Voltammetry of Cytochrome c_3 from *Desulfovibrio desulfuricans* (strain Norway) at the Graphite Electrode

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Abstract. Interfacial reactions of cytochrome c_3 at a graphite electrode were studied in a neutral medium by means of the differential pulse and cyclic voltammetry. It was found that ferricytochrome c_3 yielded three cathodic peaks C_I, C_{II} and C_{III} at potentials of -0.3 to -0.5 V (vs. Ag/AgCl reference electrode) on the voltammograms. Upon anodic polarization this protein yielded two anodic peaks A_I and A_{II} at 0.66 V and 0.89 V, respectively. It was suggested that peak C_I, appearing at relatively low concentrations of the protein (5×10^{-9} mol/l), corresponded to a catalytic reaction, in which some of haemins of adsorbed molecules of ferricytochrome c_3 were reduced to haems; these haemins could then be regenerated by chemical oxidation of the haem residues with oxygen adsorbed at the graphite surface. The next two cathodic peaks C_{II} and C_{III} apparently corresponded to diffusion-controlled reduction of haemins bound in the nonadsorbed molecules of ferricytochrome c_3. Anodic peaks A_I and A_{II} were suggested to correspond to electrode reactions identical to those corresponding to peaks A_I and A_{II} of cytochrome c. This means that the electrooxidation of tyrosine residues is responsible for the appearance of the anodic peak A_I, whereas peak A_{II} corresponds to an anodic reaction of haemin residues.

Key words: Electrochemistry — Cytochrome c_3 — Graphite electrode

Introduction

Cytochromes c_3 function as macromolecular electron carriers in the sulphate reducing bacteria of the Desulfovibrio species. These molecules (m. w. ~ 13 000) consist of a single polypeptide chain wrapped around a very compact core of four haems which present a relatively high degree of exposure to the solvent (Haser et al. 1979). The haems in the protein are nonequivalent (Bianco and Haladjian 1981; Der Vartanian and Le Gall 1974) and noninteracting electrochemically (Niki et al. 1979).

From the point of view of understanding the electron transfer reactions in which cytochromes c_3 participate in vivo, investigations of the interaction of these proteins with the electrodes used in voltammetric (polarographic) analysis have shown themselves to be useful. Up to recently particularly mercury electrodes have been used in these studies. Cytochromes c_3 are subjected at mercury electrodes to reduction-oxidation processes which correspond to rather fast electronic exchange (Bianco et al. 1979; Bianco and Haladjian 1979; Niki et al. 1977, 1979; Sokol et al. 1980).

The use of mercury electrodes makes it possible, however, to investigate the electrode processes appearing particularly at negative potentials because of the negligible anodic potential limit of these electrodes. The interfacial processes at surfaces charged positively can be followed by electrochemical techniques in connection with solid electrodes which have a significantly more positive anodic potential limit. Of the solid electrodes, particularly those made from graphite have proved useful for the investigation of anodic processes of proteins (Brabec 1980; Brabec and Mornstein 1980; Reynaud et al. 1980). Graphite electrodes have been also used in our previous work (Brabec et al. 1982) aimed at clarifying electrochemical reactions of horse heart cytochrome c containing only one haem. The goal of this report is to describe interfacial reactions of cytochrome c_3 at an impregnated graphite electrode and to compare them with those yielded under the same experimental conditions by cytochrome c.

Material and Methods

Cytochrome c_3 from *Desulfovibrio desulfuricans* (Norway strain) was a generous gift from Dr. M. Bruschi from Laboratoire de Chimie Bactérienne du C.N.R.S., Marseille. The protein was prepared and characterized as previously described (Bruschi et al. 1977). Horse heart cytochrome c (type VI) from the Sigma chemical Co. was used without further purification. The paraffin-wax impregnated spectroscopic graphite electrode (WISGE) was prepared and used as described earlier (Brabec 1979; Brabec and Mornstein 1980). Between runs the WISGE was resurfaced by polishing the end with emery polishing paper (Feuilles Papier Abrasif Emery Polishing, CEN 4/0). The WISGE had a geometric area of 7 mm^2. The supporting electrolyte for voltammetric measurements used throughout this work was phosphate buffer. It was prepared by mixing KH_2PO_4 and K_2HPO_4, both at a concentration 0.1 mol/l, to obtain pH 7.0.

Differential pulse (DP) voltammograms were obtained with a PAR Model 174A Polarographic Analyzer. All DP voltammograms were obtained with a pulse amplitude of 0.025 V and at a voltage sweep rate of 0.005 V s^{-1}. The current sampling for DP voltammetry at the graphite electrode was set with the drop time control of the PAR Model 174A at 0.5 s. Cyclic voltammetry (CV) experiments were performed with a system consisting of a PAR Model 175 Universal Programmer and a PAR Model 173 Potentiostat equipped with a PAR Model 176 Converter. Voltammetric curves were recorded on a Sefram XY recorder. In this paper all current values are reported according to the setting of current sensitivity on electrochemical analyzers. Since DP voltammetric currents were measured with the PAR Model 174A set, the DP voltammetric current was ten times amplified before its measurement. Thus the DP voltammetric current values reported here are ten times higher than the real ones. A Metrohm

silver-silver chloride (satd. NaCl) electrode (Ag/AgCl) was used as reference electrode. Throughout this report, all potentials are given against Ag/AgCl reference electrode. Unless otherwise stated oxygen was purged from solutions by bubbling U-grade nitrogen for 30 minutes before recording the voltammogram. The basic procedure for DP and cyclic voltammetry has been described previously (Brabec 1979; Brabec and Mornstein 1980; Brabec et al. 1982). Briefly, once the graphite electrode was inserted into the test solution contained in the electrochemical cell it was allowed to stand ca. 10 s without an applied potential. Then, unless stated otherwise, the initial potential (E_i) was applied for a further 120 s, after which time the voltammetric sweep was commenced. The electrochemical experiments were performed at 27°C. Other details of our measurements have been published elsewhere (Brabec 1979, 1980; Brabec and Mornstein 1980; Brabec et al. 1982).

Results

Differential pulse voltammetry

The first experiments with ferricytochrome c_3 were carried out at a relatively very low concentration of this protein (4.4×10^{-8} mol/l).Its DPV behaviour was qualitatively identical to that observed with horse heart cytochrome c (Brabec et al. 1982): If the DP voltammogram was scanned from $E_i = 0.2$ V to negative potentials (using negative pulse) ferricytochrome c_3 yielded a single well-defined peak C_I around -0.3 V (Fig. 1a). If the DP voltammogram was scanned from $E_i = 0.0$ V to positive potentials (using positive pulse), ferricytochrome c_3 yielded two peaks, A_I and A_{II}, having peak potentials (E_p) 0.66 and 0.89 V, respectively (Fig. 1b).

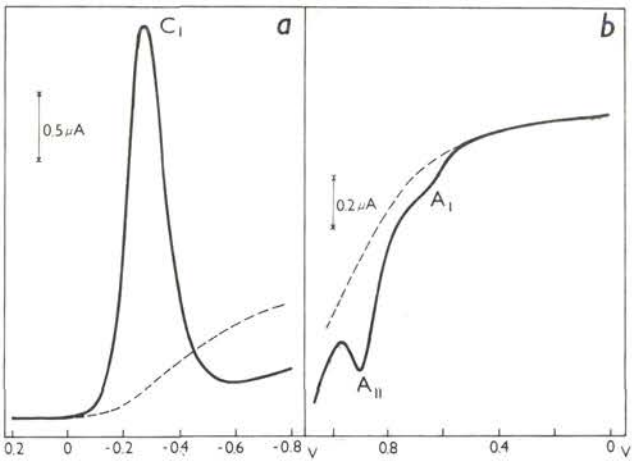

Fig. 1. Differential pulse voltammograms in 0.1 mol/l potassium phosphate, pH 7.0 of ferricytochrome c_3 at a concentration of 4.4×10^{-8} mol/l. (a) Initial potential was 0.2 V, potential sweep was cathodic and pulse amplitude was negative. (b) Initial potential was 0.0 V, potential sweep was anodic and pulse amplitude was positive. Waiting time at the initial potential was 120 s. (– – – –) supporting electrolyte curve.

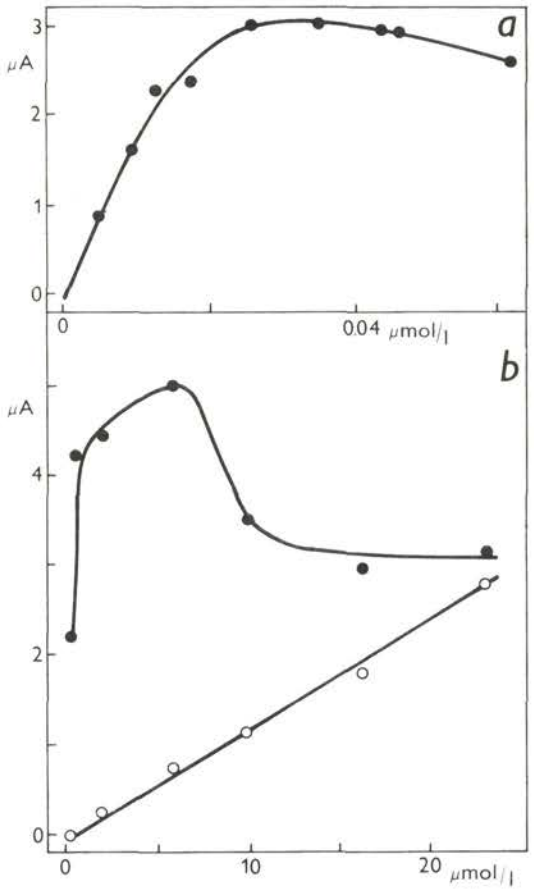

Fig. 2. Variation of the height of differential pulse voltammetric peak C_I (●) and C_{II} (○) with concentration of ferricytochrome c_3. (a) Concentration range of 0—0.06 μmol/l. (b) concentration range of 0.07—23.3 μmol/l. Other conditions were the same as in Fig. 1a.

For relatively low concentrations, the height of peak C_I, A_I and A_{II} at first increased linearly with growing ferricytochrome c_3 concentration (Figs. 2a, 3). At a concentration of ca. 3×10^{-8} mol/l these dependences reached a limiting value. The height of peak C_I, after reaching this limiting value, was slightly lowered, with a further increase of the protein concentration. The lower level of analytical utility of DP voltammetry for determination of ferricytochrome c_3 at the WISGE is about 2×10^{-9} mol/l. E_p of peak C_I was first shifted to more positive values with growing ferricytochrome c_3 concentration. After this peak had reached the first limiting height a further increase of ferricytochrome c_3 concentration led to only a slight shifting of the peak to more negative potentials. Peaks A_I and A_{II} of ferricytochro-

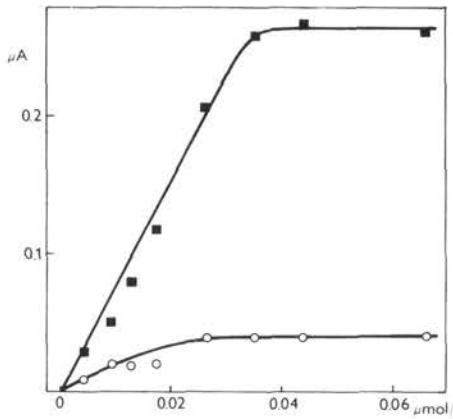

Fig. 3. Variation of the height of differential pulse voltammetric peak A_I (○) and A_{II} (■) with concentration of ferricytochrome c_3. Other conditions were the same as in Fig. 1b.

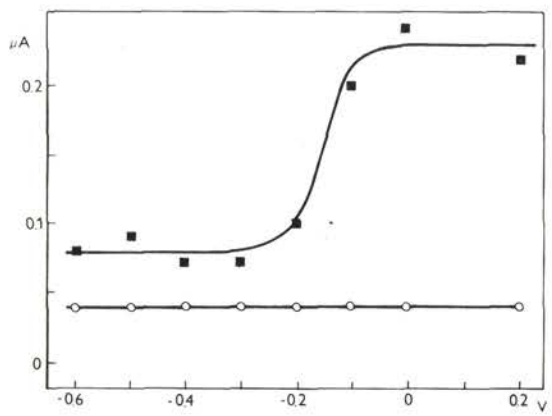

Fig. 4. Variation of the height of differential pulse voltammetric peaks A_I (○) and A_{II} (■) of ferricytochrome c_3 at a concentration of 4.6×10^{-8} mol/l with the initial potential. Other conditions were the same as in Fig. 1b.

me c_3 were shifted in the range of concentrations up to ca. 3×10^{-8} mol/l to more positive potentials.

The dependence of the height of peaks A_I and A_{II} on E_i was also recorded (Fig. 4). Whereas the height of peak A_I was independent of E_i in the whole range of E_i used (0.2 to -0.6 V), the height of the more positive peak A_{II} was independent of E_i only at E_i ranging from ca. -0.1 to 0.2 V. If the value of E_i was changed to more negative values than -0.1 V, peak A_{II} decreased. At $E_i = -0.3$ V this decrease reached a limiting value which was three times lower than a maximum

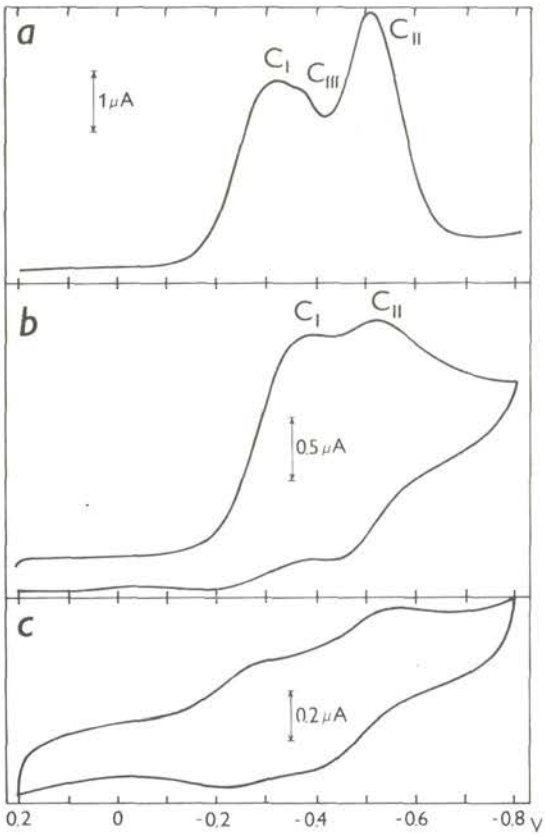

Fig. 5. Voltammograms in 0.1 mol/l potassium phosphate, pH 7.0 of ferricytochrome c_3 at a concentration of 3.9×10^{-5} mol/l (a, b) and 2.3×10^{-5} mol/l (c). (a) Differential pulse voltammogram. (b) Cyclic voltammogram. First, forward potential sweep was cathodic. (c) Steady state cyclic voltammogram (obtained after seventeenth cycle). Cyclic voltammograms were obtained at voltage scan rate of 0.05 V s^{-1}. Other conditions of voltammetric measurements were the same as in Fig. 1a.

value obtained at $E_i \geq -0.1$ V (Fig. 4). The potentials of peaks A_I and A_{II} were, however, independent of E_i.

The DP voltammetric behaviour of ferricytochrome c_3 at the WISGE described so far concerned only relatively low concentrations of this protein (up to ca. 1×10^{-7} mol/l). At higher concentrations peak C_I started to increase again (Fig. 2b). After reaching the second maximum value at a concentration of ferricytochrome c_3 of ca. 6×10^{-6} mol/l peak C_I again decreased by ca. 30% as a consequence of a further increase in ferricytochrome c_3 concentration, and then its height remained constant. The potential of peak C_I continued to shift to slightly more negative potentials even in this cytochrome c_3 concentration range. At

a concentration of about 2×10^{-6} mol/l ferricytochrome c_3 began to yield another cathodic DP voltammetric peak C_{II} at -0.5 V (Fig. 5a) which further increased linearly with growing cytochrome c_3 concentration (Fig. 2b). Its potential was independent of cytochrome c_3 concentration in the whole range of the protein concentrations used. Moreover, at relatively very high concentrations of ferricytochrome c_3 it still yielded one supplementary peak C_{III}, the height of which increased with concentration (Fig. 5a). Peak C_{III} was very close to peak C_I, so that it was difficult to follow the changes in its height as a consequence of the change of a parameter influencing the electrode process in the concentration range investigated.

Cyclic voltammetry

CV experiments described in this report were carried out with $E_i = 0.2$ V and the first, forward potential sweep was cathodic; after the potential had reached a value of -0.8 V, the voltage sweep was immediately reversed, so that E_i could be reached.

In the case of ferricytochrome c_3 at a concentration of 6.6×10^{-8} mol/l CV forward sweep gave only single, well-defined peak C_I at the potential around -0.4 V. In the course of reverse part of the current-potential curve this peak had no pronounced anodic counterpart in the whole region of potentials used in CV experiments. The height of CV peak C_I increased with growing voltage scan rate (Fig. 6a). At higher scan rates (above ca. 0.1 V s^{-1}) the CV peak C_I was independent of voltage scan rate. The peak potential was shifted with increasing scan rate to more negative potentials (Fig. 6b). Repetitive cyclic scans on the same

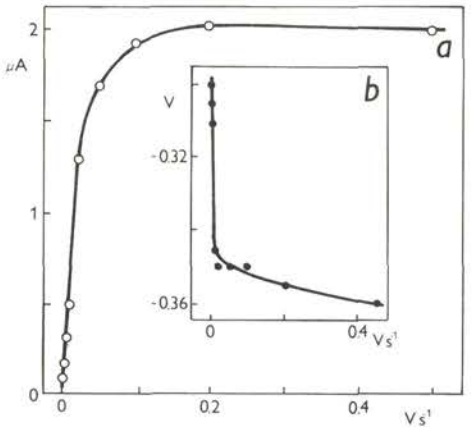

Fig. 6. Effect of voltage scan rate, v on the cyclic voltammetric behaviour of ferricytochrome c_3 at a concentration of 6.6×10^{-8} mol/l. (a) The height of peak C_I, (b) the potential of peak C_I (E_p). Other conditions were the same as in Fig. 1a.

graphite electrode surface at a constant scan rate (0.05 V s^{-1}) yielded a shift of CV peak C_I towards more positive potentials and a decrease of the peak current. For instance, the peak C_I which appeared on the CV curve corresponding to the sixth cycle was approximately seven times lower than that corresponding to the first cycle. The repetitive cyclic scans were also performed in such a way that, after recording the first cathodic part of CV curve, the WISGE was held for various times (2—20 mins) at −0.8 V, and only after this time were the anodic part of the first cycle and the second cathodic potentials sweep recorded. This procedure led to more effective lowering the height of cathodic peak C_I on the voltammetric curve corresponding to this second potential sweep.

In further CV studies of cytochrome c_3 the "film transfer method" (Kuznetsov et al. 1977; Haladjian et al. 1979) was used. The experiments described in this report were performed in deoxygenated solutions containing ferricytochrome c_3 at a concentration of 6.6×10^{-8} mol/l in the supporting electrolyte used throughout this work. At this concentration 120 s were allowed for the adsorption at the WISGE with the open current circuit. If WISGE thus equilibrated was washed and transferred to deoxygenated solution of the supporting electrolyte free of cytochrome c_3, its CV behaviour was identical to that observed in the solution of ferricytochrome c_3 at a concentration of 6.6×10^{-8} mol/l. Repetitive cyclic scans on the same graphite electrode surface coated with ferricytochrome c_3 yielded identical results as the same experiment performed in the presence of ferricytochrome c_3 at a concentration of 6.6×10^{-8} mol/l. If the coated electrode was taken out of the electrochemical cell (after the sixth cycle), exposed 60 s to air, then transferred again to deoxygenated solution free of cytochrome c_3, after new, shorter deoxygenation a CV peak C_I was obtained which was significantly higher than that obtained using preceding sixth cathodic potential sweep.

At relatively higher concentrations of ferricytochrome c_3 (2.3×10^{-5} mol/l) this protein yielded on CV curves, besides peak C_I, a further more negative cathodic peak C_{II} (Fig. 5b). At this concentration of ferricytochrome c_3 both cathodic CV peaks had anodic counterparts in the course of the reverse part of the current-potential curve. Moreover, after about seventeen cycles a steady state voltammogram was obtained on which both CV cathodic peaks C_I and C_{II} and their anodic counterparts were observable (Fig. 5c). A higher concentration of cytochrome c_3 (2.3×10^{-5} mol/l) was also used in the experiments in which the "film transfer method" was exploited. If the WISGE in the solution of ferricytochrome c_3 at a concentration of 2.3×10^{-5} mol/l was washed and transferred to the supporting electrolyte (free of cytochrome c_3), it yielded only peak C_I, whose height was approximately identical to that observed in the solution of ferricytochrome c_3 at a concentration of 2.3×10^{-5} mol/l. The identical result was obtained if the WISGE thus equilibrated was transferred to a solution containing horse heart ferricytochrome c at a concentration of 4.9×10^{-5} mol/l.

Discussion

The results of this paper indicate that cathodic peak C_I corresponds to an electrochemical reaction in which ferricytochrome c_3 participates in the adsorbed state. Peak C_I yielded by ferricytochrome c_3 at relatively low concentrations (up to ca. 5×10^{-8} mol/l) exhibits identical voltammetric characteristics to peak C of ferricytochrome c (Brabec et al. 1982). The latter fact along with the results of voltammetry of proteins differing in their composition (Brabec et al. 1982) allow one to conclude that peak C_I of ferricytochrome c_3 and peak C of ferricytochrome c (Brabec et al. 1982) correspond to the same electrochemical reaction. In the case of the latter protein this reaction is ascribed (Brabec et al. 1982) to the process during which haemin residue is reduced to haem (having iron in the bivalent state). Haemin is then regenerated with oxygen adsorbed at the WISGE surface so that a given protein molecule may be reduced at the WISGE several times during one cathodic potential sweep. As for ferricytochrome c_3, it yielded a peak C_I which was markedly higher (Fig. 2) than peak C of ferricytochrome c (Brabec et al. 1982). This could indicate that more than one haemin residue of ferricytochrome c_3 participates in this catalytic reaction.

The dependence of the peak C_I on the concentration of ferricytochrome c_3 has two limiting parts (Fig. 2). This stepwise course apparently corresponds to the fact that concentration of cytochrome c_3 in solution influences the structure of the adsorption layer. Changes in the structure of the adsorption layer might include a change in the configuration and conformation of the adsorbed molecule, allowing either an increase of the protein concentration at the electrode surface or an increase in the number of haemin residues accessible for the reaction with the electrode.

Peaks C_{II} and C_{III} appear only at higher cytochrome c_3 concentrations. The results obtained up to now (Fig. 5b,c) indicate that probably reversible electroreduction of some haemin residues of cytochrome c_3 is responsible for the formation of peaks C_{II} and C_{III}. This reduction might take place in a diffusion-controlled process through a layer of adsorbed molecules of this protein in a manner similar to that proposed for cathodic processes of this biopolymer earlier (Bianco and Haladjian 1979; Bianco et al. 1979; Bianco and Haladjian 1982).

In contrast to cytochrome c_3, haemin in cytochrome c can be reduced reversibly only at some chemically modified electrodes (Yeh and Kuwana 1977; Eddowes and Hill 1979; Lewis and Wrighton 1981). The results of this investigation indicate (see experiments in which the "film transfer method" was used) that horse heart ferricytochrome c is not reducible at a graphite electrode modified by adsorbed cytochrome c_3.

It was shown in our previous communication (Brabec et al. 1982) that both anodic peaks A_I and A_{II} of haem-containing proteins correspond to irreversible electro-

chemical reactions of the protein adsorbed at the graphite electrode: peak A_I corresponds to the electrooxidation of tyrosine residues, whereas the more positive peak A_{II} corresponds to an electrode reaction in which haemin or haem participate. The dependence of the height of anodic peak A_{II} of ferricytochrome c_3 on the initial potential (Fig. 4) had a qualitatively similar course to the same dependence obtained for ferricytochrome c (Brabec et al. 1982). In the latter case it was suggested that the reduction of ferricytochrome c at the graphite electrode charged to more negative potentials (potentials of peak C) led to a change in the conformation of the adsorbed protein. A change in the haem configuration in the electrode surface that would not be favourable to the electrode reaction responsible for the appearance of peak A_{II} might be a consequence of this conformational change. The height of peak A_{II} of ferricytochrome c_3 was decreased three times if the initial potential was changed from the value of 0—0.2 V to −0.3 − −0.6 V (Fig. 4). This fact might indicate that three haemins were electrooxidizable in the adsorbed molecule of cytochrome c_3 at potentials of peak A_{II} if the electrode was prepolarized to 0 − 0.2 V. On the other hand, the prepolarization to −0.3— −0.6 V (at which cytochrome c_3 was apparently reduced at the graphite electrode) caused such change in adsorbed protein molecule that only one haem remained accessible for electrooxidation at potentials of peak A_{II}.

In conclusion it can be summarized that the advantage of the use of graphite electrodes in investigations of interfacial reactions of haem-containing proteins consists in the possibility of obtaining new information, particularly on properties of these compounds at a positively charged surface. This work further demonstrates that graphite electrode can easily be used in a form modified by physically adsorbed biomacromolecules. Electrodes thus modified could be exploited, for instance, in biotechnology in those cases where only a small amount of a chemically or biologically active biopolymer is available.

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