DENATURATION OF PROTEINS WITH DISULPHIDE DOUBLE BONDS

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SUMMARY

The first harmonic alternating current polarography, with a selected phase angle of $\pi/2$ rad, gives structural and analytical information about the denaturation process of insulin. This method allows detecting any alteration in the composition and in the aggregational or conformational state of the protein. The reduction of the disulphide bonds and free Zn(II) gives complementary information on the capacitive contribution around the zero charge potential, for controlling the quality of the insulin samples. Kalousek K_3 and K_4 methods provide fast information about the structural state of proteins in solution. Proteins with disulphide bonds (as albumin and lysozyme) yield catalytic waves in Co(II)/amonia buffer that denote the different electrochemical activity of -SH lipophilic and hydrophilic residues.

INTRODUCTION

Polarographic techniques give fast information about the denaturation and degradation of proteins (1-3).

The globular proteins containing disulphide bonds present a double catalytic wave in Brdicka medium that is associated whit SH groups (4,5). The first catalytic wave , recorded at less negative potencials, is caused by the SH groups present in hidrophobic regions, while the other wave, recorded at more negative potentials, is caused

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by the SH groups localized in charged, hydrophilic regions (6). Although diverse mechanisms have been postulated for the catalytic currents (7-13), the proposed mechanism by P.Mader and V.Veselá (5), is based on three stages:

a) $Co(II)RS + 2\ e^- \rightarrow Co(0)RS$
b) $RS^- + Co(II) \rightarrow Co(II)RS$

Initiation

c) $Co(0)RS + DH^+ \rightarrow Co(0)RSH^+ + D$
d) $Co(0)RSH^+ + 1\ e^- \rightarrow Co(0)RS + 1/2\ H_2$

Propagation

e) $Co(0)RS \rightarrow Co(0) + RS^-$
f) $Co(0)RSH^+ \rightarrow Co\ (o) + RS^- + H^+$
g) $Co(II) + 2\ e^- \rightarrow Co(0)$

Termination

Therefore, there are, at least, three possibilities for the discharge of hydrogen, from the prewave of sodium and the two produced by the SH groups.

In this paper, experimental results obtained from polarographic methods of Kalousek (14) are summarized, with the aim of proving how catalytic waves in ammoniacal medium of cobalt are useful for controlling the quality of aqueous preparations of proteins. The electrochemical study of macromolecule-cation interaction provides structural, kinetic, thermodynamic and analytical information about the macromolecule. In the case of insulin, the Zn(II) cation is an unavoidable ion, since commercial insulin preparations and insulin present in the pancreas of mammalia, are stabilized by this cation (15-17).

It is known that disulphide bonds are reduced on *dme* and that this reaction is affected by the presence of Zn(II), in excess, or by the presence of phenformine (1). It has been proven that the biguanides are able to capture Zn(II) linked initially to the insulin (18). In this

paper, ac first harmonic waves with phase selection ($\phi = \pi/2$) of the system insulin-Zn(II)-medium is studied in the presence or absence of phenformine. In the first place, the K_3 and K_4 Kalousek methods are applied to follow the process of denaturation of the albumin and the lysozyme. The evolution with time of the catalytic waves that yield these proteins in an ammoniacal/Co(II) medium is studied. Next the process of the denaturation of insulin is addressed, throuhg alternating ac_1 current polarography (with a phase angle of $\pi/2$ rad), from the reduction of its disulphide bonds. This method seems to be very suitable in the case of insulin, since the reduction of the Zn(II) overlaps with its catalytic waves.

EXPERIMENTAL

ac polarograms have been recorded by a Metrohm E-506 polarograph, with a three electrodes system, using a thermostated cell. Potentials were referred to the $Ag/AgCl,KCl_{sat.}$ electrode. The capillary used in the study of the insulin had a mercury flow rate of $m = 2.61 \pm 0.01\ mg.s^{-1}$ at -1.0 V, when immersed in 0.1 M KCl solution. The drop time was mechanically fixed at a value of 0.6 s. Acetic-acetate buffer solutions of ionic strengh I = 0.5 were used. The pH of the solutions was measured with a pH-meter Radiometer PHM 62 Standard,that allowed reaching a precision of 0.01 pH units.

The concentration of the protein (18,1) denaturizer phenformine hydrochloride (19) has been mantained constant at 0.2 M in samples containing these biguanide. Amorphous insulin was chosen, since this allows the recording of better waves. $CoCl_2$ (A.R) of Merck, cystine hydrochoride (Fluka) and porcine insulin Velosulin-modified (Nordisk Gentofte of 40 u.i.) were used. Bovine albumin absent of fatty acids and lyophilized Lysozyme, from egg

white (98% pure), both from Sigma Chemmical Co were prepared at 0,01% in weight. The mercury and water used were bidistilled. NH_4Cl/NH_4OH (0.5M) buffer was prepared. Notable microbian contamination was proven non-existant in the denaturation of samples stocked at 280 K.

RESULTS AND DISCUSSION

The cystine polarograms $\mathbf{K_3}$ and $\mathbf{K_4}$, (Figs.1), show only the catodic component. The peak shape of the reduction waves of both cobalt and hydrogen, is due to charge contributions of the acting species. The evolution of these waves allows following the denaturation and the degradation of the albumin samples. In the albumin, the polarographic waves are smaller(Fig.2). Simultaneously with cobalt hydrolysis, the catalytic wave of the hydrophilic region as much as the catalytic wave of the hydrophobic region diminish.

In the lysozyme, it is observed, that as time evolutes, the catalytic wave decreases in size, both in $\mathbf{K_3}$ as in $\mathbf{K_4}$(Fig. 3). In the last polarogram the discharge of hydrogen, corresponds to the so-called pre-sodium wave. The coincidence of the $\mathbf{K_3}$ and $\mathbf{K_4}$ polarograms indicates that anodic contribution of the pulses does not exist. The height of the waves of this irreversible process is approximatelly equal to that of the dc polarograms The peak intensities of the catalytic waves depend on two factors: the reduction process of Co(II) complexed with the protein (controlled by diffusion) and other factor that depends on the catalytic activity of the protein centers.

The insulin yield catalytic waves overlaped with the Zn(II) reduction wave. The capacitive contribution on the first harmonic

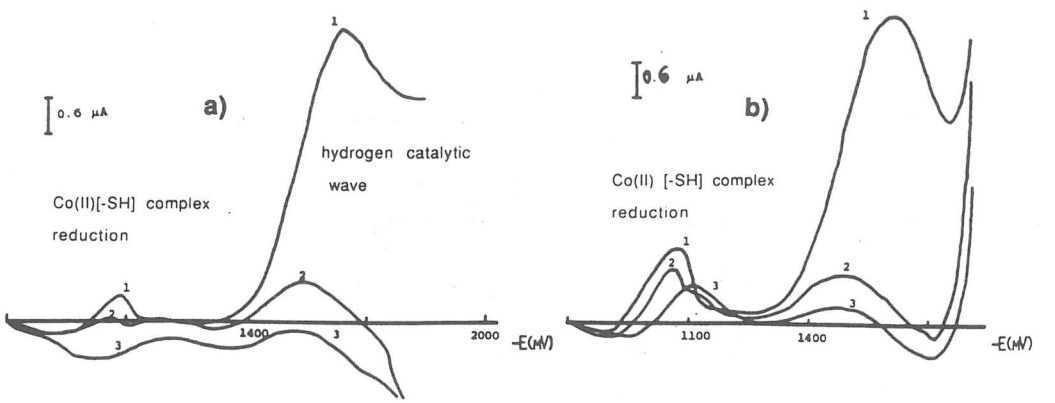

Fig. 1- Kalousek catalytic waves of cystine: a) K_3 waves; b) K_4 waves. [Cystine] = 4×10^{-5} M; [Co(II)] = 4×10^{-4} M; I = 0.5 M; pH = 8.85; T = 298K; t = 0.6 s; m = 0.88 mg s^{-1}; E_{pb} = -1200 mV; f_k = 75 Hz. 1) 0 days; 2) 6 days; 3) 27 days.

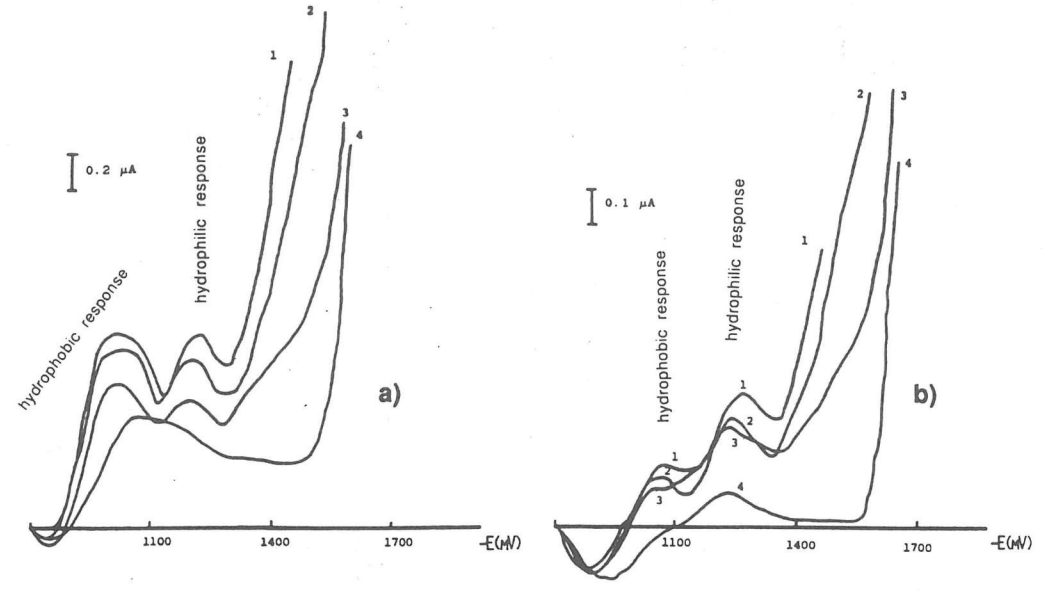

Fig. 2 - Kalousek catalytic waves of albumin: a) K_3 waves; b) K_4 waves. [albumin] = 0.01% weight; [Co(II)] = 4×10^{-4} M; I = 0.5 M; pH = 8.85; T = 298 K; t = 6 s; m = 0.88 mg s^{-1}; E_{pb} = -1200 mV; f_k = 75 Hz. 1) 0 days; 2) 6 days; 3) 29 days; 4) 147 days.

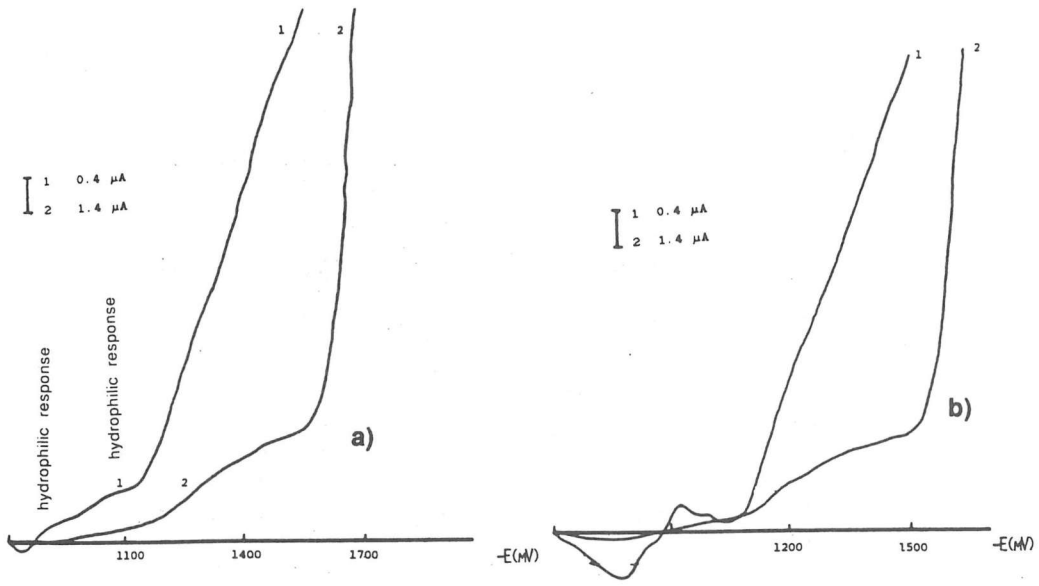

Fig. 3 -Kalousek catalytic waves of lysozyme: a) K_3 waves; b) K_4 waves. [lysozyme] = 0.01% weight; [Co(II)] = 4 x 10^{-4} M; I = 0.5 M; pH = 8.85; T = 298K; t = 0.6 s; m = 0.88 mg s^{-1}; E_{pb} = -1200 mV; f_K = 75 Hz. 1) 0 days; 2) 2 days.

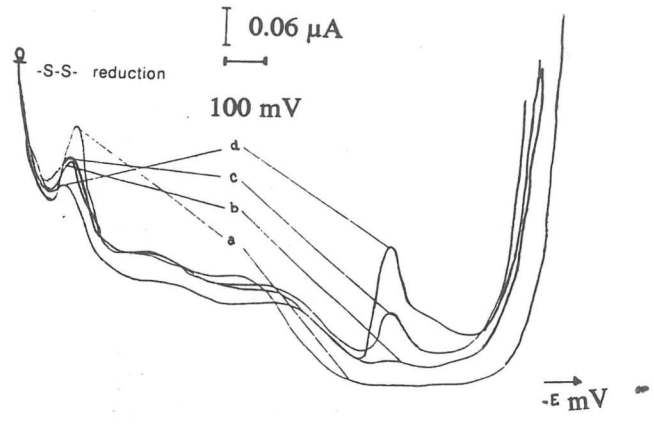

Fig. 4 - Time effect on the AC_1 waves of cystine (ø = 0 rad). [cystine] = 4 x 10^{-4} M; T = 310 K; m = 2.63 mg s^{-1}; I = 0.5 M; pH = 5.02; t = 0.6 s; ΔE = 10 mV. a) 29 min; b) 1411 min; c) 2878 min; d) 4334 min.

polarograms of insulin is recorded in a phase angle near π/2 rad, if faradaic electrochemical processes are not taking place. The wave recorded is of the sort of capacitive curves but faster to obtain(20,21). In every case, the $\mathbf{ac_1}$ (ϕ = π/2 rad) polarograms may be compared to electrocapillary curves.

It is known that proteins, like insulin, are adsorbed on the mercury electrode. Insulin adsorption on the *dme* affects the reduction of free Zn(II) present in the solution, since the insulin decreases the efective area of the electrode. However, this does not hinder the fact that a wave of analytical usefulness corresponding to the reduction of Zn(II) ions, may be recorded. ac_1 polarography with phase selection at π/2 rad, provides great information of faradaic and non-faradaic processes, complementary to other polarographic and voltammetric methods. The reduction waves of disulphide bonds were recorded, at different times, since cystine solutions were prepared. The height of the waves decreases (Fig. 4) with time This is explained, on the basis of reactions of cystine degradation, which seem favoured by the direct reaction between the cystine and the mercury electrode(22,24). The figure 5(c and d curves at ϕ = π/2 rad) involve the faradaic contribution of reduction of a new component formed so being clearly detected at ϕ = 0 rad.

When there is approximately 0.5 M Zn(II) in the medium, the disulphide wave remains exalted and it is clearly prove that its concentration decreases with time simultaneously with as the adsorption on the electrode (Fig. 6). The peaks cannot be recorded when phenformine is 0.2 M, even if Zn(II) concentration is 0.5 M.

The height of faradaic waves of insulin disulphide bonds decreases, in the course of time , more slowly than the cystine ones (Fig. 7). This fact shows a larger steric effects in the case of insulin.

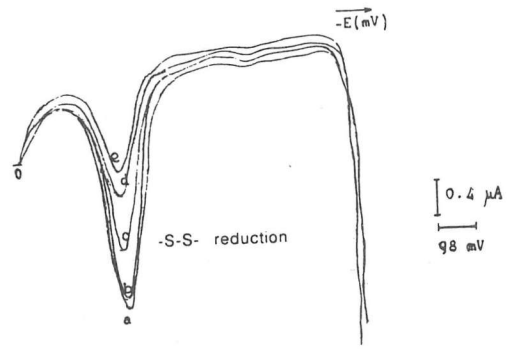

Fig. 5 - Time effect on cystine ac_1 waves ($\phi = \pi/2$ rad) in the presence of zinc. [cystine] = 4.10 x 10^{-4} M; [Zn(II)] = 0.5 M; T = 310 K; m = 2.63 mg s^{-1}; I = 0.5 M; pH = 5.02; t = 0.6 s; ΔE = 10 mV. a) 36 min; b) 1397 min; c) 2858 min; d) 4318 min; e) 8666 min.

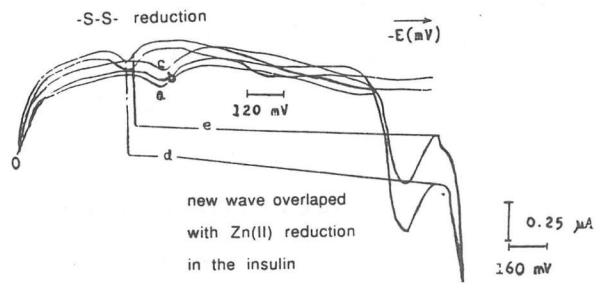

Fig. 6 - Time effect on insulin ac_1 waves ($\phi = \pi/2$ rad). [Insulin] = 6.3 x10^{-5} M; T = 308 K; m = 2.63 mg s^{-1}; I = 0.5 M; pH = 5.02; t = 0.6 s; ΔE = 10 mV. a) 106 min; b) 1453 min; c) 5833 min; d) 7209 min; e) 8674 min.

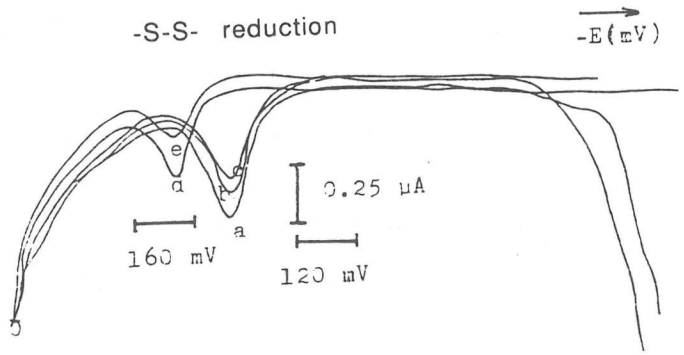

Fig. 7 - Time effect on insulin ac_1 waves ($\phi = \pi/2$ rad) in the presence of phenformine. [Insulin] = 6.3 x 10^{-5} M; [phenformine] = 0.2 M; T = 308 K; m = 2.63 mg s^{-1}; I = 0.5 M; pH = 5.02; t = 0.6 s; ΔE = 10mV. a) 22 min; b) 1222 min; c) 5536 min; d) 6959 min; e) 8394 min.

Similarly to cystine, a new faradaic wave is recorded as the primary chain of insulin is decomposed(8,11). Also, a new wave due to the biguanide-Zn complex is detected near the medium discharge.

The presence of Zn(II) 0.5 M, prevents the reduction of disulphide bonds detection, that is to say, the behavior is like the more cristalline insulin. The same effect is shown in the presence of a great excess of phenformine and zinc with respect to insulin.

The superimposed alternating current polarographic method, selecting a phase angle of $\phi = \pi/2$ rad, allows distinguishing between different types of insulins. Also, its denaturation process is allowed to be studied. All insulins are adsorbed in a large interval of potentials, centered around zero charge potential, giving a zone of constant apparent capacity of double layer. Therefore, in ac_1 ($\phi = \pi/2$ rad) polarography the zero charge potential is not clearly recorded. Insulin and cystine form chemical compounds with mercury (23,24), therefore the observed denaturation also depends on the mercury presence.

During the polarographic reduction, the helical structure and intrachain bonds remain practically intact; however the denaturation of glogular proteins by urea, guanidine or alkali causes an increase in the number of electroactive disulphide bridges and so in the height of the wave, compared with native insulin (25,26). The phenformine effect is more complicated.(Table I) Adsorption on mercury electrode and direct action on cysteinic residues yields a decrease in the height of the wave of the disulphide bonds.

Therefore, selecting a phase angle of $\pi/2$ rad, gives qualitive information about the structure of commercial insulin preparatives since the disulphide bonds and Zn(II) reduction waves and capacitive contribution of ac polarograms depend on it. A previous calibration (as form catalytical waves) is needed for obtaining quantitative

Table I.- ac_1 Polarographic disulphide bonds reduction of insulin

$\phi = \pi/2$ rad.; T = 298 K; m = 2.63 mg. s^{-1}; t = 0.6 s; I = 0.5 pH = 5.02; ΔE = 10 mV

buffer 0,5 M	porcine insulin	phenformine hydrochoride	time	$(-S-S-) \xrightarrow{2H^+ + 2e^-} 2(-SH)$		
				$-E_p$	i_p	$n\alpha$
ml	ml	ml	min	mV	μ A	
10	5	0	106	450	0,975	1,00
			1.453	456	0,825	1,07
			1.470	456	0,825	1,07
			5.833	456	0,675	1,07
			7.209	456	0,600	1,02
			7.212	456	0,600	1,13
			8.674	456	0,375	1,25
10	5	10	22	450	0,200	0,88
			37	450	0,200	0,94
			1.222	456	0,200	0,83
			1.242	462	0,200	0,88
			5.536	456	0,200	1,00
			6.959	456	0,200	0,80
			8.394	456	0,175	0,87

analytical information of the degree of insulin denaturation, However, these methods are completely useful for testing the quality of commercial samples of proteins. The information obtained from this methods are comparable with those obtained from dc or pulse polarography.

REFERENCES

1) Sanz C., Monleón C., Vicente F., Soriano C. and Vera J.; *Anales de Química.* Serie A, 83 (3) (1987), 605.

2) Trijueque J., Sanz C., Monleón C., and Vicente F., *J. Electroanal. Chem.*, 251 (1988) ,173.

3) Monleón C.. *Doctoral These*. University of Valencia (1990).

4) Mairanovskii S.G. : *Catalitic and Kinetic waves in polarography*. Plenum Press. New York (1968).

5) Mader P. and Veselá V., *Bioelectrochem. and Bioenergetics,* 4 (1977) 413.

6) Kuznetsov B.A. and Shumakovich G.P.; *Bioelectrochem. and Bioenergetics*, 2 (1975), 35.

7) Kolthoff J.M. and Mader P.; *Anal. Chem.*, 42 (14) (1970), 1762.

8) Kolthoff I.M., Yamashita K., Hie T.B. and Kanbe A.; *Proc. Nat. Acad. Sci. USA*, 70 (7), 2020 (1973).

9) Kolthoff I.M. , Yamashita K., Hie T.B., *Proc. Nat. Acad. Sci. USA*, 71 (5) (1974), 2072.

10) Palecek E., Brabec V., Jelen F. and Pechan Z.; *J. Electroanal. Chem.*; 75 (1977), 471.

11) Kolthoff I.M., Yamashita K. and Hie T.B.; *J. Electroanal.*

Chem., 63 (1975), 393.

12) Stankovich M.T. and Bard A.J.; *J. Electroanal. Chem.*, 86 (1978), 189.

13) Kolthoff I.M. and Kihara S.; *J. Electroanal. Chem.*, 96 (1979), 95.

14) Ruzic J.; *J. Electroanal. Chem.*, 39 (1972), 111.

15) H. Reuscreidt, W. Stranburger, H. glatter, A. Wolliner, G. Dodson and D.A. Marcela. *Eur. J. Biochem.*, 142(1984)7.

16) R. Bi, Z. Danter, E. Dodson, G. Dodson, F. Giordano, R. Hubbard and C. Reynolds, *Proc. Indian Acad. Sci. (Chem. Sci.)* 92(1983)473.

17) L. Dongcai, W. Zhuli, Z. Yousang and Quin-ping, *Sciencia Sinica. Serie B*, 24(1984)23.

18) F. Vicente, J. Trijueque and F. Tomás, *J. Pharm. Sci.* 72(1983)565.

19) M.E. Noelken, *Anal. Biochem.*, 104 (1980) 228.

20) K.S.G. Doss, A. Kalyanasundaran, *Proc. Indian Acad. Sci., Sect. A*, 35(1952)27.

21) H.B. Breyer and S. Jacobian, *J. Austr. Sci. Res.*, A5(1952)500.

22) J. Trijueque, F. Vicente,F. Martinez and J. Vera, *Portugaliae Electrochimica Acta*. 9(1991) 399.

23) J.M. Issa, A.A. Elsamay, R.M. Issa and Y.M. Temerik, *Electrochim. Acta*, 17(1972)1615.

24) M.T. Stancovich and J. Bard, *J. Electroanal. Chem,*. 85(1977)173.

25) C. Tanford, *Adv. Protein. Chem.*, 23(1986)121.

26) T.M. Florence, *Anal. Letters*, B11(1978)913.

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SUBJECTS