysis of proteins based on the ability of proteins, containing sulfhydryl- and/or disulphide groups, to yield catalytic hydrogen evolution currents at a mercury electrode seems best known. This electrocatalytic ability of proteins is greatly enhanced by the addition of cobalt salts. The catalytic polarographic currents of proteins in the

presence of cobalt ions were first observed by Brdička and are therefore often called Brdička's polarographic currents (for a review, see e.g. Brdička et al. 1965; Březina and Zuman 1958; Ivanov and Rachleeva 1968). The mechanism of their origin is not yet fully understood, but it is generally accepted that zero-valent cobalt which is liganded to a catalytically active group of protein to form a protein-Co(0) complex on an electrode surface may catalyze the hydrogen evolution before the complex is decomposed into Co(0) amalgam (Senda et al. 1982). It has also been shown that Brdička's currents of proteins may be dependent on the structure of proteins, and can thus be affected by conformational changes of these biopolymers (Brdička et al. 1965; Ruttkay—Nedecký et al. 1977).

It has already been shown (Ikeda et al. 1980a; Ikeda et al. 1980b) that heme containing proteins, cytochrome c and cytochrome c_3 , can yield Brdička's polarographic catalytic currents even though they contain no SS- and/or free SH-groups. For instance, cytochrome c contains two cysteine residues, both linked to the heme group, so that they are not present as SS- or SH- groups. Senda has proposed that the heme group and its sixth ligand could play a dominant role in producing Brdička's polarographic catalytic currents of cytochrome c (Ikeda et al. 1980a). The present paper is aimed at demonstrating that Brdička's catalytic currents of horse-heart ferricytochrome c can be influenced by conformational changes in this protein, leading to higher accessibility of the heme for interaction with the environment.

Materials and Methods

Horse-heart ferricytochrome c (type VI) from Sigma Chemical Co. was used without further purification. The absence of electroactive impurities was verified by the method described by Bianco and Haladjian (1977). Cytochrome c_3 from *Desulfovibrio desulfuricans* (Norway strain) was a generous gift of Dr. J. Haladjian, Université de Provence, Marseilles. All other chemicals used were of reagent grade. Most of the polarographic and spectroscopic measurements were performed in a medium containing 1.0 mmol/l CoCl_2 with 0.1 mol/l NH_4Cl plus 0.1 mol/l NH_3 , pH 9.3. This medium is referred to throughout the text as standard Brdička's solution. Moreover, the effect of addition of urea and sodium perchlorate on polarographic Brdička's currents of cytochrome c was investigated. The addition of higher concentrations of the above denaturants caused a slight shift in pH of the background electrolyte. In these cases pH was adjusted to the original value with hydrochloric acid or sodium hydroxide. The concentrations of cytochromes c and c_3 were checked spectrophotometrically (Bianco and Haladjian 1979; Haladjian et al. 1982).

Unless otherwise stated, DC sampled and DP polarograms were obtained with a PAR Model 174A polarographic analyzer. Polarographic curves of cytochrome c in the presence of sodium perchlorate, and the corresponding control sample were obtained with a PAR Model 384-B polarographic analyzer in connection with a static mercury drop electrode (medium size). AC polarography was performed with a PAR Model 174A polarographic analyzer coupled with a PAR Model 174/50 AC polarographic analyzer interface and a PAR Model 5204 lock-in analyzer. All polarograms were recorded with a voltage scan rate of 2 mV/s and a drop time of 2.0 s. DP polarograms were obtained with a pulse amplitude of -5 mV. Phase selective AC polarographic measurements were carried out

with a modulation voltage of 80 Hz and 10 mV peak-to-peak. Phase angles of 0° and 90° with respect to the applied alternating voltage were employed (in phase and out-of-phase component of the AC being measured). A Metrohm Ag/AgCl (satd. KCl) electrode was used as a reference electrode, and a platinum wire as a counter electrode. Throughout this report all potentials are given vs. Ag/AgCl reference electrode.

Absorption spectra were measured at 25°C with a Beckman DU-8 recording spectrophotometer equipped with a thermostat-controlled cuvette holder. Fluorescence measurements were carried out with an Aminco Bowman spectrofluorimeter. L-tryptophan, a Sigma product, was used as the fluorescence standard. Solutions containing cytochrome c or tryptophan were excited at 280 nm and the fluorescence recorded between 300 and 420 nm. The results were normalized with respect to the fluorescence intensity of L-tryptophan at pH 7.0, taken for 100%. All fluorescence measurements with cytochrome c were performed in standard Brdička's solution.

Unless otherwise stated, all the measurements described in this paper were performed at 25°C .

Results and Discussion

DC and DP polarography. The effect of denaturants.

Cytochrome c in standard Brdička's solution yields classical double-wave on DC polarograms with maxima A and B (Fig. 1). However, Ruttkay—Nedecký showed that under certain conditions (at 0°C and at an ammonia concentration of 0.3 mol/l and higher) cytochrome c could yield a third, more negative wave (or maximum), C (Ruttkay—Nedecký and Anderlová 1967). In analogy to DC polarographic behaviour of tobacco mosaic virus-protein in Brdička's solution (Ruttkay—Nedecký and Bezúch 1971; Ruttkay—Nedecký et al. 1977) the maximum C might be expected to be very sensitive to conformational changes in the protein that result in an unmasking of the catalytically active group in the protein. We therefore tried to find conditions under which the DC polarographic maximum C would occur for cytochrome c as well. By varying temperature, pH and the composition of the background electrolyte it was found that cytochrome c could yield maximum C even at temperatures higher than 0°C , but only if the concentration of ammonia is increased (and, consequently, also the value of pH). For instance, cytochrome c at 25°C can yield on the DC polarographic curve a maximum C at concentrations of ammonia of 1.0 mol/l (pH 10.2) and higher (Fig. 1). It was, however, interesting that on a DP polarogram, under conditions allowing maximum C to appear on the DC polarographic curve, only maxima A and B were observed (in the DP polarographic experiment a relatively small pulse amplitude was used to obtain high resolution of DP polarographic peaks). The latter result is in accordance with results obtained in DP polarographic investigation of tobacco-mosaic virus protein (Ruttkay—Nedecký and Paleček 1980).

We tried further to study effects of the unfolding of the cytochrome c molecule in the bulk solution on Brdička's polarographic catalytic currents yielded by this protein. Horse-heart cytochrome c can be unfolded by a number of agents. It

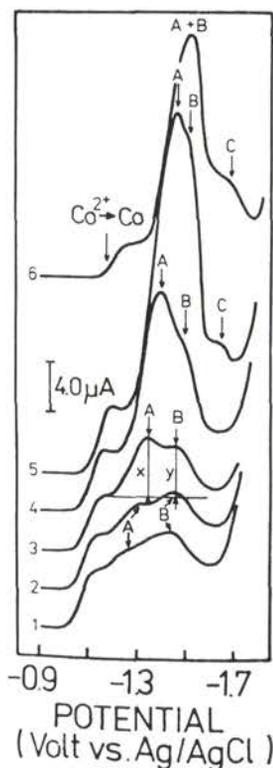


Fig. 1. DC sampled polarograms of 3 $\mu\text{mol/l}$ cytochrome c in medium containing 1.0 mmol/l CoCl_2 , 0.1 mol/l NH_4Cl and NH_3 at following concentrations (in mol/l): Curve 1 — 0.01, pH 8.2; curve 2 — 0.03, pH 8.7; curve 3 — 0.1, pH 9.3; curve 4 — 0.3, pH 9.8; curve 5 — 1.0, pH 10.2; curve 6 — 3.0, pH 10.7. Curve 6 was recorded at a half the current sensitivity of the polarographic analyzer indicated in the figure. The values of x and y were taken to represent the heights of waves A and B, respectively.

follows from earlier studies, that denaturation and unfolding of the cytochrome c molecule can also be accomplished by urea (Myer et al. 1980; Harrington 1981). Numerous reports can be found in the literature on denaturation of cytochrome c by this agent, obtained primarily using optical and hydrodynamical methods. We therefore studied first effects of the addition of urea on DC polarographic behaviour of cytochrome c under conditions allowing all the three catalytic maxima, A, B and C, to appear. Preliminary experiments revealed that, at 0 °C and even at 25 °C, the addition of 8.0 mol/l of urea, sufficient to cause the unfolding of the cytochrome c molecule, did not result in an increase in the wave (or maximum) C; however, the wave (maximum) B did increase. Nor did an increase in pH (by adding sodium hydroxide) to the value of 11–12 at 25 °C (1 mmol/l CoCl_2 with 0.1 mol/l NH_4Cl plus 3.0 mol/l NH_3 used as the background electrolyte) result in

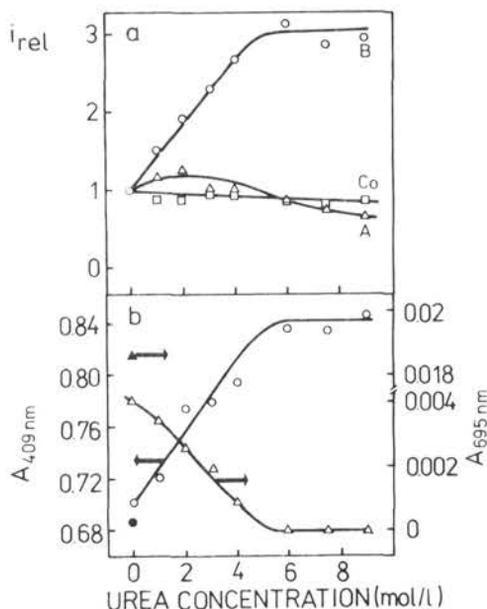


Fig. 2. The effect of urea on Brdička's currents (DC polarographic wave heights) (a) and absorbance (b) of cytochrome c in standard Brdička's solution. (a) The dependence of the relative heights of the cobalt wave (curve Co), the more positive wave A (curve A) and the more negative wave B (curve B) on urea concentration. The height of the wave in the absence of urea was taken for 1. For other conditions of the measurement, see Fig. 1. (b) Absorbance changes at 409 nm of 7 $\mu\text{mol/l}$ cytochrome c (triangles) and at 695 nm of 85 $\mu\text{mol/l}$ cytochrome c (circles) induced by increasing concentrations of urea. The medium was standard Brdička's solution (open symbols), or 0.05 mol/l sodium phosphate plus 0.2 mol/l potassium chloride, pH 7.0 (full symbols). Absorbance of the 695 nm band was determined according to the procedure previously described (Kaminsky et al. 1975).

an increase of the wave (maximum) C. As follows from the paper of Hasumi (1980), an increase in pH above 10 also causes the unfolding of the cytochrome c molecule. Qualitatively identical results were obtained if hexaammincobalt(III) chloride was used instead of CoCl_2 .

A further systematic study, aimed at clarifying the effects of agents capable of denaturing cytochrome c in the bulk solution, was performed in standard Brdička's solution at 25 °C. In order to obtain information on the course of urea denaturation of cytochrome c in the bulk solution, absorption and fluorescence spectra of cytochrome c in standard Brdička's solution containing various concentrations of urea were recorded. We used three well-characterized structural probes, the 409 nm (Soret) band, a feature linked to the exposure of the covalently linked heme moiety of the cytochrome c molecule, the 695 nm band indicating the

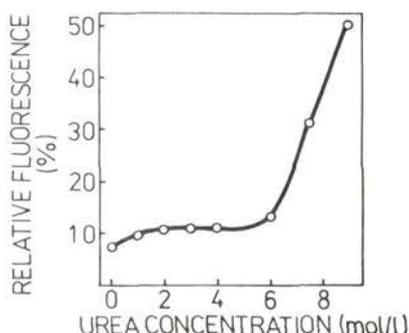


Fig. 3. The effect of urea on the fluorescence efficiency of the tryptophan residue of cytochrome *c*. Conditions: Standard Brdička's solution + varying concentrations of urea; excitation wavelength 280 nm; cytochrome *c* concentration was 1 μ mol/l. Relative fluorescence with respect to free tryptophan, 1 μ mol/l in 0.05 mol/l sodium phosphate plus 0.2 mol/l potassium chloride, pH 7.0 without urea. Values were taken at the maxima of the emission spectra (around 340 nm).

presence or absence of the methionine-80-S-iron linkage, and the fluorescence efficiency of the single tryptophan side chain, a direct measure of the conformation of the heme-tryptophan domain, i.e. the deepest part of the heme crevice (Myer et al. 1980). Changes in the Soret region were characterized by a gradual increase in absorbance at 409 nm, following the addition of urea to a final concentration of ca. 6 mol/l (Fig. 2*b*). A further increase of urea concentration remained without any effect on absorbance at 409 nm. Similarly as reported in other papers (Stellwagen 1968; Harrington 1981), this increase is attributed to an increase in exposure of the covalently linked heme moiety, which is partly buried in the hydrophobic interior of the native molecule of cytochrome *c*. Absorbance at 695 nm decreased in parallel with the increase in absorbance at 409 nm (Fig. 2*b*). A major proportion of the absorbance at 695 nm should be associated with the unfolding of the protein moiety of the cytochrome *c* molecule, also reflecting the presence or disruption of the methionine 80 linkage (Myer et al. 1980). The absence of the 695 nm band at urea concentrations of 6 mol/l and higher suggests the absence of the methionine-80-S-iron linkage under these conditions.

The effect of increasing concentrations of urea on fluorescence of a single tryptophan residue of the cytochrome *c* residue is a marked enhancement of the fluorescence efficiency, starting only when ca. 6 mol/l urea were added (Fig. 3). This result suggests that a perturbation in the deepest part of the crevice, or the tryptophan-heme domain of the cytochrome *c* molecule, appears in standard Brdička's solution only when 6 mol/l or more urea are added. This means that in standard Brdička's solution the loosening of the structures of cytochrome *c* occurred in the deepest domain of the crevice at urea concentrations significantly

higher than those inducing conformational changes responsible for absorbance changes at 409 and 695 nm.

Fig. 2a shows changes in the height of DC polarographic waves of cytochrome c in standard Brdička's solution induced by the addition of urea. Whereas the wave of cobalt and the wave (maximum) A were not significantly influenced by the addition of urea, the height of the more negative wave B increased in parallel with the increase in absorbance at 409 nm and with the decrease in absorbance at 695 nm. An identical result was obtained if Brdička's catalytic currents were measured by means of DP polarography.

A factor which could influence the phenomena observed in polarography is the adsorption of urea on the mercury electrode. The effect of urea adsorption on the polarographic behaviour of cytochrome c in standard Brdička's solution was investigated by AC polarography. It was found that urea added to standard Brdička's solution or to a solution containing only 0.1 mol/l NH_4Cl with 0.1 mol/l NH_3 lowered the out-of-phase component of AC in a broad region of potentials around the potential of zero charge (PZC). However, these AC polarographic measurements indicated that urea was not adsorbed at potentials as negative as those corresponding to Brdička's polarographic waves A and B, even at a bulk concentration of urea of 8 mol/l. The latter result is also in good agreement with reports of other authors who investigated adsorption of urea at the mercury electrode (Parsons et al. 1975). It is thus concluded that adsorption of urea at the mercury/solution interface does not influence Brdička's polarographic catalytic currents.

It is thus possible to conclude that polarographic behaviour of cytochrome c in standard Brdička's solution is dependent on the conformation of this protein in the bulk solution. The results of the experiments with urea suggest that the more negative wave (or maximum) B is sensitive to changes in conformation of cytochrome c that result, in particular, to the unfolding of the protein moiety and to higher accessibility of heme for reaction with the environment.

We also tried to support our previous conclusions using a denaturant of cytochrome c, of a substantially different chemical nature. It has been shown that the conformation of cytochrome c could also be changed in the presence of an inorganic agent, namely sodium perchlorate (Harrington 1981). This salt is known not to be specifically adsorbed at the mercury electrode, so that any interference phenomena associated with adsorption of the denaturant at the mercury electrode can be ruled out. The addition of sodium perchlorate to ferricytochrome c in standard Brdička's solution caused, similarly as with the addition of urea, an increase in the more negative wave (maximum) B (Figs. 4 and 5). The heights of the cobalt wave and the more positive Brdička's catalytic wave were not greatly influenced by the addition of sodium perchlorate. A further increase of sodium perchlorate concentration to above 1.0 mol/l resulted in no further changes in the

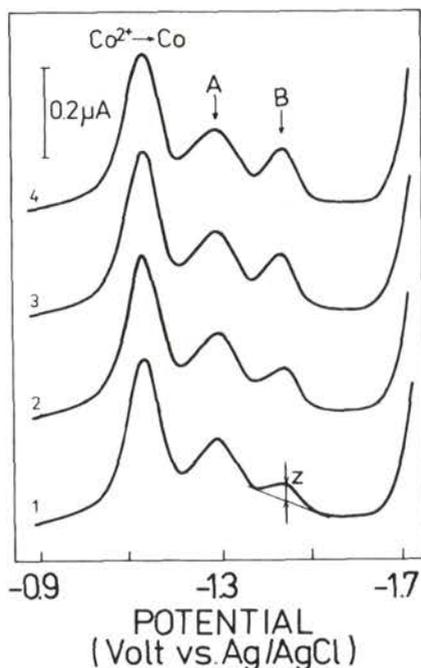


Fig. 4. DP polarograms of 3 $\mu\text{mol/l}$ cytochrome *c* in standard Brdička's solution with varying concentrations of sodium perchlorate (in mol/l): Curve 1 — 0, curve 2 — 0.1, curve 3 — 1.0, curve 4 — 3.0. The value of *z* was taken to represent the height of the most negative peak B.

height of the more negative wave (maximum) B. The dependence of the height of DP polarographic peak B of cytochrome *c* in standard Brdička's solution on sodium perchlorate concentration is shown in Fig. 5a. A qualitatively identical result was obtained if DC polarography was used. Simultaneously with polarographic curves, absorbances at 409 and 695 nm were also measured. Brdička's catalytic current corresponding to the more negative polarographic wave, or peak, B, increased in parallel with the increase in intensity of the Soret band at 409 nm, and with the decrease in absorbance at 695 nm. This parallel course thus strongly supports our conclusion that more negative Brdička's polarographic catalytic current (polarographic wave, or peak, B) also reflects the conformational state of the molecule of cytochrome *c* in the bulk solution associated, in particular, with the accessibility of the heme moiety.

DC polarographic behaviour of cytochrome *c*₃ in standard Brdička's solution was also investigated. This conjugated protein has four hemes within a single polypeptide chain. Similarly as in the recent paper by Senda (Ikeda et al. 1980a), Brdička's polarographic catalytic currents of cytochrome *c*₃ were substantially

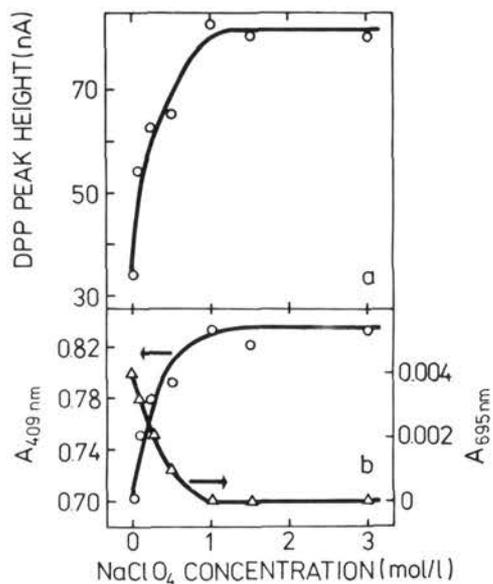


Fig. 5. The effect of sodium perchlorate on Brdička's polarographic currents (a) and absorbance (b) of cytochrome c in standard Brdička's solution. (a) The dependence of the height of the most negative DP polarographic peak B on sodium perchlorate concentration. (b) Absorbance changes at 409 nm of 7 μmol/l cytochrome c (triangles) and at 695 nm of 85 μmol/l cytochrome c (circles) induced by increasing concentrations of sodium perchlorate. For other conditions, see Fig. 2.

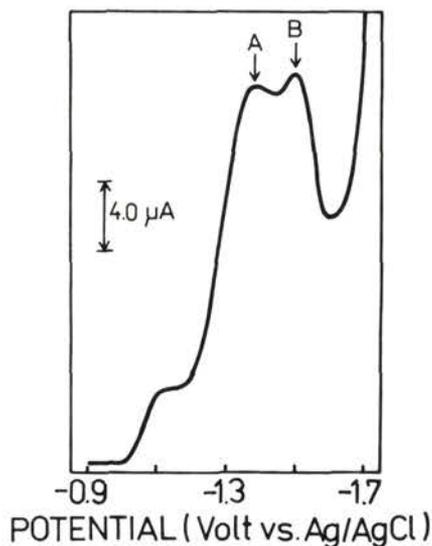


Fig. 6. DC sampled polarogram of 3 μmol/l cytochrome c, in standard Brdička's solution.

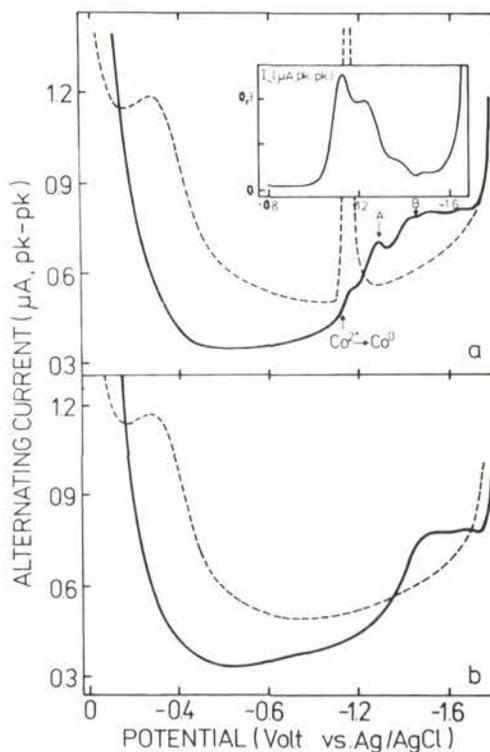


Fig. 7. AC polarograms of 0.1 mmol/l cytochrome c. (a) Medium: Standard Brdička's solution, 90° out-of-phase component of AC; insert in phase component of AC. (b) Medium: 0.1 mol/l NH_4Cl plus 0.1 mol/l NH_3 , pH 9.3 (standard Brdička's solution without CoCl_2), 90° out-of-phase component of AC. Dashed curve — background electrolyte.

higher than those of cytochrome c (Fig. 6). This result was explained by a higher content of catalytically active sites in cytochrome c_3 . The precise quantitative comparison of catalytic currents of cytochromes c and c_3 is, however, difficult because the two proteins differ not only in their contents of amino acids, but also in their molecular mass. Both these factors can greatly influence, for instance, adsorption of these biopolymers at the mercury electrode, and thus also Brdička's catalytic currents (Senda et al. 1982). An important property of the molecules of cytochrome c_3 is a large accessible surface area of the hemes compared to that in a eukaryotic cytochrome c (Niki et al. 1984). This means that, if our conclusion concerning the effect of heme accessibility on Brdička's catalytic currents is valid, the ratio of the heights of the waves B and A yielded by cytochrome c_3 should be higher than that for cytochrome c. It follows from the comparison of these ratios determined experimentally (cf. Figs. 1 and 6) that it was by 24 % higher for

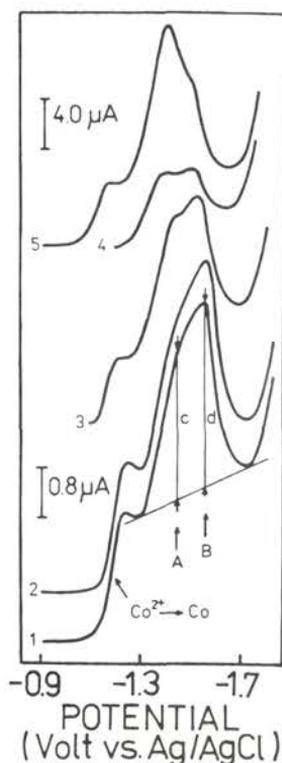


Fig. 8. NP polarograms (curves 1—4) and DC sampled polarogram (curve 5) of $3 \mu\text{mol/l}$ cytochrome c in 1.0 mmol/l CoCl_2 with 0.1 mol/l NH_4Cl plus 0.3 mol/l NH_3 , pH 9.8. Initial potential: Curve 1, -0.2 V , curve 2, -0.8 V , curve 3, -1.1 V , curve 4, -1.2 V . The values of c and d were taken to represent the heights of the NP polarographic waves A and B, respectively.

cytochrome c_3 than for cytochrome c. This result thus supports our conclusion concerning the effect on Brdička's catalytic currents of heme exposure to solvent.

NP polarography. The effect of cytochrome c adsorption.

In our studies of Brdička's catalytic currents yielded by cytochrome c, also NP polarography was used. The latter method differs from DC or DP polarography in that it uses large potential excursions during the drop life. In NP polarography a series of increasing amplitude voltage pulses, starting from an initial potential (E_i), were imposed on successive drops at the end of drop life. In our experiments the electrode was prepolarized to E_i more positive than the potentials of waves or peaks A and B yielded by cytochrome c, and the amplitudes of voltage pulses were negative. Thus the electrode was prepolarized to E_i where cytochrome c was expected to be appreciably adsorbed at the mercury electrode. Qualitative data on adsorption of cytochrome c in Brdička's solution were obtained from AC polaro-

grams (Fig. 7). It follows from these measurements that cytochrome *c* in standard Brdička's solution lowers the differential capacitance of the electrode double layer and is therefore adsorbed at potentials around PZC. As for the potential range close to that corresponding to the origin of Brdička's maxima A and B, it is apparent from Fig. 7 that cytochrome *c* is adsorbed at least up to a potential of -1.3 V. The adsorption of cytochrome *c* in Brdička's solution at more negative potentials was difficult to estimate due to the interference of faradaic catalytic currents.

Brdička's currents of cytochrome *c* were measured by NP polarography in a medium containing 1.0 mmol/l CoCl_2 with 0.1 mol/l NH_4Cl plus 0.3 mol/l NH_3 , pH 9.8. If E_i more positive than -1.0 V was used the height of the catalytic waves A and B was independent of E_i , and the wave B was higher than the wave A (Fig. 8). If E_i was selected more negative than -0.9 V both catalytic waves were lowered with decreasing value of E_i . The ratio of the heights of the waves B and A (h_B/h_A) was also lowered with decreasing E_i . The lowering of both NP polarographic waves with decreasing E_i (at E_i more negative than -0.9 V) might be due to the fact that Co(II) ion near the electrode is preelectrolyzed to Co(0) before the pulse imposition; thus, the flux of cobalt ions from the bulk solution to the electrode surface was decreased. It is generally accepted that, in addition to the surface concentration of protein adsorbed on the electrode surface, the flux of cobalt ions is a major factor, controlling Brdička's catalytic currents of proteins (Senda et al. 1982).

An attempt was made to explain changes in the value of the ratio h_B/h_A resulting from changes in E_i (Fig. 9) on the basis of our conclusion concerning the effect of the structure of cytochrome *c* on Brdička's catalytic currents of this protein. We suggest that this change in the ratio h_B/h_A might be due to surface conformational alteration in the molecules of cytochrome *c*; this would result from an interaction of this protein with the electrode polarized to potentials more positive than -1.0 V. This surface conformational change could thus result in a higher accessibility of heme because of the unfolding of the protein moiety. The unfolding of the molecules of cytochrome *c* could occur on the mercury electrode surface, due to the adsorption of this protein (Fig. 7). The unfolding of cytochrome *c* and other globular proteins adsorbed at the surface of the mercury electrode around PZC has already been observed in various media by other authors (Scheller et al. 1975). The latter interpretation is also supported by the results of NP polarography of cytochrome *c* in the presence of 6 mol/l urea or 1.5 mol/l sodium perchlorate. If measurements were performed in identical background electrolytes and in the presence of denaturants, the ratio h_B/h_A was practically independent of E_i , even if E_i more negative than ca. -1.0 V was used. Therefore, NP polarographic Brdička's currents of cytochrome *c* do not seem to be exploitable for observing the conformational properties of this protein in the bulk solution. The results of

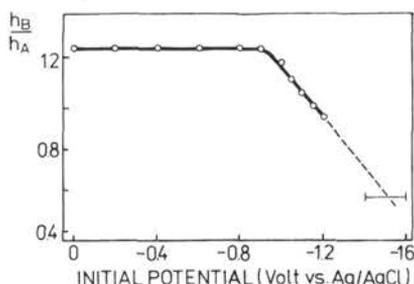


Fig. 9. The dependence of the ratio of the heights of NP polarographic waves B and A (h_B/h_A) on the initial potential. The horizontal abscissa at around -1.5 V corresponds to the range of potentials at which DC polarographic waves A and B occur, and its position related to the axis h_B/h_A indicates the ratio of the heights of DC polarographic waves B and A. For other conditions, see Fig. 8.

experiments in which cytochrome c denaturants were used (Figs. 2 and 4) suggest that the polarographic methods working with small potential excursions during the drop life (DC and DP polarography) are more suitable for this purpose. Unfortunately, NP polarography cannot be employed with E_i identical to the potentials at which waves A and B originate. If, however, the dependence of the ratio h_B/h_A on E_i was extrapolated to more negative values of E_i , corresponding to the potential range in which the waves A and B occur, this extrapolated ratio was very small compared to that obtained for E_i around PZC (Fig. 9). Considering our conclusion on the relation between Brdička's currents of cytochrome c and its conformation in the bulk solution, it is reasonable to expect that the interaction of cytochrome c with the electrode polarized only to the potentials of the origin of waves A and B does not result in an extensive unfolding comparable to that occurring around PZC. It is remarkable that the ratio h_B/h_A measured by DC polarography was approximately identical with that measured by NP polarography using the extrapolation described above (Fig. 9).

Conclusions

It can be summarized that DC or DP polarography of cytochrome c in ammoniacal buffers containing cobalt ions may provide information on conformational alterations of this protein in the bulk solution. Such alterations result in a higher exposure of heme to the solvent. It is highly probable that under conditions of our DC or DP polarographic experiments Brdička's catalytic currents of cytochrome c originate due to the interaction of the mercury electrode with the protein, the latter being not extensively unfolded at the electrode. However, the question, whether under these conditions cytochrome c is completely native, cannot be answered as yet.

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Abbreviations

AC	alternating current
DC	direct current
DP	differential pulse
E_i	initial potential
NP	normal pulse
PZC	potential of zero charge

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