Effect of pH on Brdička Currents Produced by Cytochrome c

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Effect of pH and buffer concentrations on Brdička currents produced by horse heart cytochrome c was investigated in the presence of hexaamminecobalt(III) chloride in ammonia, tris(hydroxymethyl)aminomethane, *N,N*-dimethylglycine, borate, and carbonate buffer solutions at pH varying between 8.2 and 10.7. The Brdička current-activity of the protein varied with pH and the kind and concentration of buffers, but, if corrected for the effect of the buffer salts, showed a sigmoidal pH-dependence. The pH-dependence was interpreted as due to the protonic dissociation of Lys-79 of the protein. Brdička currents of the protein were observed also in unbuffered solutions containing no proton donor except water. The current intensity was explained by taking into account the change of pH at electrode surface. The results indicate that a main proton donor in the Brdička reaction is a water molecule.

Effect of the electrolyte pH and the kind and concentration of buffers on Brdička currents produced by SS- and/or SH-containing proteins has been studied extensively.[1-6] However, an understanding of the effect of these factors is far from complete. We have shown[7] that cytochrome c gives Brdička currents in ammoniacal buffers of pH 9.4 containing cobalt salts and that the heme group and its sixth ligand are important in determining the Brdička current-activity of the protein. Since the structure at heme moiety changes with pH,[8] it seems interesting to study the effect of pH on the Brdička current produced by cytochrome c. In this study we have investigated the pH effect on the Brdička current produced by horse heart cytochrome c in the presence of hexaamminecobalt(III) chloride in buffer solutions of various kinds at pH varying between 8.2 and 10.7 and shown that the pH-dependence of the Brdička current-activity can be interpreted as due to the protonic dissociation of Lys-79 of the protein. The results and discussion are presented in this paper. Importance of water molecule as a proton donor in the Brdička reaction is also described.

Experimental

Chemicals and Apparatus. Horse heart cytochrome c (cyt-c) was a product of Sigma Chemical Co. (Type IV, Lot No 78c-7040). The purity of the cyt-c was checked by SDS-disk electrophoresis. Stock solutions, usually 0.5% in cyt-c were stored at 5 °C. The concentrations were checked spectrophotometrically.[9] Other chemicals used were of a reagent grade quality. D.c. polarograms were recorded on a Yanagimoto P8 polarograph. The characteristics of the dropping mercury electrode (DME) were $m=1.60\ \mathrm{mg\,s^{-1}}$ and $\tau=6.3$ s at open circuit in ammoniacal buffer at 70 cm height of mercury reservoir.

Electrochemical Measurements. All measurements were made in an H-type cell immersed in a thermostat controlled at 25 °C. All potentials were measured against a saturated calomel electrode (SCE). Buffer solutions (0.06—0.30 M ammonium chloride–ammonia for pH 8.4—10.1, 0.06—0.25 M hydrogen chloride–tris(hydroxymethyl)aminomethane for pH 8.2—9.2, 0.06—0.24 M boric acid–potassium hydroxide for pH 9.1—9.4, 0.05—0.20 M *N,N*-dimethylglycine–potassium hydroxide for pH 9.7—10.7, and 0.025—0.10 M sodium hydrogencarbonate–disodium carbonate for pH 9.9) were used as the base solution. The ionic strength of the base solution was adjusted to 0.2 mol dm^{-3} with potassium chloride. Other details of the electrochemical measurements have been described previously.[7]

Results

Brdička Currents in Ammoniacal Buffers. Figure 1 shows polarograms of Brdička current of 0.34 μM cyt-c in the presence of 2×10^{-4} M hexaamminecobalt(III) chloride (Co(III)) in ammoniacal buffers at various pH-values. Two Brdička waves were observed in the pH range covered in Fig. 1, the first one exhibiting a peak. The peak potential of the first wave was shifted negatively with increasing pH, that is, from −1.31 V at pH 8.4 (Fig. 1a) to −1.38 V at pH 10.1 (Fig. 1g), whereas the potential of the second peak (or a shoulder) was almost independent of pH and appeared at −1.52 V. The height of the waves increased with increasing pH. Figure 2 shows the dependence of the Brdička current on the ammonia buffer concentration, $c_{amm}=(NH_4^+)+(NH_3)$, at pH 10.1. The height of the first wave increased greatly with increasing c_{amm} from 0.06 M (Fig. 2a) to 0.30 M (Fig. 2c), whereas the height of the second one was hardly influenced by c_{amm}. With decreasing pH the effect of c_{amm} on the Brdička current became smaller.

Brdička Currents in Tris(hydroxymethyl)aminomethane Buffers (Tris Buffers). Figure 3A shows a polaro-

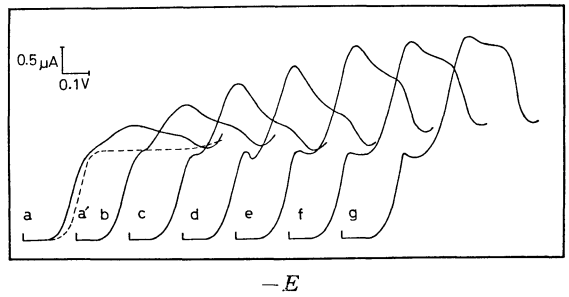

Fig. 1. Polarograms of Brdička current of 0.34 μM cyt-c in the presence of 2×10^{-4} M Co(III) in ammoniacal buffers; a) $c_{amm}=0.11$ M, pH 8.4, b) $c_{amm}=0.12$ M, pH 8.7, c) $c_{amm}=0.15$ M, pH 9.1, d) $c_{amm}=0.10$ M, pH 9.4, e) $c_{amm}=0.08$ M, pH 9.7, f) $c_{amm}=0.07$ M, pH 9.9, and g) $c_{amm}=0.06$ M, pH 10.1. Each curve starts from −0.9 V. (Broken line represents polarogram in the absence of cyt-c.)

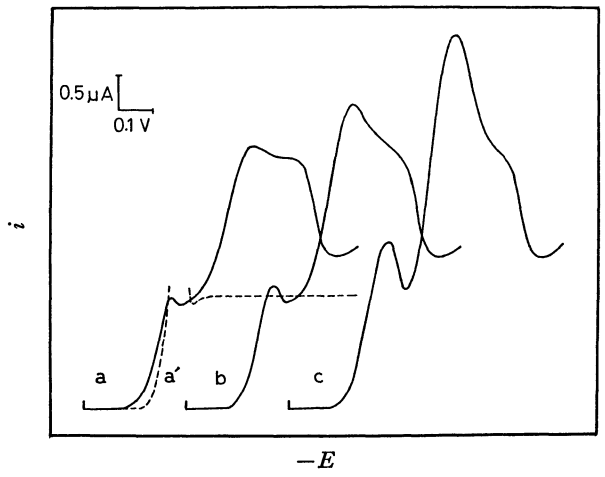

Fig. 2. Dependence of Brdička current of cyt-c on c_{amm} at pH 10.1; c_{amm}: a) 0.06 M, b) 0.12 M, and c) 0.30 M. Each curve starts from −0.9 V.

gram of Brdička current of cyt-c in the presence of Co(III) in 0.12 M tris buffer at pH 8.9. A rounded hump was observed at −1.34 V, followed by a shoulder at −1.52 V. The shape of the wave was similar at pH varying between 8.2 and 9.2, whereas the height of the wave increased with increasing pH. The shape and the height of the wave were hardly influenced by the concentration of tris buffer, c_{tris}, in the range 0.06—0.25 M at constant pH.

Brdička Currents in Borate Buffers. Figure 3B shows a polarogram of Brdička current of cyt-c in the presence of Co(III) in 0.24 M borate buffer at pH 9.1. Two waves of nearly equal wave height were observed at −1.32 and −1.52 V, respectively. At pH 9.4 the Brdička waves were similar in shape to but larger in height than those at pH 9.1. The shape and the height of the waves were hardly influenced by the concentration of borate buffer, c_{borate}, in the range 0.06—0.24 M at constant pH.

Brdička Currents in N,N-*Dimethylglycine Buffers.* Figure 3C shows a polarogram of Brdička current of cyt-c in 0.05 M *N,N*-dimethylglycine buffer at pH 10.0. Two waves observed at −1.36 and −1.52 V grew up to a certain limit with increasing pH from 9.7 to 10.7. With increasing concentration of *N,N*-dimethylglycine buffer, c_{dimet}, from 0.05 to 0.20 M at constant pH, the height of the two waves decreased slightly. In these buffer solutions the polarogram of Co(II) to Co(0) reduction step in the absence of cyt-c (Fig. 3C′) appeared at the potential more negative by about 130 mV than that in ammoniacal buffer solutions (see Fig. 2a′). Similar negative shift of the reduction step of Co(II) to Co(0) has been reported[3] in glycine buffer solutions.

Brdička Currents in Carbonate Buffers. Figure 3D shows a polarogram of Brdička current of cyt-c in 0.025 M carbonate buffer at pH 9.9. Two waves were observed at −1.36 and −1.52 V, respectively. The height of the waves decreased with increasing concentration of carbonate buffer, c_{carb}, from 0.025 to 0.10 M. The polarogram of Co(II) to Co(0) reduction step in the absence of cyt-c (Fig. 3D′) appeared at the

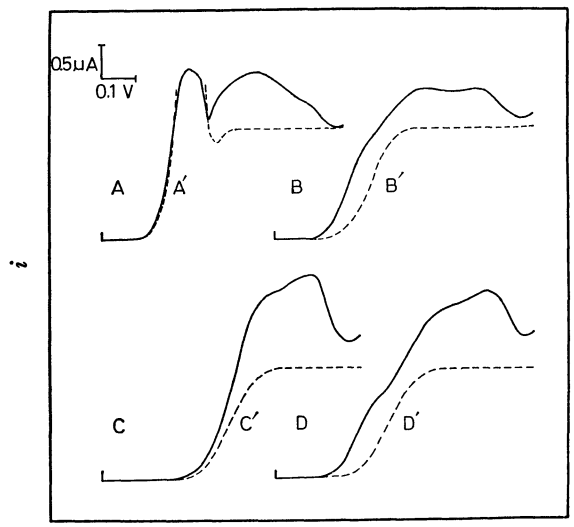

Fig. 3. Polarograms of Brdička current of 0.33 μM cyt-c in the presence of 2×10^{-4} M Co(III) in A) 0.12 M tris buffer, pH 8.9, B) 0.24 M borate buffer, pH 9.1, C) 0.05 M dimethylglycine buffer, pH 10.0, and D) 0.025 M carbonate buffer, pH 9.9. Each curve starts from −0.9 V. (Broken lines represent polarograms in the absence of cyt-c.)

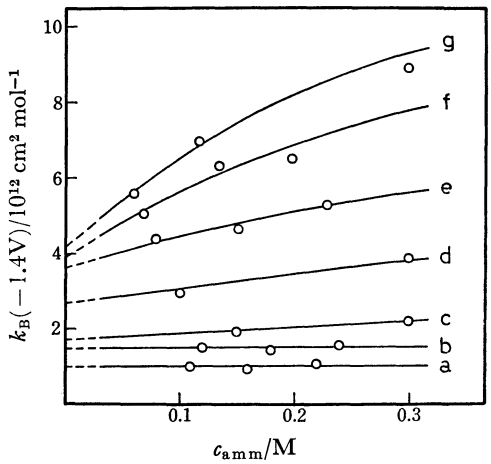

Fig. 4. Plots of k_B (−1.4 V) against c_{amm} at pH= a) 8.4, b) 8.7, c) 9.1, d) 9.4, e) 9.7, f) 9.9, and g) 10.1.

potential more negative by about 100 mV than that in ammoniacal buffer solutions.

$k_B(-1.4\,V)$-Value. It has previously been shown[7,10] that the Brdička current of cyt-c in ammoniacal buffers of pH 9.4 containing Co(III) at the concentration lower than 2×10^{-4} M can be expressed by

$$i_B = FAk_B\Gamma f_{Co}, \quad (1)$$

where F is Faraday, A the surface area of the DME, k_B a proportionality constant representing the Brdička current-activity of cyt-c, Γ the surface concentration of cyt-c adsorbed on the DME surface, and f_{Co} the flux of Co(III) ions at the electrode surface. Further it has been shown[7,10] that the adsorption of cyt-c on the DME surface at low bulk concentrations of cyt-c and at −1.4 V is cntrolled by diffusion and that

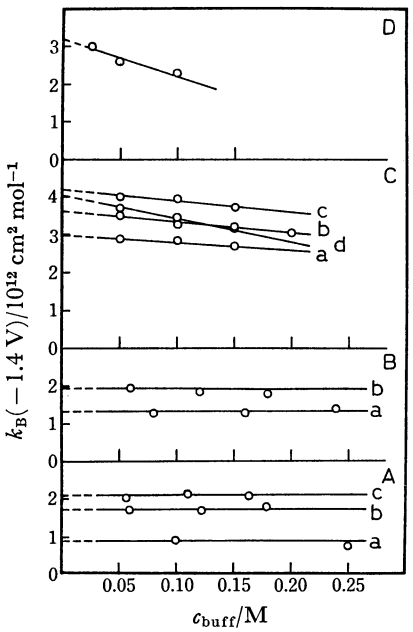

Fig. 5. Plots of k_B (−1.4 V) against A) c_{tris} at pH= a) 8.2, b) 8.9, and c) 9.2, B) c_{borate} at pH=a) 9.1 and b) 9.4, C) c_{dimet} at pH=a) 9.7, b) 10.0, c) 10.3, and d) 10.7, and D) c_{carb} at pH=9.9.

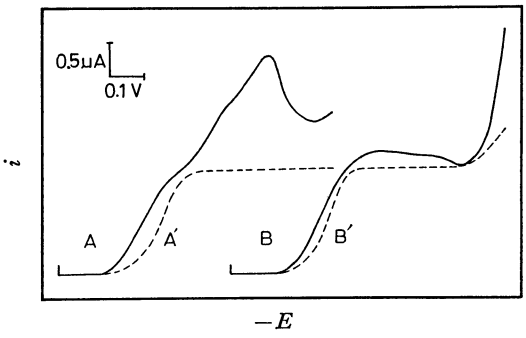

Fig. 6. Polarograms of Brdička current of 0.33 μM cyt-c in the presence of 2×10^{-4} M Co(III) in A) 0.2 M KCl solution and in B) 0.2 M NH_4Cl solution. Curves start from −0.9 V. (Broken lines represent polarograms in the absence of cyt-c.)

Γ can be estimated by Koryta's equation.[11] It was found that the Brdička currents of cyt-c measured under the present experimental conditions also obeyed Eq. 1 and that Γ at −1.4 V was able to be determined by Koryta's equation. The cobalt ion flux at the DME is given by the Ilković theory.[12] Thus, we determined the k_B-values of cyt-c in various buffer solutions by applying Eq. 1 to the Brdička currents at −1.4 V. The k_B(−1.4 V)-values are plotted against the buffer concentrations, c_{buff}, in Figs. 4 and 5.

Brdička Currents in Unbuffered Solutions. Figure 6 shows polarograms of Brdička current of 0.33 μM cyt-c in the presence of 2×10^{-4} Co(III) in 0.2 M KCl solution (Fig. 6A) and in 0.2 M NH_4Cl solution (Fig. 6B). Characteristic Brdička waves were observed in these solutions. The height of the waves in 0.2 M KCl solution was very much greater than that in 0.2 M NH_4Cl solution.

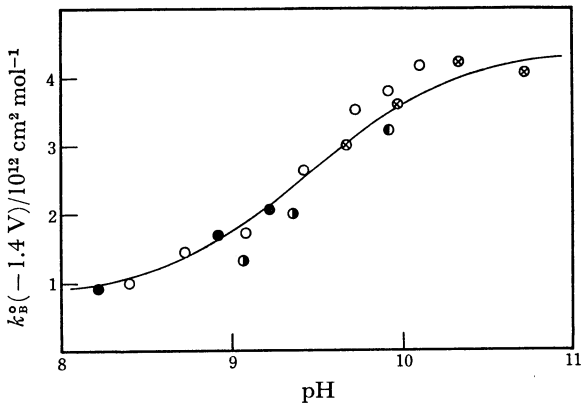

Fig. 7. Plots of k_B° (−1.4 V) against pH; data obtained in ○ ammonia buffer, ● tris buffer, ◑ borate buffer, ◐ carbonate buffer, and ⊗ dimethylglycine buffer. Solid line was calculated on the basis of equation and assumption in the texst.

Discussion

Experimental results show that the shape and the height of the Brdička waves of cyt-c vary with the electrolyte pH and the kind and concentration of buffers. Equation 1 predicts that the Brdička currents are controlled by two factors, k_B and Γ. As reported previously[7,10] at −1.4 V cyt-c is very strongly adsorbed on DME surface, so that at low bulk concentrations of cyt-c Γ can be estimated by Koryta's equation. With increasing negative potential, however, the protein becomes less strongly adsorbed on DME surface and Γ can no longer be evaluated by Koryta's equation. Accordingly, in the following we shall limit the quantitative analysis of the effects of pH and buffers on the Brdička currents to k_B-values at −1.4 V.

Figures 4 and 5 show the dependence of the k_B-(−1.4 V)-values on the buffer concentrations at various pH-values. In ammoniacal buffers, the k_B(−1.4 V) increases with increasing c_{amm} (Fig. 4). On the contrary, in *N,N*-dimethylglycine and carbonate buffers k_B(−1.4 V) decreases with their concentrations, whereas it is hardly affected in tris and borate buffers (Fig. 5). To eliminate these buffer effects, each k_B(−1.4 V) *vs.* c_{buff} plot was extrapolated to $c_{buff}=0$. Values of $k_B^\circ(-1.4\,V) \equiv \lim_{c_{buff}\to 0} k_B(-1.4\,V)$ thus estimated are plotted against pH in Fig. 7. They are on a single sigmoidal curve, independent of the kind of buffers.

In the pH range covered in Fig. 7 ferricytochrome c has two different conformational states depending on pH.[8] At the dme ferricytochrome c in the two states is adsorbed and reduced to ferrocytochrome c at the electrode surface.[7] Assuming that the ferrocytochrome c also has two states, state I and state II, and that the two states are in equilibrium as expressed by

$$\Gamma^{II}(H^+)/\Gamma^{I} = K_1, \qquad (2)$$

where Γ^{I} and Γ^{II} are the surface concentrations of state I and state II in the adsorption layer, respectively, and K_1 the protonic dissociation constant, we can get the following expression for the Brdička current;

$$i_B = FA(k_B^I \Gamma^I + k_B^{II} \Gamma^{II}) f_{Co}$$
$$= FA((k_B^I(H^+) + k_B^{II} K_1)/((H^+) + K_1)) \Gamma f_{Co}, \quad (3)$$

where k_B^I and k_B^{II} represent the Brdička current-activities of state I and state II, respectively. Thus, k_B in Eq. 1 is given by

$$k_B = (k_B^I(H^+) + k_B^{II} K_1)/((H^+) + K_1),$$

or

$$pH = pK_1 + \log((k_B - k_B^I)/(k_B^{II} - k_B)). \quad (4)$$

Solid line in Fig. 7 was calculated by Eq. 4 with $pK_1 = 9.45$, $k_B^I = 0.8 \times 10^{12}$ cm^2 mol^{-1}, and $k_B^{II} = 4.4 \times 10^{12}$ cm^2 mol^{-1}.

Previously we have shown that heme moiety is important in determining the Brdička current-activity of cyt-c.[7] Ferricytochrome c undergoes a transition with an apparent pK, pK_a, of 9.35, which corresponds to the heme-linked deprotonation of ε-amino group of Lys-79.[8] The value of pK_a has been reported to decline with the ionic strength, I, from 9.2 at $I=0.02$ to 8.9 at $I=0.3$.[13] In case of ferrocytochrome c the pK value for the deprotonation of Lys-79 has not been reported so far as we know, but it should have a higher value than the pK_a value of ferricytochrome c, since Lys-79 of ferrocytochrome c is free from bonding to the heme iron.[8] Accordingly, we may conclude that the equilibrium of Eq. 2 corresponds to the equilibrium for the deprotonation of Lys-79 in ferrocytochrome c adsorbed on the DME surface and that the lysine residue together with heme group contributes to the Brdička current-activity of cyt-c.

It has been supposed that the effect of buffer concentration on Brdička currents is due to an acidic component which works as a proton donor.[6,12] This implies that k_B increases with the concentration of an acidic component of buffer salt. However, as shown in Fig. 4 the $k_B(-1.4\text{ V})$-values of cyt-c are almost independent of c_{amm} at pH's lower than 9 at which the concentration of the acidic component, (NH_4^+), is in large excess over that of the basic component, (NH_3), indicating that the effect of (NH_4^+) is negligibly small. Tris and borate buffers also have negligible effect on the $k_B(-1.4\text{ V})$-values (Figs. 5 A and B). Even a decreasing effect is found in dimethylglycine and carbonate buffers (Figs. 5 C and D). As will be reported elsewhere,[14] Brdička current of a protein can be interpreted as due to the catalytic action of protein–Co(0) complex[12] which is transiently formed on electrode surface and the k_B-value depends on the catalytic activity constant, k_c, and the stability constant, k_f/k_d, k_f, and k_d being the formation and decomposition rate constants of the protein–Co(0) complex, respectively. The complicated effects of the buffers described above should be explained by taking into account the contribution of the buffers (probably the basic components) to the k_c and/or the k_f/k_d.

The results that $k_B^\circ(-1.4\text{ V})$ increases with increasing pH and that $k_B(-1.4\text{ V})$ is hardly influenced by the acidic components of the buffer salts indicate that a main proton donor in the Brdička reaction is a water molecule.

In order to get further evidence for the validity of this indication we measured polarograms of Brdička current of cyt-c in unbuffered solutions. As shown in Fig. 6A, in 0.2 M KCl solution containing only 2×10^{-4} M Co(III) cyt-c gave distinct Brdička waves of which $k_B(-1.4\text{ V})$-value was estimated as 3.0×10^{12} cm^2 mol^{-1} by Eq. 1. In 0.2 M NH_4Cl solution containing 2×10^{-4} M Co(III) the protein gave rather small Brdička waves (Fig. 6 B), of which $k_B(-1.4\text{ V})$-value was estimated as 0.8×10^{12} cm^2 mol^{-1}. In an unbuffered solution, pH of the solution in the vicinity of electrode surface must be different from that in the bulk of solution, since Brdička reaction (catalytic hydrogen evolution reaction) produces hydroxide ions, OH^-, and simultaneously the reduction of hexaamminecobalt(III) ions liberates NH_3 molecules. The OH^- and NH_3 molecules diffuse into the solution, so that their surface concentrations, $(OH^-)^\circ$ and $(NH_3)^\circ$, are related[15] to the Brdička current and the limiting current of Co(III), $i_{lim.Co}$, respectively, by the Ilković equations:

$$i_B = \kappa_{OH^-}(OH^-)^\circ \quad (5\text{-a})$$

and

$$i_{lim.Co} = \kappa_{Co}(Co(III))^* = \kappa_{NH_3}(NH_3)^\circ, \quad (5\text{-b})$$

where κ_{OH^-}, κ_{Co}, and κ_{NH_3} are the Ilković constants for OH^-, Co(III), and NH_3, respectively, depending on their respective diffusion coefficients, D_{OH^-}, D_{Co}, and D_{NH_3}, and $(Co(III))^*$ the bulk concentration of Co(III). By Eq. 5-a with $D_{OH^-} = 5.2 \times 10^{-5}$ cm^2 s^{-1} [13] and $i_B(-1.4\text{ V}) = 0.97$ μA (Fig. 6A), $(OH^-)^\circ$ is estimated as 9.5×10^{-5} M, which corresponds to pH 10.0. By Eq. 5-b $(NH_3)^\circ$ is estimated as 7.0×10^{-4} M at $(Co(III))^* = 2 \times 10^{-4}$ M, where $D_{Co} = 7.5 \times 10^{-6}$ cm^2 s^{-1} [10] is used; D_{NH_3} is assumed to be equal to the diffusion coefficient of NH_4^+: $D_{NH_4} = 1.9 \times 10^{-5}$ cm^2 s^{-1}.[12] The pH of 7.0×10^{-4} M NH_3 solution is calculated to be approximately 10. Accordingly, it will be safe to estimate the electrode surface pH as about 10, under the condition under which the Brdička current in Fig. 6 A is observed. In 0.2 M NH_4Cl solution OH^- ions produced by the Brdička reaction at the DME surface can react with NH_4^+ ions to form NH_3 molecules. The reduction of Co(III) ions also liberates NH_3 molecules. Thus, using Eqs. 5-a and 5-b, the ratio $(NH_4^+)/(NH_3)$ may be estimated as 2.5×10^2 at the electrode surface. This value of $(NH_4^+)/(NH_3)$ ratio corresponds approximately to pH 7. The results that $k_B(-1.4\text{ V}) = 3.0 \times 10^{12}$ cm^2 mol^{-1} in 0.2 M KCl (electrode surface pH$\approx$10) and $k_B(-1.4\text{ V}) = 0.8 \times 10^{12}$ cm^2 mol^{-1} in 0.2 M NH_4Cl (electrode surface pH$\approx$7) can reasonably be explained by the $k_B^\circ(-1.4\text{ V})$-pH curve in Fig. 7.

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References

1) R. Brdička, *Collect. Czech. Chem. Commun.*, **5**, 112 (1933).
2) G. J. Miller, *Biochem. J.*, **53**, 393 (1953).
3) V. Kalous, *Collect. Czech. Chem. Commun.*, **21**, 1227 (1956).
4) B. Alexander, M. Brezina, and V. Kalous, *Collect. Czech. Chem. Commun.*, **28**, 210 (1963).

5) M. Ito, *Mie Medical Journal*, **8**, 149 (1964).
6) a) I. M. Kolthoff, K. Yamashita, and Tan Boen Hie, *Proc. Natl. Acad. Sci. U. S. A.*, **71**, 2072 (1974); b) I. M. Kolthoff, K. Yamashita, Tan Boen Hie, and A. Kanbe, *J. Electroanal. Chem.*, **53**, 417 (1974); c) I. M. Kolthoff, K. Yamashita, Tan Boen Hie, and A. Kanbe, *J. Electroanal. Chem.*, **58**, 375 (1975).
7) T. Ikeda, H. Kinoshita, Y. Yamane, and M. Senda, *Bull. Chem. Soc. Jpn.*, **53**, 112 (1980).
8) R. E. Dickerson and R. Timkovich, "The Enzyme XI," ed by P. D. Boyer, Academic Press, New York (1975) p. 397.
9) W. D. Butt and D. Keillin, F. R. S., *Proc. R. Soc. London, Ser. B*, **156**, 429 (1962).
10) H. Kinoshita, Ph. D. Thesis, University of Kyoto, 1979.
11) J. Koryta, *Collect. Czech. Chem. Commun.*, **18**, 206 (1953).
12) J. Heyrovský and J. Kůta, "Principles of Polarography," Academic Press, New York and London (1966).
13) C. Greenwood and M. T. Wilson, *Eur. J. Biochem.*, **22**, 5 (1971).
14) M. Senda, T. Ikeda, and T. Kakutani, *Bioelectrochem. Bioenerg.*, in press.
15) T. Ikeda and M. Senda, *Bull. Chem. Soc. Jpn.*, **46**, 2107 (1973).