Studies on polarographic catalytic hydrogen currents of proteins

Author(s)
Kano, Kenji

Citation
Kyoto University (京都大学)

Issue Date
1983-01-24

URL
https://doi.org/10.14989/doctor.k2864

Type
Thesis or Dissertation

Textversion
author
STUDIES ON POLAROGRAPHIC CATALYTIC HYDROGEN CURRENTS OF PROTEINS

KENJI KANO

1982
STUDIES ON POLAROGRAPHIC CATALYTIC
HYDROGEN CURRENTS OF PROTEINS

KENJI KANO

1982
CONTENTS

Introduction ......................................................... 1

Part I. Fundamental Studies on Brdicka Current ................. 4
  Chapter 1. Theory of Brdicka Current ......................... 4
    Theory .......................................................... 4
    Summary ...................................................... 8
  Chapter 2. Experimental Verification of Theoretical Equation .. 9
    Experimental ................................................ 9
    Results and Discussion ..................................... 10
    Summary ..................................................... 18
  Chapter 3. Effects of the Concentration of Buffer Components,
    pH and Temperature ........................................ 19
    Experimental ................................................ 19
    Results and Discussion .................................... 19
    Summary ..................................................... 29
  Chapter 4. Pulse Polarographic Studies on Brdicka Current . . 30
    Experimental ................................................ 30
    Equations of Brdicka Current in Pulse Polarography .. 31
    Results and Discussion ..................................... 33
    Summary ..................................................... 43

Part II. Analytical Applications of Brdicka Current ............. 44
  Chapter 5. Trace Analysis of Proteins by Differential Pulse
    Polarographic Technique .................................. 44
    Experimental ................................................ 44
    Results and Discussion .................................... 45
    Summary ..................................................... 48
  Chapter 6. Polarographic Study on Enzyme-Inhibitor Interaction 49
    Experimental ................................................ 49
    Results and Discussion .................................... 51
    Summary ..................................................... 69
Chapter 7. Polarographic Study on Interaction between Human IgG and Sheep Antihuman IgG Antiserum and its Analytical Application
Experimental
Results and Discussion
Summary
Conclusion
Acknowledgements
References
List of Frequently Used Symbols

A: electrode surface area
C: concentration in bulk phase
D: diffusion coefficient
E: electrode potential
F: Faraday constant

$f_{Co}$: flux of cobalt ion at electrode surface
h: height of mercury reservoir of dropping mercury electrode

$i_B$: Brdicka current intensity
K: dissociation constant

$k_B$: Brdicka current constant of protein

$k_c$: intrinsic catalytic activity constant of protein-cobalt complex

$k_d$: rate constant for decomposition of protein-cobalt complex

$k_f$: rate constant for formation of protein-cobalt complex

$N_c$: number of Brdicka-active groups in a protein molecule

$N_S$: number of cysteine or half-cystine residues in a protein molecule

$n_c$: number of the total sites, on which protein-cobalt complex can be formed, in a protein molecule

R: gas constant
T: absolute temperature

$t$: a) time

b) Celsius temperature

v: potential sweep rate

$\Gamma$: surface concentration of protein adsorbed on electrode surface

$\kappa$: proportional constant converting protein concentration in bulk phase to Brdicka current intensity

$\tau$: drop time
INTRODUCTION

Proteins containing sulphydryl- and/or disulfide-groups possess a marked ability for producing catalytic hydrogen evolution current at mercury electrode.\textsuperscript{1,2)} The characteristic feature of the SS/SH-containing proteins as the hydrogen evolution catalyst is the sharp increase in their catalytic ability upon addition of cobalt salts to the solution. This was first observed by Brdicka\textsuperscript{3)} some fifty years ago, and is usually referred to as polarographic protein wave or Brdicka current after its discoverer. Recently, it has been found that heme proteins, such as cytochromes c containing heme\textsuperscript{4)} and myoglobin containing protoheme,\textsuperscript{5)} and S-(ethylsuccinimide)-protein\textsuperscript{6)} also produce Brdicka current. These catalytic currents are observed on d.c. polarogram succeeding the limiting current of cobalt ion, as shown in Fig.1.

Immediately after its discovery, the Brdicka current was applied first to serum protein analysis for diagnosis of cancer and other patients, then to other fields of protein analysis, such as the evaluation of irradiated food.

Fig.1 D.c. polarograms of
A) ribonuclease-A, B) bovine serum albumin, c) cytochrome c,
D) myoglobin, and E) subtilisin BPN' at 80 \( \mu \text{g cm}^{-3} \) in ammoniacal buffer of pH 9.5 containing 2 x \( 10^{-4} \text{ mol dm}^{-3} \) Co(NH\textsubscript{3})\textsubscript{6}Cl\textsubscript{3}. 
proteins (for reviews, see references 7,8). Recently, increased attention has been paid to fundamental aspects of Brdicka current with the intention to develop its analytical applicability (for recent reviews, see references 9-13). From the analytical chemistry point of view, the Brdicka current is based on the measurement of the rate (i.e., current) of the electrochemical reaction catalyzed by trace amount of proteins present at the mercury electrode surface. Furthermore, the protein are concentrated at the electrode surface by adsorption. Thus, the Brdicka current has a considerably high sensitivity as a tool for protein analysis,12) together with the comparatively high precision and the usability of the polarographic methods of analysis with the dropping mercury electrode. On the other hand, the Brdicka current is produced only but almost indiscriminately by SS and/or SH containing proteins (and a few other kinds of proteins 4-6). This feature of the Brdicka current can be an advantage or disadvantage as analytical demands may be. Also the Brdicka current is sensitive to some extent to the structure of protein and its conformational change.13-16) Accordingly, combination of the high sensitivity of Brdicka current with the selectivity or ability of proteins to recognize other molecules, as seen in enzyme-inhibitor complex formation or antigen-antibody complex formation, may lead to a novel technique of trace analysis of proteins.

In spite of a large number of papers devoted to the study of the fundamentals of Brdicka current, the mechanism of Brdicka current has not fully been understood yet. To advance the analytical application of Brdicka current furthermore, the elucidation of the mechanism is important. Thus the development of the basic theory of Brdicka current which may relate the Brdicka current intensity with various experimental factors in a quantitative way is highly desirable. Recent studies12,17-19) have revealed that the Brdicka current is controlled by the rate of supply of cobalt ions to the electrode surface, as well as the surface concentration of proteins adsorbed on electrode surface. Thus, Senda et al.20) has proposed a basic equation of the Brdicka current, in which a zero-valent cobalt - protein complex is considered
as an active center to produce Brdicka current.

In this thesis, contribution of the present author to the study on fundamentals and applications of polarographic Brdicka current has been summerized in two parts. Part I deals with fundamental studies on Brdicka current. In chapter 1, the extended theoretical equation of Brdicka current which allows to explain the characteristics of Brdicka current over the wide range of experimental conditions is proposed. Chapter 2 describes experimental verification of the theoretical equation. By this theoretical equation, dependence of Brdicka current on the conditions of electrode system as well as on the concentrations of protein and of cobalt (III) or (II) salt can well be explained.

In chapter 3, effects of the concentration of buffer components, pH and temperature on Brdicka current are quantitatively analyzed and the mechanism of Brdicka reaction is discussed. Chapter 4 is concerned with the study on some basic properties of Brdicka current in normal- and differential-pulse polarography. These are reasonably explained by the above theoretical equation. In part II, some applications of Brdicka current to protein analysis are described. Chapter 5 deals with the important features of Brdicka currents obtained with differential pulse polarographic technique. The method allows to detect trace amount of proteins as low as few ng per cm$^3$ under certain conditions. In chapter 6, using d.c.- and differential pulse-polarographic techniques, the complex formation of proteinase (subtilisin BPN') with protein proteinase inhibitors (Streptomyces subtilisin inhibitor or plasminostreptin) is studied and the dissociation constants of the (dimeric) enzyme-inhibitor complexes, as low as $10^{-10}$ mol dm$^{-3}$, are determined. In determining the dissociation constants the multiple equilibrium involving microscopically distinct forms of the complexes has been taken into account. In the final chapter, polarographic study of the interaction between antigen (human IgG) and antibody (sheep antihuman IgG antiserum) is described, and the polarographic immunoassay based on the Brdicka current is discussed.
PART I. FUNDAMENTAL STUDIES ON BRDICKA CURRENT

CHAPTER 1. THEORY OF BRDICKA CURRENT\textsuperscript{a, b)}

Recent studies\textsuperscript{12,17-19)} on fundamentals of Brdicka current have revealed that Brdicka current is controlled by the surface concentration of protein adsorbed on electrode surface and the flux of cobalt ion from the bulk of the solution to electrode surface. Also it is generally accepted\textsuperscript{12,19,21)} that cobalt (II) ion is reduced to zero valent cobalt, Co(0), at the potential where Brdicka current is observed and that Co(0), which is liganded to a Brdicka-active group of protein to form a protein-Co(0) complex on electrode surface, may catalyze the hydrogen evolution before the complex is decomposed into Co(0)-amalgam. In this chapter, the author presents an equation of Brdicka current in which the Brdicka current is expressed by a function of the surface concentration of protein adsorbed on electrode surface, the bulk concentration of cobalt ion and two parameters representing the properties of the complex which catalyzes the hydrogen evolution.

THEORY

The mechanism of Brdicka current is not yet fully understood, but it is generally accepted\textsuperscript{12,19,21)} that zero-valent cobalt, Co(0), which is liganded to a Brdicka-active group of protein to form a protein-Co(0) complex on electrode surface, may catalyze the hydrogen evolution before the complex is decomposed into Co(0)-amalgam (see Fig.1-1). The surface concentration (number of moles per cm\textsuperscript{2}) of the protein-Co(0) complex is expressed by \( n_c \Gamma_0 \), where \( n_c \) the number of sites on which the protein-Co(0) complex can be formed in a molecule of protein,
The proposed mechanism of Brdicka current.

\[ i_B = \frac{F A n k \Theta \Gamma}{c_c} \]  

(1-1)

where \( F \) is Faraday constant, \( A \) the electrode surface area, and \( k_c \) the constant representing the intrinsic activity of the complex to catalyze the hydrogen evolution. If \( f_{Co} \) is the flux of cobalt ion at the electrode surface and \((1-\Theta)\) is the fraction of the sites which is not occupied, the rate of formation of the complex will be proportional to \( f_{Co} n \Gamma(1-\Theta) \), and hence is given by \( k_f f_{Co} n \Gamma(1-\Theta) \), where \( k_f \) is the (constant) proportion which forms the complex. The rate of decomposition of the complex into Co(0)-amalgam may be given by \( k_d n \Gamma \Theta \), where \( k_d \) is the constant. At the stationary state we have \( k_f f_{Co} n \Gamma(1-\Theta) = k_d n \Gamma \Theta \), or \( \Theta = \frac{k_f f_{Co}}{k_d} \Gamma \). Accordingly, from Eq.(1-1) we get an equation of Brdicka current,

\[ i_B = \frac{F A n k \Gamma(k_f/k_d) f_{Co}}{1 + (k_f/k_d) f_{Co}} \]  

(1-2)

In this derivation, it is assumed that all the protein-Co(0) complexes
in a protein molecule are identical in their catalytic activity of hydrogen evolution as well as in their kinetics of formation and decomposition. If they are not identical, Eq.(1-2) should be replaced by an exact equation,

\[ i_B = FAF_C \sum \frac{k_C^i (k_f^i / k_d^i)}{1 + (k_f^i / k_d^i)c_{Co}} \]  

(1-3)

where \( k_C^i, k_f^i \) and \( k_d^i \) (i = 1, 2, ..., n_c) are the constants for the i-th complex. This consideration indicates that in Eq.(1-2) the constants \( k_C^i, k_f^i \) and \( k_d^i \) should be interpreted as the average constants of all the complexes.

At extremely low concentration of cobalt ion \((k_f^i / k_d^i)c_{Co} \ll 1 \) (or \( H_2 > Co \)), see Eq.(1-7)), so that we have

\[ i_{B'Co} = FAF_{BCo} \]  

(1-4)

with

\[ k_B = n \sum k_C^i (k_f^i / k_d^i) = \sum k_C^i (k_f^i / k_d^i) \]  

(1-5)

At the dropping mercury electrode, \( f_{Co} \) is given by Ilkovic equation,

\[ f_{Co} = (7D_{Co} / 3\pi t)^{1/2}_{Co} \]  

(1-6)

where \( t \) is the time, \( D_{Co} \) and \( C_{Co} \) are the diffusion coefficient and concentration of cobalt ion, respectively. On substituting Eq.(1-6) into Eq.(1-2), we get

\[ i_B = H_1 C_{Co} / (H_2 + C_{Co}) \]  

(1-7)

with
\[ H_1 = FA_0 n k_c \Gamma \] (1-8)

and

\[ H_2 = \left( \frac{k_d}{k_f} \right) \left( \frac{3\pi t}{7D_c} \right)^{1/2} \] (1-9)

The surface area of the drooping mercury electrode is given by \( A = 0.0085m^{2/3}t^{2/3} = A_0 t^{2/3} \), \( A_0 \) being a constant at a constant mercury flow rate. Exact mathematical expression to predict \( \Gamma \) for a given system is very complicated, except Koryta's treatment\(^{22}\) of the limiting case of extremely strong adsorption of protein. If Koryta's treatment is valid, at low concentration of protein we have

\[ \Gamma = \left( \frac{12D_p}{7\pi} \right)^{1/2} C_p \] (1-10)

where \( D_p \) and \( C_p \) are the diffusion coefficient and concentration of protein. Thus we have at low concentration of protein

\[ H_1 = FA_0 n k_c \left( \frac{12D_p}{7\pi} \right)^{1/2} t^{7/6} C_p \] (1-11)

then at extremely low concentration of cobalt ion \( (C_{Co} << H_2) \)

\[ i_{B,Co \rightarrow 0} = FA_0 n k_c \left( \frac{k_f}{k_d} \right) \left( \frac{2}{7\pi} \right) \left( \frac{D_p D_{Co}}{C_p C_{Co}} \right)^{1/2} t^{2/3} C_p C_{Co} \] (1-12)

and at extremely high concentration of cobalt ion \( (C_{Co} >> H_2) \)

\[ i_{B,Co \rightarrow \infty} = FA_0 n k_c \left( \frac{12D_p}{7\pi} \right)^{1/2} t^{7/6} C_p \] (1-13)

At high concentration of protein, the surface of drooping mercury electrode may be considered as saturated with adsorbed protein for the whole life of a mercury drop, that is, \( \Gamma = \Gamma_{max} \). Hence we have

\[ H_1 = FA_0 n k_c \Gamma_{max} t^{2/3} \] (1-14)
then, at extremely low concentration of cobalt ion ($C_{\text{Co}} \ll H_2$)

\[ i_{B, \Gamma = \Gamma_{\text{max}}}, C_{\text{Co}} \rightarrow 0 = F A_0 n_c c (k_f/k_d)^{\text{max}} (7D_{\text{Co}}/3\pi)^{1/2} t^{1/6} C_{\text{Co}} \]  

(1-15)

and at extremely high concentration of cobalt ion ($C_{\text{Co}} \gg H_2$)

\[ i_{B, \Gamma = \Gamma_{\text{max}}}, C_{\text{Co}} \rightarrow \infty = F A_0 n_c c t^{2/3} \]  

(1-16)

**SUMMARY**

Basic equation of Brdicka current is presented, in which Brdicka current is expressed by a function of the surface concentration of protein, the bulk concentration of cobalt ion and two parameters, $n_c k_c$ and $k_f/k_d$, where $n_c$ is the number of the total sites, on which the complex can be formed, in a protein molecule, and $k_c$ and $k_f/k_d$ are the (average) constants representing the intrinsic catalytic activity and the life time, respectively, of the complex.
CHAPTER 2. EXPERIMENTAL VERIFICATION OF THEORETICAL EQUATION$^{b,c}$

In this chapter, dependence of Brdicka current on the conditions of electrode system as well as on the concentrations of protein and of cobalt (III) or (II) salt has experimentally been investigated to verify the prediction of the theoretical equation derived in the preceding chapter. Method to determine the two parameters $n_k c c$ and $k_f/k_d'$ representing the properties of the complex to catalyze the hydrogen evolution, is proposed.

EXPERIMENTAL

Materials:

Bovine pancreas ribonuclease-A (RNase, Type I-A, lot No. 58C-0116) and horse heart cytochrome c (Cyt-c, Type VI, lot No. 78C-7040) were products of Sigma Chemical Co. and used without further purification. S-(ethylsuccinimide)-ribonuclease-A (NEM-RNase) was prepared according to Smith et al.$^{23}$ The concentrations of proteins were checked spectrophotometrically.$^{24,25}$ Other chemicals used were of reagent grade quality. Triply distilled water was used to prepare the electrolysis solution.

Apparatus:

Polarograms and current-time curves were recorded with a Fuso potentiostat 311, equipped with a Yokogawa X-Y recorder 3077. All measurements were made under potentiostatic conditions with a three-electrode system consisting of a dropping mercury working electrode (dme), a platinum wire auxiliary electrode, and a saturated calomel reference electrode (SCE). The characteristics of the dme were $m = 0.687 \text{ mg s}^{-1}$ and $T = 9.80 \text{ s}$ at $h = 21.0 \text{ cm Hg}$ and $E = -1.40 \text{ V vs. SCE}$ in an ammoniacal
Electrochemical Measurements:

All measurements were made in an H-type cell immersed in a thermostat controlled at 25.0 ± 0.5 °C. Buffer solution containing 0.1 mol dm⁻³ NH₃, 0.1 mol dm⁻³ NH₄Cl and 0.1 mol dm⁻³ KCl (pH 9.5, ionic strength 0.2 mol dm⁻³) was used as the base solution. Ten ml of the base solution was transferred into the polarographic cell and deaerated for 15 to 20 min by passing nitrogen gas, which had previously been passed through a solution of the same composition as the buffer solution. Then, aliquot of solutions of proteins and Co(NH₃)₆Cl₆ were introduced into the deaerated base solution with a microsyringe under nitrogen atmosphere. In the current measurement, instantaneous currents were taken and the catalytic currents were measured from the cobalt limiting currents.

RESULTS AND DISCUSSION

Experimental Verification of Theory:

Fig.2-1 shows polarograms of RNase in the base solution containing different concentration of hexaminecobalt (III) chloride, Co(III), In the following we shall discuss the Brdicka current at -1.4 V, where most proteins are so strongly adsorbed on mercury electrode surface that \( \Gamma \) can be calculated by Koryta equation. \(^{20,22,26}\)

Fig.2-2 shows the parabolic dependence of Brdicka current, \( i_B \), on the concentration of Co(III), \( C_{Co(III)} \). Fig.2-3, in which \( 1/i_B \) is plotted against \( 1/C_{Co(III)} \), most clearly demonstrated that the dependence of \( i_B \) on \( C_{Co(III)} \) is well reproduced by Eq.(1-7), that is, \( 1/i_B = 1/H_1 + (H_2/H_1)(1/C_{Co}) \). This had empirically been demonstrated by Klumpp as early as in 1940 s. Fig.2-4 shows the dependence of \( i_B \) on \( C \); \( i_B \) first increases linearly with increasing \( C \) (Eqs.(1-11) to (1-13)), but approaches a saturation value at high \( C \) (Eqs.(1-14) to

- 10 -
Fig. 2-1. D.c. polarograms of 4 μg cm⁻³ RNase in the base solution containing Co(III) of a) 1, b) 5, c) 10, and d) 20 x 10⁻⁴ mol dm⁻³.

Fig. 2-2. Variation of the Brdicka current of 4 μg cm⁻³ RNase with the concentration of Co(III) at the dme: t = a) 2, b) 3, c) 4, d) 6 and e) 8 s.
Fig. 2-3. Plots of the reciprocal of the Brdicka current against the reciprocal of Co(III) concentration for the Brdicka current of RNase at $t = a) 2, b) 3, c) 4, d) 6, and e) 8$ s. The data are the same as those in Fig. 2-2.

The parameters $H_1$ and $H_2$ were determined by fitting the $i_B$ vs. $C_{Co(III)}$ curves to Eq. (1-7) with the aid of a Facom M-200 Computer in the Data Processing Center of Kyoto University for $C_p = 4 \mu$g cm$^{-3}$ and $t = 2$ to $8$ s, where $\Gamma$ can be evaluated by Koryta equation (Fig. 2-5), and for

Fig. 2-4. Variation of the Brdicka current with the concentration of RNase in the base solution containing a) 2, b) 5, c) 10, and d) $20 \times 10^{-4}$ mol dm$^{-3}$ of Co(III).
Fig. 2.6. Time dependence of A) $H_1$ and B) $H_2$ parameters for the Brdicka current of 50 $\mu$g cm$^{-3}$ RNase.

Fig. 2.5. Time dependence of A) $H_1$ and B) $H_2$ parameters for the Brdicka current of 4 $\mu$g cm$^{-3}$ RNase.
$C_p = 50 \mu g \text{ cm}^{-3}$ and $t = 2$ to $8 \text{ s}$, where $\Gamma = \Gamma_{\text{max}}$ (Fig.2-6). Regression analysis was performed by means of SALS program (copyright: SALS group$^{28}$). As seen on Figs.2-5 and 2-6 the $H_2$ parameters depend linearly on $t^{1/2}$ for both $C_p = 4$ and $50 \mu g \text{ cm}^{-3}$, whereas the $H_1$ parameters depend linearly on $t^{7/6}$ or $t^{2/3}$ at $C_p = 4$ or $50 \mu g \text{ cm}^{-3}$, respectively. The results agree well with the prediction of Eqs.(1-9), (1-11) and (1-14).

Similarly, good agreement between theory and experimental results for the dependence of $H_1$ and $H_2$ parameters on the time was obtained also with the Brdicka current of Cyt-c.

According to Eq.(1-8), $H_1$ should vary with $C_p$, since $\Gamma$ depends on $C_p$ for a given system. Fig.2-7 shows the dependence of $H_1$ on $C_p$ for the Brdicka current of RNase. At relatively low $C_p$, $H_1$ increases linearly with $C_p$, and the slope of the linear part of $H_1$ vs. $C_p$ plots is well explained by Koryta equation or Eq.(1-11) (see Fig.2-5A), giving the $n_k$ value of the protein if its $D_p$ is known. On the other hand, $H_2$ depends linearly on $t^{1/2}$ in the concentration range between $C_p = 4$ and $50 \mu g \text{ cm}^{-3}$ (see Figs.2-5B and 2-6B), giving the $k_f/k_d$ value of the protein by Eq.(1-9) if $D_{\infty}$ is known. At extremely low concentration of protein ($C_p = 1 \mu g \text{ cm}^{-3}$), however, the linearity of $H_2$ vs. $t^{1/2}$ plots was not extremely valid. This apparent disagreement between theory and experimental results is likely to be explained by an advanced theory based on more elaborated reaction mechanism, but we shall not go here.

![Fig.2-7. Dependence of $H_1$ parameter on the concentration of RNase for the Brdicka current of RNase at $t = 1\) 2, 2) 3, 3) 4, 4) 6, and 5) 8 s.](image)
into the details.

Many authors had reported the drop time dependence of the Brdicka current or the value of the exponent $x$ in the expression of $i_B = (\text{const.})t^x$. When $C_{Co}$ is extremely small, $x$ is $2/3$ (kinetic control characteristic) at small $C_p$ and becomes to be $1/6$ (diffusion control characteristic) with increase in $C_p$. $^{16,19,29}$ These reported values are well explained by Eqs. (1-12) and (1-15). $^{16,20}$ On the other hand, when $C_{Co}$ is large, $x$ varies from $7/6$ to $2/3$ with increase in $C_p$. $^{30,31}$ If $H_2 \ll C_{Co}$, Eq. (1-7) can be reduced to $i_B = H_1$, which predicts $x = 7/6$ (surface kinetic control characteristic $^{2})$ at small $C_p$ (Eqs. (1-11) and (1-13)) and $x = 2/3$ (kinetic control characteristic) at large $C_p$ (Eqs. (1-14) and (1-16)). Accordingly, Eq. (1-2) can also predict the general dependence of the Brdicka current on the drop time.

**Brdicka Current Constants of Brdicka Active Groups:**

Table 2-1 shows the $n_kN_c$ and $k_f/k_d$ values at -1.40 V of three proteins having different kinds of Brdicka-active groups in 0.1 mol dm$^{-3}$ NH$_3$, 0.1 mol dm$^{-3}$ NH$_4$Cl, and 0.1 mol dm$^{-3}$ KCl (pH 9.5) at 25°C. Preliminary experiment showed that the $n_kN_c$ and $k_f/k_d$ values of other SH- and/or SS-containing proteins were nearly equal to those of RNase. Myoglobin, which contains protoheme group, is Brdicka active, $^5$ but its catalytic activity ($k_B = 0.22 \times 10^{12}$ cm$^2$ mol$^{-1}$) is small and hence could not be evaluated by the present method. Other -SR groups, such as $-\text{SCH}_3$, $^-\text{SC}_2\text{H}_5$, $^-\text{SC}_2\text{COOH}$, $^-\text{SC}_2\text{CONH}_2$, $^-\text{SCH}_2\text{C}_6\text{H}_5$, $^-\text{SO}_3\text{H}$ are known to be Brdicka inactive. In this table the $n_kN_c$ values of the three proteins containing different Brdicka-active groups are nearly constant. This fact indicates that the difference in the Brdicka current activity as expressed by $k_BN_c$ of the Brdicka-active groups should first be attributed to the difference in the stability (or lifetime) of the complexes. This result also indicated that the Brdicka-active groups may play the most important role as a ligand to form the complex, though other groups such as amino, carboxyl, and peptide groups also may function as ligands to form the complex.
Table 2-1. Brdicka Current Constants of Proteins with Cobalt(III) or (II) at $E = -1.4\, V$ in 0.1 mol dm$^{-3}$ NH$_3$, 0.1 mol dm$^{-3}$ NH$_4$Cl and 0.1 mol dm$^{-3}$ KCl (pH 9.5) at 25°C.

<table>
<thead>
<tr>
<th>Protein Cobalt</th>
<th>Active Group</th>
<th>$n_{k_c}/N_c$</th>
<th>$k_{f}/k_d$</th>
<th>$k_{f}/k_d$</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNAse Co(III)</td>
<td>$-SH$</td>
<td>8</td>
<td>2.1</td>
<td>1.2</td>
</tr>
<tr>
<td>NEM-RNAse Co(III)</td>
<td>$-SH$</td>
<td>8</td>
<td>1.8</td>
<td>4.6</td>
</tr>
<tr>
<td>Cyt-c Co(III)</td>
<td>Heme-$b^-$</td>
<td>1</td>
<td>0.28</td>
<td>3.6</td>
</tr>
<tr>
<td>RNAse Co(III)</td>
<td>$-SH$</td>
<td>8</td>
<td>0.61</td>
<td>0.09</td>
</tr>
</tbody>
</table>

a) Number of Brdicka-active groups in a protein molecule.
b) The 6th ligand is methionine.
Brdicka Current Constants of Co(III) and Co(II):

Brdicka current was also observed in the presence of cobalt (II) chloride, Co(II), instead of Co(III), but the Brdicka current of Co(II) was less than that of Co(III). Table 2-1 also shows the $n_{c_c}$ and $k_f/k_d$ values of RNase with Co(II). The small Brdicka current or small $k_B$ values of Co(II) was revealed to result from small $n_{c_c}$ value. The difference between the $n_{c_c}$ values of Co(II) and Co(III) must be attributed to the difference in the ligand-substitution lability of cobalt complex ion, that is, Co(II) is substitution labile and Co(III) is substitution inert. Above result indicates that the intrinsic activity and the stability of the protein-Co(0) complex are also influenced by the ligands of cobalt complex ion in solution, and that different kinds of protein-Co(0) complexes, different ligand complexes, are generated on electrode surface from Co(III) and Co(II).

Effect of Electrode Potential:

Fig.2-8 shows the dependence of the $n_{c_c}$ and $k_f/k_d$ of RNase on the electrode potential, $E$, $\Gamma$ being estimated by Koryta equation. Plot A in Fig.2-8 indicates that the $n_{c_c}$ can be expressed by $n_{c_c} = (\text{const.}) \exp(-\alpha_n FE)/RT$ with $\alpha_n = 0.3$. Also, plot B in Fig.2-8 shows that the $k_f/k_d$ value of RNase changes with the electrode potential and decreases with increasing negative potential. The dependence of $k_f/k_d$ on $E$ should in part be due to the conformational change of protein.
adsorbed on electrode surface.

In conclusion we can state that the Brdicka current is well described by Eqs.(1-2) to (1-16), in which the current is expressed as a function of the surface concentration (or bulk concentration) of protein and the bulk concentration of cobalt ion with two parameters, \( n_c^k_c \) and \( k_f/k_d \), or three constants, that is, \( n_c \) the number of the total sites (or Brdicka-active groups), on which a protein-Co(0) complex can be formed, in a protein molecule; \( k_c \) the (average) constant representing the intrinsic activity of the complex to catalyze the hydrogen evolution; and \( k_f/k_d \) the (average) constant representing the stability or the lifetime of the complex.

**SUMMARY**

The theoretical equation derived in chapter 1 has been experimentally verified on the dependence of Brdicka current on the conditions of electrode system as well as on the concentrations of protein and cobalt (III) or (II) salt. The method to determine the two parameters \( n_c k_c^k_c \) and \( k_f/k_d \), representing the properties of the complex to catalyze the hydrogen evolution, has been established.

Brdicka current activities \( k_B = n_c k_c^k_c (k_f/k_d) \) or \( n_c k_c^k_c \) and \( k_f/k_d \) of SS (or SH) group, heme-c group and S-(ethylsuccinimide) group were determined. The difference in their activity between these three groups has been found mainly due to the difference in the stability.
CHAPTER 3. EFFECTS OF THE CONCENTRATION OF BUFFER COMPONENTS, pH AND TEMPERATURE

Effects of various experimental factors, such as the concentration of buffer components, ionic strength, pH, and temperature, on Brdicka current have been studied by many workers (for review, see references 2, 36, 37), but their analysis and interpretation have been limited to qualitative ones. This is probably due to the lack of theoretical equation of Brdicka current. In this chapter, the effects of the concentration of buffer components, pH and temperature on Brdicka current have quantitatively been investigated on the basis of the theoretical equation of Brdicka current derived in chapter 1, and the mechanism of the Brdicka reaction has been discussed in some details.

EXPERIMENTAL

As the base solutions, 0.05 to 0.3 mol dm⁻³ NH₃-NH₄Cl buffers of pH 7.91 to 10.32 were used. The ionic strength of the base solution was adjusted to 0.2 mol dm⁻³ with KCl. For recording the current, the drop time was regulated at τ = 4.08 s, with a Yanagimoto drop controller P-8-RT. Temperature was controlled thermostatically at 10.0 to 40.0 °C. Other details of experimental procedures were described in chapter 2.

RESULTS AND DISCUSSION

Effect of Buffer Concentration:

Fig. 3-1 shows the dependence of the Brdicka wave of RNase (4 µg cm⁻³) at three different concentration of ammoniacal buffer, Cₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐₐ¢
[NH₄⁺] = 0.1, 0.2 and 0.3 mol dm⁻³ and at pH = 9.5 and I = 0.2 mol dm⁻³. The height of the first wave increases with increasing Cₐmm as reported by many workers. The values of kₐ at -1.4 V obtained according to Eq.(1-4) are plotted against Cₐmm as shown in Fig.3-2. The kₐ value increases with Cₐmm, which agrees with the results of Cyt-c obtained by Ikeda et al. In order to strictly investigate the effect of

Fig.3-1. D.c. polarograms of 4 µg cm⁻³ RNase in the presence of 2 x 10⁻⁴ mol dm⁻³ Co(III) in ammoniacal buffer at pH 9.5 and Cₐmm = a) 0.1, b) 0.2 and c) 0.3 mol dm⁻³.

Fig.3-2. Dependence of kₐ on the ammoniacal buffer concentration. The kₐ values were estimated at Cₐ = 4 µg cm⁻³, pH 9.5, E = -1.4 V and 25 °C.

the concentration of Cₐmm on the Brdicka current, the values of nₙ and kₙ/kₚ at various Cₐmm were obtained by analyzing the dependence of the Brdicka current on Cₐ Co(III), according to Eqs.(1-7) to (1-11), and plotted against Cₐmm (Fig.3-3). The nₙ value, referring to the intrinsic activity of protein-Co(0) complex, increases linearly with Cₐmm from 0.1 to 0.3 mol dm⁻³. Whereas the kₙ/kₚ value, referring to the stability of the complex, somewhat decreases with Cₐmm.
Fig. 3-3. Dependence of $n_c k_c$ and $k_f/k_d$ on the ammoniacal buffer concentration.
These values were estimated at $C_p = 4 \mu g \ cm^{-3}$, pH 9.5, $E = -1.4 \ V$ and 25 $^oC$.

The increase in $n_k$ with $C_{amn}$ could be interpreted as the increase in acidic component of buffer salt, i.e. ammonium ion, which works as a proton doner in the Brdicka reaction. This interpretation agrees well with those of many workers (see references 2, 36, 37). Moreover, the extrapolated $n_k$ value in $n_k$ $vs.$ $C_{amn}$ plot to the intercept, $(n_k)_0$, should correspond to the catalytic activity of protein-Co(0) complex in the Brdicka reaction where a proton doner is water molecule, according to Ikeda et al. 39) Then, on assuming that $n_c$ value is independent of $C_{amn}$, the present results on the effect of $C_{amn}$ on $n_k$ can be expressed by the following empirical formula,

$$k_c = k_{c1}^H_2O + k_{c4}^{NH^+} [NH_4^+]$$

(3-1)
where, $k_{cH_2O}^0$ is the constant representing the intrinsic catalytic activity of protein-Co(O) complex in the catalytic reaction where water molecule plays as a proton donor, and $k_{cNH_4}^{+}$ the proportional constant representing the intrinsic catalytic activity of the complex in the reaction where ammonium ion plays as a proton donor.

The slight decrease in $k_f/k_d$ value with $C_{amm}$ suggests that an ammonia molecule, which is a ligand of cobalt complex ion in solution, stabilizes the cobalt amine complex and participates competitively in the complex formation between protein and Co(0). This is supported by observation that the reduction step of Co(II) to Co(0) shifts negatively with increase in $C_{amm}$ (Fig.3-1). An advanced theory based on more elaborated reaction mechanism was proposed, but we shall not go here into the details. The extrapolated $k_f/k_d$ value in the $k_f/k_d$ vs. $C_{amm}$ plot to the intercept, $(k_f/k_d)_0$, should represent the stability of protein-Co(O) complex at $[NH_3] = 0$, although the effect of ammonia molecules coordinated to cobalt complex ion in bulk solution is involved.

**Effect of pH:**

Fig.3-4 shows d.c. polarograms of the protein wave of 4 μg cm$^{-3}$ RNase in 0.2 mol dm$^{-3}$ ammoniacal buffer containing $2 \times 10^{-4}$ mol dm$^{-3}$ Co(III) at pH 8.4, 9.5 and 10.3. The catalytic current increases to a great extent with increasing pH. The reduction step of Co(II) to Co(0) shifts to more negative potential with increase in pH, which is interpreted.

Fig.3-4. D.c. polarograms of 4 μg cm$^{-3}$ RNase in the presence of $2 \times 10^{-4}$ mol dm$^{-3}$ Co(III) in ammoniacal buffer at pH = a) 10.3, b) 9.5, and c) 8.4.
Fig. 3-5. Plots of \( (n^c k^c)_0 \) and \( (k^f/k^d)_0 \) against pH; data obtained in ammoniacal buffer containing 4 \( \mu \)g cm\(^{-3} \) RNase at -1.4 V and 25 °C. Solid lines are regression curves (see text).

as due to the increase in the concentration of ammonia molecule, which may stabilize cobalt complex ion in solution, as described in previous section. The observed pH-dependence of Brdicka current involves evidently a complicated origin, since the above experimental procedure alters the concentrations of the buffer components as well as pH. In order to eliminate the effect of buffer components, \(^{39}\) \( n^c k^c \) and \( k^f/k^d \) vs. \( c^\text{amm} \) plots were extrapolated to \( c^\text{amm} = 0 \), and the extrapolated values \( (n^c k^c)_0 \) and \( (k^f/k^d)_0 \) were obtained at various pH from 7.9 to 10.3. Fig. 3-5 shows the results: the \( (n^c k^c)_0 \) and \( (k^f/k^d)_0 \) value increased sigmoidally with pH, but \( (n^c k^c)_0 \) began to increase at lower pH than in the case of \( (k^f/k^d)_0 \).

We assume that the pH effect of \( (n^c k^c)_0 \) and \( (k^f/k^d)_0 \) was attributed to the ionization of the functional group in RNase (probably electro-chemically reduced SH group, see below), which participates in the complex formation between protein and cobalt, and proposed a scheme as shown in Fig. 3-6. Two forms of ionization state of the functional group in protein, protonated and non-protonated, participate in the Brdicka reaction \(^{38,41}\) with different values of \( k^f, k^d \) and \( k^c \), and the equilibrium
of the protonic dissociation of the functional group is affected by the interaction of the functional group with cobalt.

Here the surface concentration of protein-SH, protein-S\textsuperscript{−}, protein-SH-Co(0) complex, and protein-S\textsuperscript{−}-Co(0) complex in Fig.3-6 are expressed by \( n_{c\,u} \), \( n_{c\,v} \), \( n_{c\,w} \), and \( n_{c\,x} \). Then we have

\[
\Theta_u + \Theta_v + \Theta_w + \Theta_x = 1 \tag{3-2}
\]

and

\[
K_1 = \frac{[n_{c\,v}] [H^+]}{[n_{c\,u}]} \tag{3-3}
\]

\[
K_2 = \frac{[n_{c\,x}] [H^+]}{[n_{c\,w}]} \tag{3-4}
\]

At stationary state, we have

\[
n_{c\,u} \cdot k_{f} \cdot k_{Co} = n_{c\,w} \cdot k_{d} \tag{3-5}
\]

where \( k_{f} \) and \( k_{d} \) are the pH-independent proportions which forms and
decomposes the complex at $C_{amm} = 0$. From the Eqs.(3-2) to (3-5), we can get

\[ n_c \Gamma^0_w = n_c \frac{k_f [H^+] f_{Co}}{k_d [H^+] + k_d K_1 + f_{Co} k_f [H^+] + K_2} \]  

(3-6)

\[ n_c \Gamma^0_x = n_c \frac{k_f K_2 f_{Co}}{k_d [H^+] + k_d K_1 + f_{Co} k_f [H^+] + K_2} \]  

(3-7)

Assuming that the intrinsic catalytic activity of protein-$S^-$-Co(O) complex is $\alpha$ times of that of protein-$SH$-Co(O) complex, the Brdicka current at $C_{amm} = 0$ can be expressed by

\[ i_B, C_{amm} \to 0 = \frac{FA(n_c k_c') \Gamma^0 w + \alpha \Gamma^0 x}{n_c k_c'} \]  

(3-8)

where $k_c'$ is the constant representing the intrinsic catalytic activity of the complex at $C_{amm} = 0$. Upon substituting Eqs.(3-6) and (3-7) into Eq.(3-8), we can get

\[ i_B, C_{amm} \to 0 = \frac{FA(n_c k_c') \Gamma^0 f_{Co} f_{Co} + (k_f/k_d) \Gamma^0 f_{Co}}{1 + (k_f/k_d) \Gamma^0 f_{Co}} \]  

(3-9)

where

\[ (n_c k_c')_0 = (n_c k_c') \frac{K_2 + [H^+]}{[H^+] + K_2} \]  

(3-10)

\[ (k_f/k_d)_0 = \frac{K_2 + [H^+]}{K_1 + [H^+] + K_2} \]  

(3-11)

The values of $K_1$, $K_2$ and $\alpha$ were determined by fitting the $(n_c k_c')_0$ and $(k_f/k_d)_0$ vs. pH plots to the curves represented by Eqs.(3-10) and (3-11), respectively. The results were $pK_1 = 9.48$, $pK_2 = 8.88$ and $\alpha = 4.55$. The solid lines in Fig.3-5 are regression curves.

These results indicate that the complex formation of protein with Co(O) promotes the tendency of the ionizable group to be deprotonated.
and that the catalytic activity of protein-S\(^{-}\)-Co(0) complex is larger than that of protein-SH-Co(0). The value of pK\(_1\) obtained here may correspond with the sulfhydryl group's pK of adsorbed RNase, but is slightly higher than pK of sulfhydryl group of cysteine molecule in solution (pK = 8.33\(^{42}\)).

**Effect of Temperature:**

Fig.3-7 shows d.c. polarograms of 4 µg cm\(^{-3}\) RNase at 10, 25, and 40 °C in the base solution of 0.1 mol dm\(^{-3}\) NH\(_3\), 0.1 mol dm\(^{-3}\) NH\(_4\)Cl and 0.1 mol dm\(^{-3}\) KCl containing 2 x 10\(^{-4}\) mol dm\(^{-3}\) Co(III) and broken lines represent polarograms in the absence of RNase at corresponding temperature. The diffusion current of cobalt ion increases with temperature, because of the increase in the diffusion coefficient of cobalt ion.

The Brdicka currents at -1.3, -1.4 and -1.5 V are plotted against temperature in Fig.3-8. The value of i\(_B\) (-1.3 V) increases with temperature from 10 to 30 °C but above 30 °C i\(_B\) (-1.3 V) decreases with temperature. The values of i\(_B\) (-1.4 V) and i\(_B\) (-1.5 V) decrease monotonously with temperature in the temperature range investigated. This behavior had been reported by many workers.\(^{30,37-39,43}\)

As previously described, the Brdicka current is a function of \(\Gamma\) and \(f\)\(_{Co}\), which are also the function of temperature. Then, we corrected the temperature effect.
Fig. 3-8. Temperature dependence of the Brdicka current of 4 μg cm$^{-3}$ RNase in 0.2 mol dm$^{-3}$ Co(III) at A) -1.5, B) -1.4 and C) -1.3 V. The $f_{CO}$ value at a given temperature was estimated from the diffusion current of cobalt ion according to Ilkovic equation (broken lines in Fig. 3-7). If the adsorption process is controlled by diffusion, the temperature effect of $\Gamma$ is reduced to that of $D_p$, according to Koryta equation. The value of $D_p$ were calculated using the relation $D_p(T) = (\text{const.})T/\eta(T)$, where $D_p(T)$ and $\eta(T)$ are diffusion coefficient of protein and viscosity of water at $T$ °K, respectively.

The relative values of $k_B$ at a given temperature to that obtained at 25 °C are plotted against temperature as shown in Fig. 3-9. The values of $k_B$ decrease monotonously with increasing temperature in the temperature range investigated at all the measurement potentials. Although Brezina and Gultjaj attributed an increase in the first wave height with temperature to an increase in reaction rate of catalytic active complex formation, above results indicate that the apparent increase in $i_B$ (-1.3 V) from 10 to 30 °C is attributed to the increase in $f_{CO}$ and/or $\Gamma$ with temperature.
We also determined the $n_{kc}$ and $k_f/k_d$ values at various temperature. Fig.3-10 shows the dependence of $n_{kc}$ on temperature. The value of $n_{kc}$ increases with increasing temperature at all the measurement potential. Fig.3-11 shows the temperature dependence of $k_f/k_d$. The $k_f/k_d$ value decreases with temperature. In other words, the intrinsic catalytic activity of protein-Co(O) complex increases with temperature, but in contrast, the complex becomes less stable or the surface concentration of the complex decreases with temperature. Many investigators$^{30,38,43}$ suggested that negative temperature effect of the Brdicka current is attributed to the desorption of protein. However, $\Gamma$ must depend positively on temperature, because the adsorption is controlled by diffusion under these conditions. The results described here indicate that the monotonous decrease in over-all catalytic activity constant $k_B$ is attributed to the decrease in the stability of protein-Co(O) complex.

The slope of linear lines in Fig.3-10 give the apparent activation energy of the hydrogen evolution reaction on electrode surface, $(E^0)_{app}$. 

![Graph](image1)

Fig.3-10. Dependence of $n_{kc}$ on temperature at A) -1.3, B) -1.4, and C) -1.5 V; data obtained in 0.2 mol dm$^{-3}$ ammoniacal buffer containing 4 $\mu$g cm$^{-3}$ RNase.

![Graph](image2)

Fig.3-11. Dependence of $k_f/k_d$ on temperature at A) -1.3, B) -1.4, and C) -1.5 V; data obtained in 0.2 mol dm$^{-3}$ ammoniacal buffer containing 4 $\mu$g cm$^{-3}$ RNase.
These values were \((E^a)_{\text{app}}\) (at -1.3 V) = 12 kcal mol\(^{-1}\), \((E^a)_{\text{app}}\) (at -1.4 V) = 5 kcal mol\(^{-1}\) and \((E^a)_{\text{app}}\) (at -1.5 V) = 1 kcal mol\(^{-1}\), indicating that almost linearly decrease in \((E^a)_{\text{app}}\) with negatively increasing potential.

The interpretation of the effect of temperature on the Brdicka current or the apparent activation energy of the hydrogen evolution reaction is complicated by the temperature effect of pH of ammoniacal buffer. In order to analyze strictly the effect of temperature on \(n_{c}k_{c}\), the change in pH due to the temperature change has to be taken into consideration. We tried to correct pH effect using the relation,

\[
\frac{\partial(n_{c}k_{c})}{\partial T} = \left(\frac{\partial(n_{c}k_{c})}{\partial pH}\right)_T \frac{\partial pH}{\partial T} + \left(\frac{\partial(n_{c}k_{c})}{\partial T}\right)_{\text{pH}}
\]

where \(\left(\frac{\partial(n_{c}k_{c})}{\partial pH}\right)_T\) was estimated tentatively from the \((n_{c}k_{c})_o\) vs. pH plot in Fig.3-5 and \(\frac{\partial pH}{\partial T} = -0.0303\) in the ammoniacal buffer at 25 °C. The corrected activation energy, \(\left(\frac{\partial(n_{c}k_{c})}{\partial T}\right)_{\text{pH}}\) at -1.4 V was estimated to be 7 kcal mol\(^{-1}\).

**SUMMARY**

Effects of ammonia buffer concentration, \(C_{\text{amm}}\), pH and temperature on \(n_{c}k_{c}\) and \(k_{f}/k_{d}\) have been investigated. The dependence of \(n_{c}k_{c}\) on \(C_{\text{amm}}\) indicates that water molecule as well as ammonium ion participates in the catalytic reaction as a proton donor. The \(k_{f}/k_{d}\) value slightly decreases with \(C_{\text{amm}}\), suggesting participation of ammonia molecule in the protein-Co(O) complex formation. The sigmoidal pH-dependence has been found for \(n_{c}k_{c}\) and \(k_{f}/k_{d}\) values corrected for the effect of buffer salts. This has been interpreted as due to the ionization of functional group (probably sulfhydryl group) in adsorbed protein. With increasing temperature, the \(n_{c}k_{c}\) value increases, but \(k_{f}/k_{d}\) value decreases. The decrement of \(k_{f}/k_{d}\) is larger than the increment of \(n_{c}k_{c}\), resulting in the decrease in overall catalytic activity with temperature. The apparent activation energy of the electrochemical hydrogen evolution has been estimated.
CHAPTER 4. PULSE POLAROGRAPHIC STUDIES ON BRDICKA CURRENT\textsuperscript{47)}

Pulse polarographic techniques were first introduced by Barker\textsuperscript{47)} and now two types of the techniques, \textit{i.e.} normal pulse polarography (NPP) and differential pulse polarography (DPP) are used. NPP is well suited to the study of the electrode process involving adsorption of proteins, since the electrode potential, on which the adsorption may be dependent, can be controlled stepwise. On the other hand, DPP is nowadays the most widely used polarographic technique for trace analysis, because DPP shows an improved sensitivity due to the better resolution of the current-voltage curves at very low concentration. In this chapter, the author investigated the characteristics of Brdicka current in NPP and DPP on the basis of the theoretical equation described in chapter 1.

EXPERIMENTAL

Materials:

\textit{Streptomyces} subtilisin inhibitor (SSI) was a gift of Prof. K.Hiromi. Other chemicals used were described in chapter 2.

Apparatus:

Normal pulse (NP)-, differential pulse (DP)- and d.c.-polarograms were recorded with Yanagimoto voltammetric analyzer P-1000, equipped with Watanabe X-Y recorder WX-4401. Characteristics of a dropping mercury electrode (dme) were \( m = 0.852 \, \text{mg s}^{-1} \) and \( t = 11.08 \, \text{s} \) at mercury reservoir height \( h = 50.0 \, \text{cm Hg} \) at \( E = -1.5 \, \text{V} \) in an ammoniacal buffer. Drop time was regulated usually at \( t = 1.67 \, \text{s} \). In NP polarographic mode, a series of increasing amplitude voltage pulses starting from an initial potential, \( E_{i}^{\text{NP}} \), were imposed on successive drops at the end of drop life. The potential pulse is of 50 ms duration. The current was sampled over a
16.6 ms interval toward the end of the pulse duration. In DP polarographic mode, small finite amplitude ($\Delta E_{DP}^P$) pulses of 50 ms duration, superimposed on a conventional d.c. ramp voltage, are applied to the dme near to the end of drop lifetime. The current output is amplified at two time intervals (16.6 ms); immediately on the ramp prior to the imposition of the pulse and then again at the end of the pulse. The difference in these two currents is displayed.

**Electrochemical Measurements:**

All of electrochemical measurements were described in chapter 2.

**EQUATIONS OF BRDICKA CURRENT IN PULSE POLAROGRAPHY**

**NORMAL PULSE POLAROGRAPHIC BRDICKA CURRENT**

In chapter 1, the author showed that the Brdicka current in d.c. polarographic mode can be well explained by Eq.(1-4) at relatively low concentration of cobalt ion. In NP polarographic mode, protein is adsorbed at a given initial potential $E_{NP_i}^P$, and cobalt ion should be pre-electrolyzed at a certain $E_{NP_i}^P$. If the adsorption of protein on mercury electrode is strong at $E_{NP_i}^P$ and the adsorption and/or desorption are negligibly small during the pulse duration, $\Gamma$ can be estimated by Eq.(1-10) and is independent of $E_{NP_i}^P$ and $E_m^P$. When we use Co(III), electrochemical state of cobalt during the pre-electrolysis is Co(III), Co(II) or Co(0) depending on $E_{NP_i}^P$. Then, the flux of cobalt ion at the electrode surface, $f_{NP_i}^{NP_i}$ on pulse imposition is generally expressed by

\[
\begin{align*}
    f_{Co}^{NP_i} &= f_{Co(III)}^{NP_i} + f_{Co(II)}^{NP_i} \\
    \text{(4-1)}
\end{align*}
\]

The value of $f_{Co}^{NP_i}$ can be evaluated in three different $E_{NP_i}^P$ regions for the
case of Co(III) ion. At the \( E_{i}^{NP} \) value more positive than the reduction potential of Co(III) to Co(II), \( f_{Co(III)}^{NP} \), can be given by Cottrell equation,

\[
f_{Co(III)}^{NP} = \left( \frac{D_{Co}}{\pi t_{p}} \right)^{1/2} C_{Co(III)}^{NP}\tag{4-2}
\]

and \( f_{Co(II)}^{NP} = 0 \), where \( t_{p} \) is the pulse duration time. Note that \( f_{Co(III)}^{NP} \) is about 5 to 10 times larger than \( f_{DC}^{Co(III)} \) expressed by Eq. (1-6). Second; in the \( E_{i}^{NP} \) region of the limiting current of Co(III) to Co(II) reduction, where Co(III) ion concentration near the electrode surface is depressed and Co(II) ion is generated by pre-electrolysis at \( E_{i}^{NP} \), \( f_{Co(III)}^{NP} \) is given by Ilkovic equation (Eq. (1-6)) and \( f_{Co(II)}^{NP} \) is given, from the theory of reverse pulse polarography, \(^{48}\) by

\[
f_{Co(II)}^{NP} = \left( \frac{7D_{Co}}{3\pi t_{p}} \right)^{1/2} \left( \frac{3\pi}{7t_{p}} \right)^{1/2} C_{Co(III)}^{NP}\tag{4-3}
\]

Note that sum of \( f_{Co(II)}^{NP} \) and \( f_{Co(III)}^{NP} \) in Eqs. (4-3) and (1-6) are equal to Cottrell equation (Eq. (4-2)). Third; in the \( E_{i}^{NP} \) region of the limiting current of Co(II) to Co(0), \( f_{Co(III)}^{NP} \) is given by Ilkovic equation (Eq. (1-6)). Under these conditions, the difference in Brdicka currents in NP- and d.c.-polarographic mode should be attributed to the difference in the adsorption potential of protein.

Because the Brdicka current activity constant of Co(II), \( k_{B,Co(II)}^{NP} \), is different from that of Co(III), \( k_{B,Co(III)}^{NP} \), as described in chapter 2, NP polarographic Brdicka current at extremely low concentration of Co(III) can be expressed by

\[
f_{B,C_{Co(III)}^{NP}}^{NP} = FA_{r}(k_{B,Co(II)}^{NP} f_{Co(II)}^{NP} + k_{B,Co(III)}^{NP} f_{Co(III)}^{NP})\tag{4-4}
\]

**DIFFERENTIAL PULSE POLAROGRAPHIC BRDICKA CURRENT**

When the drop time, \( \tau \), is long in comparison with the pulse duration, \( t_{p} \), the electrode surface area, \( A \), is practically constant during the
first and second current sampling or at $\tau - t_p$ and $\tau$ in DP polarographic mode. In the limiting current region of Co(II) to Co(0), the $f_{\text{Co}}^\text{DP}$ in DP polarographic mode, $f_{\text{Co}}^\text{DP}$, is also practically constant at $\tau - t_p$ and $\tau$, and given by Ilkovic equation. Further, if protein is adsorbed strongly on electrode surface and we can neglect the desorption of protein during the small amplitude ($\Delta E_p^\text{DP}$) imposition, $f$ is practically constant at $\tau - t_p$ and $\tau$, and given by Koryta equation. Then, the Brdicka current in DP polarographic mode at small $\Delta E_p^\text{DP}$ and extremely low $C_{\text{Co}}^{\text{NP}}$ can be expressed by

$$i_{B,C_{\text{Co}}}^{\text{DP}} \rightarrow 0 = i_B(E_m - \Delta E_p^\text{DP}, \tau) - i_B(E_m, \tau - t_p)$$

$$= i_B(E_m - \Delta E_p^\text{DP}, \tau) - i_B(E_m, \tau)$$

$$= \frac{di_B(E_m, \tau)}{dE_m} \Delta E_p^\text{DP}$$

$$= \frac{d}{d E_m} \frac{k_{\text{Co}}^\text{NP} dE_m}{\Delta E_p^\text{DP}}$$

RESULTS AND DISCUSSION

NORMAL PULSE POLAROGRAPHIC BRDICKA CURRENT

Fig. 4-1 shows normal pulse (NP) polarogram of SSI in the base solution containing $2 \times 10^{-4}$ mol dm$^{-3}$ hexaaminecobalt (III) chloride, Co(III), as well as d.c. polarogram. The second wave of Brdicka current is higher than the first one on the NP polarogram, contrary to the d.c. polarogram. In the following, we shall discuss the NP Brdicka current at -1.4 and -1.6 V, $i_B^{\text{NP}}(-1.4 \text{ V})$ and $i_B^{\text{NP}}(-1.6 \text{ V})$, corresponding to the first and second Brdicka waves, respectively.

We investigated the dependence of $i_B^{\text{NP}}(-1.4 \text{ V})$ and $i_B^{\text{NP}}(-1.6 \text{ V})$ on
the drop time, \( \tau \), at \( h = 36.6 \) cm Hg and \( \tau = 0.85 \) to 8.25 s, and on the
mercury reservoir height, \( h \), at \( \tau = 1.67 \) s and \( h = 17.8 \) to 81.3 cm Hg,
both at \( C_{\text{Co(III)}} = 2 \times 10^{-5} \) mol dm\(^{-3}\) and \( E_{i}^{\text{NP}} = -0.1 \) V. Table 4-1 shows
the results: the exponent \( x \) and \( y \) in the expression of \( i_{\text{NP}} = (\text{const.})\tau^{x} \)
and \( i_{\text{NP}}^{B} = (\text{const.})h^{y} \), respectively. At \( E_{i}^{\text{NP}} = -0.1 \) V, \( f_{\text{NP}}^{\text{Co(III)}} \) is given
by Eq. (4-2) and \( f_{\text{NP}}^{\text{Co(II)}} = 0 \). Then, from Eqs. (1-10), (4-2) and (4-4), \( x \)
and \( y \) are expected to be 7/6 and 2/3, respectively. Note that \( A=(\text{const.})\tau^{2/3} \)
at constant \( h \), and \( A=(\text{const.})h^{2/3} \) at constant \( \tau \). The values in Table 4-1
are in accordance with the theoretical value within the experimental
error, taking into account that both values of \( x \) and \( y \) for the cobalt
limiting diffusion current in NP polarographic mode, \( i_{\text{NP}}^{\text{Co}(\text{III})} \), are theoretically expected to be 2/3.

The values of \( i_{\text{NP}}^{B} (-1.4 \) V), \( i_{\text{NP}}^{B} (-1.6 \) V) increased linearly with the
protein concentration, \( C_{\text{P}} \), up to 60 \( \mu \)g cm\(^{-3}\) at \( E_{i} = -0.1 \) V and \( \tau = 1.67 \) s: the result is well explained by Eqs. (4-4) and (1-10). The \( i_{\text{NP}}^{B} \) first
increased with the cobalt concentration, \( C_{\text{Co(III)}} \), and showed a parabolic
dependence on \( C_{\text{Co(III)}} \) at higher \( C_{\text{Co(III)}} \). The linear relationship held
up to \( C_{\text{Co(III)}} = 2 \times 10^{-6} \) mol dm\(^{-3}\) for \( i_{\text{NP}}^{B} (-1.4 \) V) and \( 1.5 \times 10^{-5} \) mol dm\(^{-3}\)
for \( i_{\text{NP}}^{B} (-1.6 \) V) is well explained by Eqs. (4-2) and (4-4). The upper
limit of linear relation between \( i_{\text{NP}}^{B} \) and \( C_{\text{Co(III)}} \) is about one tenth of

---

Fig.4-1. (a) Normal pulse polarogram
and (b) d.c. polarogram of 2 \( \mu \)g cm\(^{-3}\)
SSl in ammoniacal base solution (pH 10.0)
containing \( 2 \times 10^{-4} \) mol dm\(^{-3}\) Co(III).
Table 4-1. Dependence of Normal- and Differential-Pulse Polarographic Brdicka Currents and Cobalt Reduction Currents on the Drop Time and the Mercury Reservoir Height.

<table>
<thead>
<tr>
<th>Method</th>
<th>Current</th>
<th>1) $\tau$</th>
<th>2) $h$</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPP</td>
<td>$i_B^{NP}$ (-1.4V)</td>
<td>1.1</td>
<td>0.80</td>
</tr>
<tr>
<td></td>
<td>$i_B^{NP}$ (-1.6V)</td>
<td>1.1</td>
<td>0.78</td>
</tr>
<tr>
<td></td>
<td>$i_{Co}^{NP}$</td>
<td>0.76</td>
<td>0.78</td>
</tr>
<tr>
<td>DPP</td>
<td>$i_B^{DP}$ (I)</td>
<td>0.73</td>
<td>0.76</td>
</tr>
<tr>
<td></td>
<td>$i_B^{DP}$ (II)</td>
<td>0.83</td>
<td>0.74</td>
</tr>
<tr>
<td></td>
<td>$i_{Co}^{DP}$</td>
<td>0.34</td>
<td>0.78</td>
</tr>
</tbody>
</table>

1\) Expressed in $i = (\text{const.})\tau^x$ at constant $h$.
2\) Expressed in $i = (\text{const.})h^y$ at constant $\tau$.

the case in d.c. polarography. This result can be explained by the dependence of $i_B$ on $f_{Co}^{NP}$ as expressed by Eq.(1-2) and the fact that $f_{Co(III)}^{NP}$ expressed by Eq.(4-2) is about ten times larger than $f_{Co(III)}^{DC}$ expressed by Eq.(1-6). From above results, the Brdicka current in NP polarographic mode is fundamentally expressed by Eq.(4-4) at the low concentration of cobalt ion.
The Brdicka current in NPP was dependent on the initial potential. Fig. 4-2 shows the \( i_{B}^{NP} \) (-1.4 V) and \( i_{B}^{NP} \) (-1.6 V) plotted against \( E_{i}^{NP} \). The \( i_{B}^{NP} \) values first decreased with decreasing \( E_{i}^{NP} \) from -0.1 V to -0.4 V, second, became constant from \( E_{i}^{NP} = -0.4 \) V to -0.9 V where wave form was independent of \( E_{i}^{NP} \), third, decreased again with the decreasing \( E_{i}^{NP} \) from -0.9 V to -1.2 V, and finally became constant within the potential range of \( E_{i}^{NP} = -1.2 \) V to -1.4 V.

The first decrease in \( i_{B}^{NP} \) around -0.2 V, where the reduction of Co(III) to Co(II) begins, should be attributed to the decrease in \( f_{Co(III)}^{NP} / f_{Co(II)}^{NP} \), because \( k_B \) value of Co(II), \( k_B,Co(II) \), is smaller than that of Co(III), \( k_B,Co(III) \), as described in chapter 2 and the decrease in \( i_{B}^{NP} \) in this \( E_{i}^{NP} \) region was not observed on using Co(II) instead of Co(III).

In the \( E_{i}^{NP} \) region from -0.4 V to -0.9 V (constant current region), adsorption of protein is very strong\(^{12,16,26} \) and is independent of \( E_{i}^{NP} \), and \( f_{Co(II)}^{NP} \) and \( f_{Co(III)}^{NP} \) are given by Eqs. (4-3), (1-6), respectively.

![Graph](image)

**Fig. 4-2.** Initial potential dependence of normal pulse polarographic Brdicka current at \( E_{m} = -1.4 \) V (O) and -1.6 V (●); data obtained in the ammoniacal base solution (pH 9.5) containing 4 \( \mu \)g cm\(^{-3} \) SSI and 3 \( x 10^{-5} \) mol dm\(^{-3} \) Co(III).
and are also independent of $E_i^{NP}$. Then, $i_B^{NP}$ is expected to be independent of $E_i^{NP}$, according to Eq. (4-4).

Here we shall discuss $k_{B,Co(II)}$ and $k_{B,Co(III)}$. From above discussions (see Eqs. (1-6), (4-2), (4-3), (4-4)), the ratio of NP polarographic Brdicka currents at $E_i^{NP} = -0.5$ V to that at $E_i^{NP} = -0.1$ V can be expressed by

$$\frac{i_B^{NP}(E_i^{NP} = -0.5 \text{ V})}{i_B^{NP}(E_i^{NP} = -0.1 \text{ V})} = \frac{k_{B,Co(II)}}{k_{B,Co(III)}} \left(1 - \frac{7t}{3\tau}\right) + \frac{7t}{3\tau} \tag{4-6}$$

According to Eq. (4-6), we tried to evaluate $k_{B,Co(II)}/k_{B,Co(III)}$ from $i_B^{NP}(E_i^{NP} = -0.5 \text{ V})/i_B^{NP}(E_i^{NP} = -0.1 \text{ V})$ at $\tau = 1.67$ and 8.25 s. The results are $k_{B,Co(II)}/k_{B,Co(III)}$ (at -1.4 V) = 0.65 at $\tau = 1.67$ s and 0.67 at $\tau = 8.25$ s, and $k_{B,Co(II)}/k_{B,Co(III)}$ (at -1.6 V) = 0.43 at $\tau = 1.67$ s and 0.43 at $\tau = 8.25$ s. The $k_{B,Co(II)}/k_{B,Co(III)}$ (at -1.4 V) estimated by above method is in agreement with the value estimated by d.c. polarographic method ($= 0.69/1.2 = 0.58$, see Table 2-1) within experimental error. These results indicate the validity of the theory, and that $k_B$ is dependent on the measurement electrode potential, but not $E_i^{NP}$.

Although the disulfide groups in protein should be reduced around -0.75 V at pH 9.5, the $k_B$ value was independent of $E_i^{NP}$ at least from -0.4 V to -0.9 V. This result indicates that the adsorption state or electrochemical state of proteins on electrode surface at initial potential before the pulse imposition scarcely, if any, affect the $k_B$ value or the Brdicka current in NP polarographic mode.

The decrease in $i_B^{NP}$ around $E_i^{NP} = -1.1$ V had previously been reported by Palecek et al. They suggested that this phenomenon was attributed to the conformational change of adsorbed protein. However, this phenomenon around $E_i^{NP} = -1.1$ V, where the reduction of Co(II) to Co(0) becomes appreciable, should be attributed to the decrease in the flux of cobalt ion (compare Eq. (1-6) with Eq. (4-2)).

Fig.4-3 shows the NP polarographic Brdicka waves at various $E_i^{NP}$. 

- 37 -
more negative than -1.3 V and at $C_{\text{Co(III)}} = 2 \times 10^{-4}$ mol dm$^{-3}$, in contrast to the d.c. polarographic Brdicka wave (broken line). Under these conditions, Co(III) ion near the electrode surface is preelectrolyzed to Co(0) before the pulse imposition and the flux of cobalt ion in NP polarographic mode is equal to that in d.c. polarographic mode. On the other hand, protein is adsorbed at $E_{\text{m}}^{\text{NP}}$ in NP polarography, but at the measurement potential $E_{\text{m}}$ in d.c. polarography. When $E_{\text{i}}^{\text{NP}}$ is less negative than -1.4 V, $i_{\text{B}}^{\text{NP}}$ is nearly equal to $i_{\text{B}}^{\text{DC}}$ at $E_{\text{m}}$ less negative than -1.4 V; under these conditions the second Brdicka wave is larger than the first wave in NP polarogram contrary to d.c. polarogram (see also Fig.4-1). When $E_{\text{i}}^{\text{NP}}$ is more negative than -1.45 V, $i_{\text{B}}^{\text{NP}}$ decreases sharply with negatively increasing $E_{\text{i}}^{\text{NP}}$, in accordance with the sharp decrease in d.c. polarographic wave, though $i_{\text{B}}^{\text{NP}}$ is larger than $i_{\text{B}}^{\text{DC}}$. These facts indicate that the adsorption of SSI at the potential less negative than -1.4 V is very strong and irreversible; the SSI molecule adsorbed at less negative potential than -1.4 V is hardly desorbed on the duration of more negative potential pulse, and that the adsorptivity becomes to decrease with negatively increasing the electrode potential than -1.4 V. The shape of ordinary d.c. polarographic Brdicka wave is determined by the potential dependence of $k_{\text{B}}$ and $\Gamma$, and the shape of NP polarographic Brdicka wave is determined mainly by the potential dependence of $k_{\text{B}}$. The appearence of the double wave in NP polarogram

![Fig.4-3. Initial potential dependence of normal pulse polarogram of 2 µg cm$^{-3}$ SSI in ammoniacal base solution containing 2 x 10$^{-4}$ mol dm$^{-3}$ Co(III). $E_{\text{i}}^{\text{NP}} = a) -1.3$ V, b) -1.4 V, c) -1.5 V, d) -1.55 V, e) -1.6 V, and f) -1.7 V. Broken line represents d.c. polarogram recorded in the same sample solution.](image-url)
(or the dependence of $k_B$ on the electrode potential) suggests a very large conformational change of adsorbed SSI molecule at about -1.45 V.

DIFFERENTIAL PULSE POLAROGRAPHIC BRDICKA CURRENT

Fig. 4-4 shows differential pulse (DP) polarogram of SSI in the base solution containing $2 \times 10^{-4}$ mol dm$^{-3}$ Co(III), as well as d.c. polarogram. The Brdicka wave on DP polarogram is almost a differential form of d.c. polarographic Brdicka wave (see below). The first peak of DP polarogram is higher than the second one, in good accordance with the relation of the heights of both d.c. polarographic waves. In the following current measurement, difference between the current of the first peak (at about -1.3 V) or the second peak (at about -1.5 V) and the current of the hollow (at about -1.4 V) was measured and represented as $i_{B}^{DP}$ (I) or $i_{B}^{DP}$ (II), respectively.

The peak heights of DP polarographic Brdicka current, $i_{B}^{DP}$ (I) and $i_{B}^{DP}$ (II), increase linearly with the pulse amplitude, $\Delta E_{p}^{DP}$, up to -50 mV, and tend to level off at higher $\Delta E_{p}^{DP}$ (Fig. 4-5). The linearity between $i_{B}^{DP}$ and $\Delta E_{p}^{DP}$ can be well explained by Eq. (4-5). The peak potentials of the first peak, $E_{p}^{DP}$ (I), and the second peak, $E_{p}^{DP}$ (II), have a tendency to shift less negative with $\Delta E_{p}^{DP}$, as well as the relation of cobalt reduction peak (Fig. 4-6). The relation between $E_{p}^{DP}$ (I) or $E_{p}^{DP}$ (II) and $\Delta E_{p}^{DP}$ was empirically expressed by

$$E_{p}^{DP} = E_{p,0}^{DP} - \Delta E_{p}^{DP}/2$$  \hspace{1cm} (4-7)

where $E_{p,0}^{DP}$ is the extrapolated value of $E_{p}^{DP}$ (I) of $2 \mu g$ cm$^{-3}$ SSI in the base solution (pH 10.0) containing $2 \times 10^{-4}$ mol dm$^{-3}$ Co(III).
Fig. 4-5 Pulse amplitude dependence of the first- (a) and second- (b) peak heights of differential pulse polarographic Brdicka current. $C_{SSI} = 2 \mu g \text{ cm}^{-3}$, $C_{Co(III)} = 5 \times 10^{-3} \text{ mol dm}^{-3}$, $\tau = 1.67 \text{ s}$, $h = 32.6 \text{ cm Hg}$, the presence of SSI.

or $E_{DP}^{P(II)}$ to $\Delta E_{DP}^{P} = 0$. In the following, $\Delta E_{DP}^{P}$ was set to be $-50 \text{ mV}$.

At constant mercury reservoir height ($h = 36.6 \text{ cm Hg}$), the dependence of $i_{DP}^{P}(I)$ and $i_{DP}^{P}(II)$ on the drop time was investigated in the drop time region of $\tau = 0.83$ to $8.25 \text{ s}$. Results are expressed by exponent $x$ in $i_{DP}^{P} = (\text{const.}) \tau^{x}$, and given in Table 4-1. We also investigated the mercury reservoir height dependence of $i_{DP}^{P}$ at $\tau = 1.67 \text{ s}$ in the $h = 17.8$ to $81.3 \text{ cm Hg}$ region. The results are given in Table 4-1, by the expression of exponent $y$ in $i_{DP}^{P} = (\text{const.}) h^{y}$. From Eqs. (1-6), (1-10) and (4-5), both $x$ and $y$ are expected to be $2/3$. The values in Table 4-1 are in agreement with the theoretical value within the experimental error, though the deviation of the experimental values for $i_{DP}^{P}(II)$ from the theoretical values was larger than for $i_{DP}^{P}(I)$. 

Fig. 4-6 Plots of peak potential against pulse amplitude; 1) the first peak potential $E_{DP}^{P}(I)$, 2) the second peak potential $E_{DP}^{P}(II)$, 3) cobalt reduction potential in $-3$ dm, $h = 32.6 \text{ cm Hg}$. 

- 40 -
Fig.4-7 Dependence of a) \( i_B^{DP}(I) \) and b) \( i_B^{DP}(II) \) on the SSI concentration; data Fig.4-8 Dependence of a) \( i_B^{DP}(I) \) obtained at \( \Delta E_p^{DP} = -50 \text{ mV}, h = 50 \text{ cm Hg} \), and b) \( i_B^{DP}(II) \) on \( C_{Co(III)} \):

\[
C_{Co(III)} = 2 \times 10^{-4} \text{ mol dm}^{-3}, \quad v = 2 \text{ mV s}^{-1}, \quad C_{SSI} = 2 \mu g \text{ cm}^{-3}.
\]

Fig.4-7 shows the dependence of \( i_B^{NP}(I) \) and \( i_B^{DP}(II) \) on the protein concentration. The peak height, \( i_B^{DP}(I) \) and \( i_B^{DP}(II) \), increased linearly with the SSI concentration up to 4 \( \mu g \text{ cm}^{-3} \), and tended to reach certain maximum values at higher concentration of SSI. The linear relation can be explained by Eqs.(1-10) and (4-5). The maximum value may be corresponded to the current at \( \Gamma = \Gamma_{\text{MAX}} \). However, the highest \( C_p \) value in linear relation region was about one fourth of that in d.c. polarographic Brdicka current. This result can not be explained at the present time.

We also investigated the dependence of the concentration of \( Co(III)_p \), \( C_{Co(III)} \), on \( i_B^{DP}(I) \) and \( i_B^{DP}(II) \). Fig.4-8 shows the result. The \( i_B^{DP} \) values increased at first linearly with \( C_{Co(III)} \) up to \( 3 \times 10^{-5} \text{ mol dm}^{-3} \) and parabolically at higher \( C_{Co(III)} \). The linear relation between peak height and \( C_{Co(III)} \) is predicted by Eqs.(1-6) and (4-5). In conclusion, the above results indicate that the DP polarographic Brdicka current can be fundamentally expressed by Eq.(4-5).
Here we tried to calculate the difference in d.c. polarographic current at the potentials $E$ and $E + \Delta E$, $\Delta i_{\text{DC}} = i_{\text{DC}}^{\text{E+AE}} - i_{\text{DC}}^{\text{E}}$.
The $\Delta i_{\text{DC}}$ vs. $E$ curve at $\Delta E = -50$ mV, calculated from the d.c. polarogram in Fig.4-4, was shown in Fig.4-9 (curve a). We also calculated the differential curve of d.c. polarogram by a numerical differentiation using a moving second order polynomial fit selecting five points as one set. Curve b in Fig.4-9 shows $(d i_{\text{DC}} / d E) \Delta E$ vs. $E$ curve at $\Delta E = -50$ mV. The $\Delta i_{\text{DC}}$ vs. $E$ curve and the $(d i_{\text{DC}} / d E) \Delta E$ vs. $E$ curve are nearly same shape, though the peak potential of $\Delta i_{\text{DC}}$ vs. $E$ curve is less negative by about 25 mV and peak height of $\Delta i_{\text{DC}}$ is smaller than $(d i_{\text{DC}} / d E) \Delta E$.

If $\Gamma$ is independent of $E$ and $\Delta E_{\text{DP}}$ is very small, Eq.(4-5) predicts that DP polarographic Brdicka current is almost equal to $\Delta i_{\text{DC}}$ or $(d i_{\text{DC}} / d E) \Delta E$. The real DP polarographic Brdicka wave at $\Delta E_{\text{DP}} = -50$ mV as shown by broken line in Fig.4-9, was almost in agreement with $\Delta i_{\text{DC}}$ vs. $E$ curve or $(d i_{\text{DC}} / d E) \Delta E$ vs. $E$ curve in the potential range from -1.2 to -1.45 V, however, at more negative potential than -1.45 V, the deviation became large. These results show the following: at less negative potential than -1.45 V, the adsorption of SSI is strong and controlled by diffusion, then DP polarographic Brdicka current is, as a first
approximation, expressed by Eq. (4-5). With negatively increasing the potential, however, the adsorption becomes weak and \( \Gamma \) can no longer be estimated by Koryta equation and depends on the electrode potential, then \( i_{B}^{DP} \) deviates from the simple equation such as Eq. (4-5). This interpretation is in good agreement with the result of the initial potential dependence of NP polarographic Brdicka current described in previous section.

SUMMARY

On the basis of the basic equation of Brdicka current (chapter 1), theoretical equations of normal pulse (NP)- and differential pulse (DP)-polarographic Brdicka currents have been derived. These equations well explain the experimental results of NP- and DP-polarographic Brdicka currents.

Pulse polarographic study on Brdicka current has revealed that at the less negative potential than \(-1.4\) V the protein SSI is adsorbed strongly and irreversibly on mercury electrode and the surface concentration is controlled solely by diffusion. At more negative potential than \(-1.5\) V the adsorption of SSI becomes weak with increasing negative potential and the surface concentration is controlled by both diffusion and adsorption. The latter is dependent on the electrode potential.
Chapter 5. TRACE ANALYSIS OF PROTEINS BY DIFFERENTIAL PULSE POLAROGRAPHIC TECHNIQUE

Differential pulse (DP) polarographic study on Brdicka current as described in chapter 4 has suggested that the polarographic technique based on Brdicka current may become a remarkably sensitive method for trace analysis of Brdicka active proteins. Palecek and co-workers have applied DP polarographic method to the Brdicka current of proteins and shown that trace analysis of protein as low as 50 ng cm$^{-3}$ can be determined DP polarographically. In this study the author has shown that trace amount of proteins as low as a few ng cm$^{-3}$ can be determined using the DP polarographic technique by selecting suitable experimental conditions. The result is described in this chapter.

EXPERIMENTAL

Electrochemical Measurements:

Apparatus of experiments has been described in chapter 4. On the basis of the results described in chapter 4, electrochemical measurements were done as follows. As the base solution, 0.2 mol dm$^{-3}$ NH$_3$-NH$_4$Cl buffer solution (pH 10.0) containing 2 x 10$^{-4}$ mol dm$^{-3}$ Co(NH$_3$)$_6$Cl$_3$ was used. The ionic strength of the base solution was adjusted to 0.2 mol dm$^{-3}$ with KCl. Ten cm$^3$ of the base solution was transferred into a polarographic cell and deaerated by passing nitrogen gas through the base solution for 15 to 20 min unless otherwise stated. Then an aliquot of protein stock solution was introduced into the deaerated base solution with a microsyringe and the solution was stirred with a magnetic stirrer for 3 min. After solution was allowed to stand for 3 min, differential
pulse polarograms were recorded with sweep rate, \( v = 1.0 \text{ mV s}^{-1} \), the pulse amplitude, \( \Delta E_p^{DP} = -50 \text{ mV} \) and drop time, \( \tau = 3.3 \text{ s} \). In the case of current measurements at a fixed potential, the current was recorded after enough time had elapsed from the memory circuits of the instrument to become fully charged \(^{54,55}\) (more than 15 drops of Hg in our case). All measurements were made under nitrogen atmosphere at 25.0 ± 0.5 °C in a thermostat.

RESULTS AND DISCUSSION

The differential pulse (DP) polarogram of SSI in the presence of Co(III) has been described in chapter 4. DP polarographic method is, generally, considered to be suitable for trace analysis, because the influence of depolarizers reduced at more positive potential is small at DP polarography than other method, e.g. d.c. polarography. For the purpose of trace analysis of proteins by DP polarographic Brdicka current, it is sufficient, in principle, that the output voltage representing the current in the catalytic current region is amplified. Fig.5-1A shows a DP polarogram of 50 ng cm\(^{-3}\) SSI recorded from \( E_p^{DP} = -1.23 \text{ V} \), where the DP polarographic current of cobalt reduction becomes relatively small.

![Fig.5-1 A) Differential pulse polarograms of a) the base solution and b) (a)+50 ng cm\(^{-3}\) SSI. \( E_p^{DP} = -1.23 \text{ V} \). B) Differential pulse Brdicka current vs. potential curve. The current was obtained by subtracting curve a from curve b in Fig.5-1A.](image)
Fig. 5-1B shows the DP polarographic Brdicka current, which is the current corrected for the base current (curve a in Fig. 5-1A). DP polarographic Brdicka current has two peaks at about -1.3 and -1.5 V. Although, at lower protein concentration than 50 ng cm$^{-3}$, the first peak at positive potential was not very well defined in DP polarogram, but the increase in peak current with increasing SSI concentration was found to be more sensitive at the first peak than at the second peak, and the adsorption process of protein is more simple at less negative potential. Then we tried to perform amperometry at a fixed potential of $E_{\text{max}}$, which corresponds with the potential of the first peak of the DP polarographic Brdicka current. $E_{\text{max}}$ was determined to be -1.28 V for SSI by manually recording the polarogram, according to Myers and Osteryoung.\textsuperscript{55} In determining the Brdicka current intensity, $i_{\text{B}^{\text{DP}}}(-1.28 \text{V})$, the current was corrected for the base current (Method A). The value of $i_{\text{B}^{\text{DP}}}(-1.28 \text{V})$ increased linearly with the protein concentration $C_p$ from 40 ng cm$^{-3}$ (Fig. 5-2). At $C_p < 40$ ng cm$^{-3}$, however, the calibration curve deviated upward from the straight line. We considered that the upward deviation of the calibration curve at low $C_p$ is attributed to depression of protein in bulk solution due to the adsorption of protein on the glass wall of cell and/or the surface of electrolysis solution (air/water interface).

To prevent the depression of the protein of interest (in our case SSI), amperometry at the fixed potential ($E_m = -1.28 \text{V}$) was performed under the conditions where 2 to 3 $\mu$g cm$^{-3}$ of gelatine or $\alpha$-amylase (Bacillus subtilis),\textsuperscript{56} which contains no SS, SH groups and are known to be Brdicka inactive protein, coexists in the base solution (Method B). The concentration of Fig. 5-2 Calibration curve of SSI obtained by Method A (see text).
coexisting protein (e.g. $\alpha$-amylase) was decided to be about 100 times larger than the intercept of extrapolation of the linear portion of calibration curve in Fig.5-2 to the abscissa, $C_p (i_B^0 \rightarrow 0) \approx 20 \text{ ng cm}^{-3}$, which should correspond to the amounts of protein adsorbed on solution/glass and/or solution/air interfaces. Under the conditions in Method B, the depression amount of the protein of interest due to the adsorption should decrease to one hundredth of the case in Method A. The Brdicka current was corrected for the base current measured in the presence of $\alpha$-amylase, though under these conditions, the change in the current due to the addition of $\alpha$-amylase was merely recognizable. Fig.5-3 shows the calibration line of SSI in the presence of $\alpha$-amylase for the coexisting protein. Good linear relation between $i_{B}^{DP} (-1.28 \text{ V})$ and $C_p$ can be obtained in the $C_p$ range from 5 to 2000 $\text{ ng cm}^{-3}$. The correlation coefficient of the calibration line in Fig.5-3 was 0.9999. The resulting precision for replicate determination of SSI sample solution (5 times) was 1.73% of coefficient of variation at $C_p = 51.1 \text{ ng cm}^{-3}$. The sensitivity of our method is much higher than that of the most often used methods of estimation of proteins such as a color reaction based on the Folin-Ciocalteu phenol reagent or the biuret reagent and estimation of proteins on the basis of absorption of UV light, i.e. the order of 1 to 1000 $\mu \text{g cm}^{-3}$. Radio isotope method and fluorescence technique are also known to be trace analysis of proteins. Our electroanalytical method does not have inconveniences arising from use of isotope, and is not subject to the interference from coloration or turbidity of sample solution.

This polarographic method based on the Brdicka current can not be
applied to the estimation of proteins having no SH, SS group nor heme group, which becomes, contrarily, advantageous in preventing the adsorption of the protein of interest by coexisting such Brdicka-inactive proteins.

We also attempt the amperometric analysis under non-deaerated conditions (Method C), because the peak potential of oxygen reduction is less negative by about 0.3 V than the potential of the first peak (-1.28 V) and then effect of reduction current of oxygen in DP polarography should be much less than in d.c. polarography. Linear relationship between $i_B^{DP} (-1.28 V)$ and $C_p$ in the $C_p$ range from 0.2 to 2.0 $\mu g \ cm^{-3}$ could be obtained. Detection of SS1 as low as 20 ng cm$^{-3}$ was also successful by this procedure (i.e. Method C), though the calibration curve at $C_p = 20$ to 200 ng cm$^{-3}$ was slightly vent upward and the signal to noise ratio was less than that of Method A or B. From the view of the considerable saving in effort, Method C may offer a useful analytical method of proteins in very small amount of the base solution less than 1 cm$^3$ and/or in continuous sample treatment.\(^{59}\)

We applied this DP polarographic analysis of proteins based on Brdicka current to the determination of the enzyme-inhibitor complex dissociation constant as low as $10^{-10}$ mol dm$^{-3}$ and to the study on antigen-antibody interaction, as described in the following chapters.

**SUMMARY**

Differential pulse (DP) polarographic technique based on Brdicka current has been developed. Amperometry has been performed by applying a fixed potential of $E_{max} = (-1.28 \ V)$, which corresponds with the potential of the first maximum of the DP polarographic Brdicka current of the protein. The Brdicka current at $E_{max}$ is linearly depends on the protein concentration in the range between 3 $\mu g \ cm^{-3}$ and 5 ng cm$^{-3}$. Amperometry under non-deaerated conditions has also been studied, in which protein as low as 20 ng cm$^{-3}$ can be detected.
Chapter 6. POLAROGRAPHIC STUDY ON ENZYME-INHIBITOR INTERACTION

Streptomyces subtilisin inhibitor (SSI) and plasminostreptin (PS) are protein proteinase inhibitors of microbial origin. These inhibitors, both containing SS-groups, are known to consist of two identical subunits and are able to bind one molecule of subtilisin BPN' (S.BPN', EC 3.4.21.14) per one subunit of the inhibitor to form S.BPN'-inhibitor complexes. The dissociation constants of the complexes are as low as $10^{-10}$ to $10^{-8}$ mol dm$^{-3}$, and their accurate determination has been a subject of intense studies. In this chapter the author reports some basic features of Brdicka currents of SSI and PS and present a novel method for direct determination of the dissociation constants of the enzyme-inhibitor complexes as low as $10^{-10}$ mol dm$^{-3}$. In determining the dissociation constants the multiple equilibrium involving microscopically distinct forms of the complexes has been taken into account.

EXPERIMENTAL

Chemicals:
Lyophilized preparations of Streptomyces subtilisin inhibitor (SSI) and plasminostreptin (PS) were provided by Prof. S.Murao of Osaka Prefecture University and Prof. K.Hiromi of Kyoto University, and Dr. A. Kakinuma of Takeda Chemical Industries, Ltd., respectively. Subtilisin BPN' (S.BPN') was purchased from Sigma Chemical Co. (lot No. 67C-0003). The purity of the enzyme preparation was determined to be 80.5 % according to Inouye et al's method. All chemicals were of reagent grade quality.
Apparatus:

D.c. polarograms were recorded with a Yanagimoto polarograph P-8 or with a Yanagimoto potentiostat PE21-TB2S equipped with a Yokogawa-Hewlett-Packard function generator 3310 B and a Yokogawa X-Y recorder 3077. Two dropping mercury electrodes (dme) were used. Their characteristics were $m = 0.447 \text{ mg s}^{-1}$ and $\tau = 8.59 \text{ s}$ at $-0.7 \text{ V}$ and $h = 90 \text{ cm Hg}$ for dme I and $m = 1.448 \text{ mg s}^{-1}$ and $\tau = 5.43 \text{ s}$ at $-0.7 \text{ V}$ and $h = 44.6 \text{ cm Hg}$ for dme II both in ammoniacal buffer. For recording d.c. polarographic waves, the drop time was regulated at $4.07 \text{ s}$, unless otherwise stated, with a Yanagimoto drop controller P-8-RT. Voltage sweep voltammograms on a hanging mercury drop electrode (hmde) were recorded with a Yanagimoto potentiostat PE21-TB2S equipped with a Yokogawa X-Y recorder 3077. A Metrohm mercury drop electrode E410 was used, its surface area being $0.0187 \pm 0.0003 \text{ cm}^2$. Differential pulse (DP) and taste polarograms were recorded with a Yanagimoto voltammetric analyzer P-1000, equipped with a Watanabe X-Y recorder WX-4401. The characteristics of dme were $m = 0.852 \text{ mg s}^{-1}$ and $\tau = 11.08 \text{ s}$ at $h = 50.0 \text{ cm Hg}$ and $E = -1.50 \text{ V}$ in ammoniacal buffer.

Electrochemical Measurements:

As the base solutions, $0.02 \text{ mol dm}^{-3} \text{ NaH}_2\text{PO}_4-\text{Na}_2\text{HPO}_4$ (pH 7.0), $0.1 \text{ mol dm}^{-3} \text{ tris-HCl}$ (pH 7.5 - 8.9), and $0.2 \text{ mol dm}^{-3} \text{ NH}_3-\text{NH}_4\text{Cl}$ (pH 9.0 - 10.2), containing $2 \times 10^{-4} \text{ mol dm}^{-3} \text{ Co(NH}_3)_6\text{Cl}_3$ were used. The ionic strength of the base solutions was adjusted to $0.2 \text{ mol dm}^{-3}$ with KCl. Protein concentrations were determined spectrophotometrically using values of $E_{1%}^{1\text{cm}} (276 \text{ nm}) = 8.29$ at pH 7.0 for SSI, $E_{1%}^{1\text{cm}} (276 \text{ nm}) = 8.29$ at pH 7.0 for SSI (M.W. of dimer = 22,800), $E_{1%}^{1\text{cm}} (279 \text{ nm}) = 8.7$ at pH 7.5 for PS (M.W. of dimer = 22,800), and $E_{1%}^{1\text{cm}} (278 \text{ nm}) = 10.63$ at pH 7.0 for S.BPN (M.W. = 27,500). All measurements were made under nitrogen atmosphere at $25.0 \pm 0.5 \text{ °C}$ in a thermostat. Other details of the electrochemical measurements are described in previous chapters.
RESULTS AND DISCUSSION

SUBTILISIN BPN' - STREPTOMYCES SUBTILISIN INHIBITOR INTERACTION

Brdicka Current of SSI:
SSI gave well-defined Brdicka waves in d.c. polarography at mercury electrode in buffers of pH 7.0 to 10.0 (see Fig.6-1). Analysis of the polarograms has revealed that the catalytic currents, i_B, at -1.35 to -1.50 V are explained by Eq.(1-4) at the concentration of hexaaminecobalt (III) chloride, Co(III), lower than $2 \times 10^{-4}$ mol dm$^{-3}$. At -1.35 to -1.50 V it is controlled solely by diffusion and given by Koryta equation (eq.(1-10)), at the protein concentration less than $2 \times 10^{-7}$ mol dm$^{-3}$ and $\tau = 4.07$ s, in ammoniacal buffer of pH 9.5. At dme $f_{Co}$ is given by Ilkovic equation (Eq.1-6)). Accordingly $k_B$ values of SSI dimer can be calculated from Eq.(1-4); the results are given in Table 6-1. In this

Fig.6-1. D.c. polarograms of $1.7 \times 10^{-7}$ mol dm$^{-3}$ SSI in the base solution of pH (A) 7.5, (B) 8.5, and (C) 9.5. Each curve starts from -0.8 V. The drop time was not regulated. Broken lines represent d.c. polarograms in the absence of SSI.
Fig.6-2. Dependence of $1/i_B$ on the concentration of Co(III) for the Brdicka currents of (A) $6.2 \times 10^{-8}$ mol dm$^{-3}$ (SSI)$_2$, and (B) (A) + $1.23 \times 10^{-7}$ mol dm$^{-3}$ S.BPN' in the base solution of pH 9.5.

Table, $N_S$ is the number of cysteine residues per one SSI dimer. Note that SSI exists as a dimer, (SSI)$_2$, in the test solution. 

As described in chapter 1, the Brdicka current is expressed as a function of $f_{Co}$ over a wide range of concentration of cobalt ion by Eq.(1-2) or (1-7). Fig.6-2 (plot A) shows that the Brdicka current of SSI is expressed by Eq.(1-7); from the plot, the values of $n_{cCo}$ and $k_f/k_d$ of SSI were estimated. The results are given in Table 6-1.

Fig.6-3. D.c. polarograms of (a) $4.7 \times 10^{-8}$ mol dm$^{-3}$ (SSI)$_2$, (b) (a) + $6.1 \times 10^{-8}$ mol dm$^{-3}$ S.BPN', (c) (a) + $1.13 \times 10^{-8}$ mol dm$^{-3}$ S.BPN', and (d) $6.4 \times 10^{-8}$ mol dm$^{-3}$ S.BPN' in the base solution of pH 9.5. Curve e (dotted line) represents d.c. polarogram in the absence of protein.
Table 6-1. Brdicka Current Constants of SSI and S.BPN'-SSI Complex at -1.4 V in Ammoniacal Buffer of pH 9.5 (at 25 °C)

<table>
<thead>
<tr>
<th>Protein</th>
<th>M.W.</th>
<th>(D_p) (10^{-7} \text{ cm s}^{-1})</th>
<th>(N_s)</th>
<th>(k_f/k_d) (10^9 \text{ cm s} \text{mol}^{-1})</th>
<th>(n_k_c) (10^4 \text{ s}^{-1})</th>
<th>(k_B) (10^{13} \text{ cm mol}^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>SSI</td>
<td>11500 x 2</td>
<td>9.02</td>
<td>4 x 2</td>
<td>0.9</td>
<td>1.7</td>
<td>1.5</td>
</tr>
<tr>
<td>S.BPN'-SSI</td>
<td>39000 x 2</td>
<td>6.30</td>
<td>4 x 2</td>
<td>1.1</td>
<td>1.0</td>
<td>1.1</td>
</tr>
</tbody>
</table>

a) Calculated according to Svedberg equation, using reported values of sedimentation coefficient and partial specific volume.\(^{62,67}\)

Direct Polarographic Titration of SSI with S.BPN' (or vice versa):

S.BPN', which contains neither cysteine nor cystine residues, did not give Brdicka wave (see curve d in Fig.6-3), but when S.BPN' was added to an SSI solution, the Brdicka current of SSI was reduced with addition of S.BPN', as shown in Fig.6-3, until a constant Brdicka wave was produced in the presence of a large excess of S.BPN' (curve c in Fig.6-3). On the contrary no change of the Brdicka waves of SSI was obtained when bovine milk \(\alpha_{\text{S1}}\)-casein (M.W. = 23,500), an independent protein containing neither SH- nor SS-group,\(^{68}\) was added up to about \(1.7 \times 10^{-7} \text{ mol dm}^{-3}\) to a \(2.4 \times 10^{-8} \text{ mol dm}^{-3}\) SSI solution. Accordingly, the decreasing of the Brdicka current heights of SSI with addition of S.BPN' must be attributed to the formation of complex of SSI with S.BPN', which is actually a dimeric complex, \((\text{S.BPN'})(\text{SSI})_2\)\((\text{S.BPN'})\), according to Inouye et al,\(^{67}\)

\[
2 \text{S.BPN'} + (\text{SSI})_2 = (\text{S.BPN'})(\text{SSI})_2(\text{S.BPN'})
\] (6-1)
Fig. 6-4 shows the Brdicka current heights at -1.40 and -1.50 V plotted against added amounts of S.BPN', indicating that SSI can be titrated amperometrically with S.BPN'. Conversely, S.BPN' can be titrated with SSI. The equivalent point of the complex formation was determined as [S.BPN']:[(SSI)$_2$] = 2:1.01 (titration at -1.40 V) or 2:1.04 (titration at -1.50 V) in an ammoniacal buffer of pH 9.5 (Fig. 6-4) and 2:1.02 in a phosphate buffer of pH 7.0. D.c. polarographic method could also be applied to direct titration of $2.4 \times 10^{-8}$ mol dm$^{-3}$ of (SSI)$_2$ with S.BPN'. SSI can similarly be titrated with S.BPN' by potential sweep voltammetry based on the Brdicka current at hmde at the concentration level as low as $2.2 \times 10^{-9}$ mol dm$^{-3}$ of (SSI)$_2$ (see Fig. 6-5).

Fig. 6-4. Dependence of the Brdicka current heights at (A) -1.40 V and (B) -1.50 V of $4.74 \times 10^{-8}$ mol dm$^{-3}$ (SSI)$_2$ on addition amount of S.BPN' in the base solution of pH 9.5.

Fig. 6-5. Voltage sweep voltammograms of (A) the base solution of pH 9.5, (B) (A) + $2.30 \times 10^{-9}$ mol dm$^{-3}$ (SSI)$_2$, and (C) (B) + $6.0 \times 10^{-9}$ mol dm$^{-3}$ S.BPN'. The hmde was exposed to the test solution for 2 min under stirring and for 1 min at stationary state, then the voltammograms were recorded at $v = 0.2$ V s$^{-1}$. 
Brdicka Current of \((S.BPN')(SSI)_2(S.BPN')\) Complex:

The Brdicka current of SSI in the presence of a large excess of S.BPN', which is assigned to the S.BPN'-SSI complex as discussed above, was analyzed by Eqs. (1-2) to (1-11) to give the values of \(k_c\), \(n_c\), and \(k_f/k_d\) of the complex (see plot B in Fig. 6-2). The \(n_c\) value of S.BPN'-SSI complex is about one half the value of SSI, whereas the \(k_f/k_d\) values of S.BPN'-SSI complex and SSI are nearly of the same magnitude. Since it is reasonable to presume that \(k_c\) value of S.BPN'-SSI complex is of the same magnitude as that of SSI, these results can be interpreted as that the \(n_c\) value of S.BPN' value of S.BPN'-SSI complex is one half the value of SSI; that is one of two SS bonds of SSI monomer may become hardly accessible to Brdicka reaction by the complex formation. This interpretation is in harmony with a proposed structure of the S.BPN'-SSI complex based on x-ray crystallography; one SS bond (Cys(71)-Cys(101)) of SSI is in close vicinity of the reactive site of SSI (Met(73)-Val(74)) by which S.BPN' is strongly bound to form the S.BPN'-SSI complex, so that it may become hardly accessible to the Brdicka reaction, whereas another SS bond (Cys(35)-Cys(50)) of SSI is located far from the reactive site and may remain to be accessible to the Brdicka reaction.

Theory on Multiple Equilibrium of Dimeric Enzyme-Inhibitor Complex Dissociation:

When a bifunctional dimeric inhibitor, \(I_2\), binds successively two molecules of the enzyme, \(E\), to form enzyme-inhibitor complexes. The complex formation is described by the following scheme,
In this scheme, $K_1'$, $K_1''$, $K_2'$, and $K_2''$ are the elemental dissociation constants (or microconstants of dissociation), which involve the microscopically distinct forms:

$$K_1' = \frac{[E][I_2]}{[EI_2]} \quad K_2' = \frac{[E][I_2]}{[EI_2]}$$

$$K_1'' = \frac{[E][I_2]}{[I_2]} \quad K_2'' = \frac{[E][I_2]}{[EI_2]}$$

Also the overall dissociation constants (or macroconstants of dissociation), $K_1$ and $K_2$ are defined by

$$K_1 = \frac{[E][I_2]}{([EI_2] + [I_2])} = \frac{K_1'K_1''}{K_1' + K_1''}$$

$$K_2 = \frac{[E]([EI_2] + [I_2])}{[EI_2]} = K_2' + K_2''$$

where $[E]$, $[I_2]$, $[EI_2]$, $[I_2]E$, and $[EI_2]E$ stand for the concentrations of free $E$, free $I_2$, $EI_2$, $I_2E$, and $EI_2E$, respectively. In our case two microscopic forms $EI_2$ and $I_2E$ are physically indistinguishable, therefore we have

$$K_1' = K_1'' = 2K_1$$

$$K_2' = K_2'' = K_2/2$$

We also have

$$[E]_0 = [E] + [EI_2] + [I_2] + 2[E]E$$

$$[I_2]_0 = [I_2] + [EI_2] + [I_2]E + [I_2]E + [EI_2]E$$
where $[E]_0$ and $[I_2]_0$ are the analytical concentrations of E and I$_2$. The Brdicka current intensity is proportional to the concentration of protein, as described in chapter 1. Therefore the total Brdicka current intensity, $i_t$, observed during the course of the titration of I$_2$ with E or vice versa, can be expressed as

$$i_t = K_E [E] + K_{I_2} [I_2] + K_{EI_2} ([EI_2] + [I_2E]) + K_{E_2I} [E_2I]$$

(6-8)

where $K_E$, $K_{I_2}$, $K_{EI_2}$ and $K_{E_2I}$ are the proportional constants converting the protein concentrations to the Brdicka current intensities for E, I$_2$, EI$_2$ (or I$_2$E) and E$_2$I, respectively. The $K_p$ is characteristic of the protein P for a given electrode system.

We define $\Delta i$, the change in the Brdicka current intensity due to the complex formation, by

$$\Delta i = K_E [E]_0 + K_{I_2} [I_2]_0 - i_t$$

(6-9)

Upon substituting Eqs. (6-6) to (6-8) into Eq. (6-9) and assuming that

$$K_{EI_2} = (K_{I_2} + K_{E_2I})/2$$

(6-10)

we get

$$\Delta i = (1/2)(2K_E + K_{I_2} - K_{E_2I})([EI_2] + [I_2E] + 2[E_2I])$$

$$= (1/2)(2K_E + K_{I_2} - K_{E_2I})([E]_0 - [E])$$

(6-11)

Also, $\Delta i$ may be expressed by a relative change in Brdicka current, $\Delta I$, defined by

$$\Delta I = \Delta i/(K_{I_2} [E]_0/2)$$
\[
= \Delta \kappa ([E]_0 - [E])/[E]_0
\]

(6-12)

where

\[
\Delta \kappa = \frac{(2K_2' + \kappa_2 - \kappa E_2 E_2')/\kappa_2'}{\kappa_2} \quad (6-13)
\]

On the other hand, \([E]\) is the solution of Eq. (6-14), which is derived from Eqs. (6-3) to (6-7),

\[
[E]^3 + (2[I_2]_0 - [E]_0 + 2K_2')[E]^2 + K_2'(2[I_2]_0 - 2[E]_0 + K_1')[E] - K_1'K_2'[E]_0 = 0
\]

(6-14)

Differential Pulse Polarographic Determination of Dissociation Constants of S.BPN'-SSI Complex:

The author has applied to the determination of dissociation constants of S.BPN'-SSI complex differential pulse (DP) polarographic method, which is more sensitive by about two orders of magnitude than d.c. polarography, as shown in chapter 5. In DP polarography, SSI gave a well defined Brdicka current polarogram or protein wave having two peaks (Fig. 6-6, curve b). The height of the protein wave at the potential of the first peak, \(E_{max} = -1.28 \text{ V at pH 10.0}\), increased linearly with increasing concentration of SSI solution. When S.BPN' was added to an SSI solution, the protein wave decreased in height as shown, for example, by curve d in Fig. 6-6. The decrease in the current height of SSI with addition of S.BPN' should be attributed to the complex formation of SSI with S.BPN'. S.BPN' also gave a DP polarographic protein wave, though its peak height was very much smaller than that of SSI (Fig. 6-6, curve c). Fig. 6-7 shows a \(\Delta i \text{ vs. potential curve obtained at [E]}_0 = 9.4 \times 10^{-8} \text{ mol dm}^{-3} \text{ and [I}_2\text{]}_0 = 4.7 \times 10^{-8} \text{ mol dm}^{-3} \).

S.BPN' contains neither cysteine nor cystine residue and gave no Brdicka current in d.c. polarography. Accordingly, S.BPN' was expected to give no protein wave also in DP polarography. Thus, the
Fig. 6-6 (left) Differential pulse polarograms of (a): the base solution of pH 10.0, (b): (a) + 4.7 x 10^{-8} \text{ mol dm}^{-3} \text{ SSI}, (c): (a) + 9.4 x 10^{-8} \text{ mol dm}^{-3} \text{ S.BPN'} and (d): (a) + 4.7 x 10^{-8} \text{ mol dm}^{-3} \text{ SSI} and 9.4 x 10^{-8} \text{ mol dm}^{-3} \text{ S.BPN'}. Polarograms were run from \textit{E}_i = -1.23 \text{ V} at \nu = 1 \text{ mV s}^{-1}, \Delta \text{E} = -50 \text{ mV}, and \tau = 1.67 \text{ s}.

Fig. 6-7 (right) \Delta i vs. potential curve for the complex formation between 9.4 x 10^{-8} \text{ mol dm}^{-3} \text{ S.BPN'} and 4.7 x 10^{-8} \text{ mol dm}^{-3} \text{ SSI}.

small DP polarographic wave observed with S.BPN' may most probably be attributed to trace impurities in the S.BPN' preparation. $\alpha$-Amylase (\textit{Bacillus subtilis}), which contains neither SH nor SS group, gave no DP poalrographic protein wave. Following discussion on the dissociation constants is not affected, whether the small DP polarographic wave is due to the impurities in the S.BPN' preparation ($K_e = 0$), or the enzyme
results can also be interpreted as one of two SS bonds of SSI monomer becomes inaccessible to Brdicka reaction by the complex formation.
Fig. 6-9. Polarographic titration curve (ΔI vs. \( \frac{[I_2]_0}{[E]_0} \) plot) of S.BPN' with SSI at the S.BPN' analytical concentration of 9.64 x \( 10^{-9} \) mol dm\(^{-3} \). The solid line is a regression curve calculated on values of \( K_1' = 0.5 \times 10^{-9} \) mol dm\(^{-3} \), \( K_2' = 9.4 \times 10^{-9} \) mol dm\(^{-3} \) and \( ΔK = 0.40 \).

Titration of S.BPN' with SSI was carried out also at \([E]_0 = 9.64 \times 10^{-9} \) mol dm\(^{-3} \), as shown in Fig. 6-9. The titration curve changes smoothly near the equivalent point (\( \frac{[I_2]_0}{[E]_0} = 1 \)), indicating that the dissociation of S.BPN'-SSI complex should not be negligible at such a low concentration of S.BPN'. Thus we tried to fit to the ΔI vs. \( \frac{[I_2]_0}{[E]_0} \) plot the curve represented by Eqs. (6-12) and (6-14) by adjusting three parameters \( K_1' \), \( K_2' \) and \( ΔK \) (three-parameter model) using a Facom M-200 computer in Data Processing Center of Kyoto University. The results of the least-squares analysis using SALS program\(^{28} \) were \( K_1' = (0.5 \pm 0.4) \times 10^{-9} \) mol dm\(^{-3} \), \( K_2' = (9.4 \pm 2.2) \times 10^{-9} \) mol dm\(^{-3} \) and \( ΔK = 0.40 \pm 0.02 \) at pH = 10.0, \( I = 0.2 \) mol dm\(^{-3} \), and \( t = 25 \) °C. Solid line in Fig. 6-9 is a regression curve calculated on values of \( K_1' = 0.5 \times 10^{-9} \) mol dm\(^{-3} \), \( K_2' = 9.4 \times 10^{-9} \) mol dm\(^{-3} \) and \( ΔK = 0.40 \).
This $\Delta K$ value agrees well with $\Delta I_{\text{max}} = \Delta K = 0.37$ estimated from the titration curve at $[E]_0 = 9.4 \times 10^{-8}$ mol dm$^{-3}$ (Fig. 6-8).

We also tried to fit to the $\Delta I$ vs. $2[I_2]_0/[E]_0$ plot in Fig. 6-9 the theoretical curve derived on assuming that all four elemental dissociation constants are equal; $K' = K'_1 = K'_2 = K''_1 = K''_2$, by adjusting two parameters, $K'$ and $\Delta K$ (two-parameter model). The results were $K' = (6.9 \pm 1.1) \times 10^{-9}$ mol dm$^{-3}$ and $\Delta K = 0.54 \pm 0.02$. For purpose of statistical model identification, a minimum Akaike's information criterion (AIC) estimate$^{73}$ was employed. According to Akaike,$^{73}$ AIC is defined by $AIC = N \ln S + 2M$, where $N$, $M$ and $S$ are the number of data, the number of parameters and the residual sum of squares, respectively, and when there are several competing models, the fitting model which gives the minimum of $AIC$ is a statistically maximum likelihood one. In our case, the $AIC$ value of the three-parameter model was 52.3, whereas it was 68.7 for the two-parameter model. The former is appreciably smaller than the latter. Also, the $\Delta K$ value for two-parameter model deviates appreciably from $\Delta I_{\text{max}} = 0.37$. These results indicate that the three-parameter model is statistically better one than two-parameter model; in other words, $K'_1$ differs from $K'_2$ significantly. The ratio $K'_1/K'_2 = 0.053$ implies that the intrinsic free energy change of the first binding of the enzyme to the inhibitor is 1.7 kcal mol$^{-1}$ larger than that of the second binding.

In conclusion, the above results show that the polarographic method based on Brdicka current can be applied for direct determination of the enzyme-inhibitor complex dissociation constants as low as $10^{-10}$ mol dm$^{-3}$. So far as the author knows, this is the first report describing that the intrinsic free energy change in the first binding of S.BPN' to dimeric SSI differs significantly from that of the second binding.
SUBTILISIN BPN' - PLASMINOSTREPTIN INTERACTION

Brdicka Currents of PS and S.BPN'-PS Complex:

PS is a protein proteinase inhibitor containing two SS bonds per monomer, of which the amino acid sequence is identical with that of SSI at about 70% of the positions, and gave well defined Brdicka waves at mercury electrode in buffers of pH 7.5 to 10.2 (see Fig.6-10). The d.c. or taste polarographic Brdicka current at -1.35 V increased linearly with increasing concentration of the protein, up to 8.0 × 10^-7 mol dm^-3 in a tris buffer of pH 8.5 with dme of τ = 1.52 s. When S.BPN' was added to a PS solution, the Brdicka current of PS decreased with addition of S.BPN' and vice versa, as shown in Fig.6-11. The Broken line in this Figure shows Δi (defined by Eq.(6-9)) vs. potential curve, obtained at [E]_0 = 2.0 × 10^-7 mol dm^-3 and [I_2]_0 = 1.0 × 10^-7 mol dm^-3 in taste polarographic mode.

Titration of 2.86 × 10^-7 mol dm^-3 S.BPN' with PS was performed amperometrically at E_m = -1.35 V, τ = 1.25 s by taste polarography.

Fig.6-10. D.c. polarograms of 1.75 × 10^-7 mol dm^-3 (PS)_2 in the base solution of pH (A) 7.5, (B) 8.5 and (C) 9.5. Each curve starts from -0.8 V. The drop time was not regulated. Broken lines represent d.c. polarograms in the absence of PS.
Fig. 6-11. Tast polarographic protein wave of (1) $2.0 \times 10^{-7}$ mol dm$^{-3}$ S.BPN', (2) $1.0 \times 10^{-7}$ mol dm$^{-3}$ (PS)$_2$ and (3) $2.0 \times 10^{-7}$ mol dm$^{-3}$ S.BPN' and $1.0 \times 10^{-7}$ mol dm$^{-3}$ (PS)$_2$ in the base solution of pH 8.0. The current was measured from the limiting current of the base solution. Dotted line (curve 4) represents $\Delta I$ vs. potential curve for the complex formation between $2.0 \times 10^{-7}$ mol dm$^{-3}$ S.BPN' and $1.0 \times 10^{-7}$ mol dm$^{-3}$ (PS)$_2$.

Fig. 6-12. Tast polarographic titration curve ($\Delta I$ vs. $2[I_2]_0/[E]_0$ plot) of S.BPN' with PS at the S.BPN' analytical concentration of $2.86 \times 10^{-7}$ mol dm$^{-3}$. The current was measured at $E_m = -1.35$ V.
Fig. 6-12 shows the plot of $\Delta I$ (defined by Eq. (6-12)) against the amount of PS or $2[I_2^0]/[E]^0$ at pH 8.5. The $\Delta I$ values initially increase linearly with $2[I_2^0]/[E]^0$ and reach a certain maximum value, $\Delta I_{\text{max}} = 0.55$, with an inflection point at $2[I_2^0]/[E]^0 = 1.0$, indicating that PS binds one molecule of S.BPN' per one subunit of PS to form S.BPN'-PS complex. The value of $\Delta I_{\text{max}} = \Delta K = 0.55$ can be interpreted as one of two SS bonds in PS monomer, which may be Cys(67)-Cys(97) in close vicinity of the reactive site of PS (Lys(67)-Gly(70)), may become inaccessible to Brdicka reaction by the complex formation.

**Determination of Dissociation Constants of S.BPN'-PS Complex:**

Titration of S.BPN' with PS was carried out also at $[E]^0 = 4.56 \times 10^{-8}$ mol dm$^{-3}$ and pH = 8.5 as shown in Fig. 6-13. PS exists as a dimer in tris buffer of pH 7.5. Upon assuming that S.BPN'-PS complex is dimeric, $(S.BPN')(PS)_2(S.BPN')$, the author determined the dissociation constants of S.BPN'-PS complex by the same way as in the case of S.BPN'-SSI complex. The results were $K_1' = (6.7 \pm 4.8) \times 10^{-9}$ mol dm$^{-3}$, $K_2' = (1.2 \pm 0.7) \times 10^{-8}$ mol dm$^{-3}$, and $\Delta K = 0.55 \pm 0.01$. The solid line in Fig. 6-13 is a regression curve calculated on values of $K_1' = 6.7 \times 10^{-9}$ mol dm$^{-3}$ and $K_2' = 1.2 \times 10^{-10}$ mol dm$^{-3}$ and $\Delta K = 0.55$, according to Eqs. (6-12) and (6-14). This $\Delta K$ value also agree well with $\Delta I_{\text{max}} = \Delta K = 0.55$ estimated from the titration curve at $[E]^0 = 2.86 \times 10^{-7}$ mol dm$^{-3}$ (Fig. 6-12).

Fig. 6-13. Tast polarographic titration curve ($\Delta I$ vs. $2[I_2^0]/[E]^0$ plot) of S.BPN' with PS at the S.BPN' analytical concentration of $4.56 \times 10^{-8}$ mol dm$^{-3}$. The solid line is a regression curve calculated on values of $K_1' = 6.7 \times 10^{-9}$ mol dm$^{-3}$, $K_2' = 1.2 \times 10^{-10}$ mol dm$^{-3}$ and $\Delta K = 0.55$. 

- 65 -
As in the case of S.BPN'-SSI complex. The ratio $K_1'/K_2' = 0.56$ implies that the intrinsic free energy of the first binding of the enzyme to the inhibitor is 0.34 kcal mol$^{-1}$ larger than that of the second binding.

*Effect of pH on Dissociation Constants of S.BPN'-PS Complex:*

The author also determined the intrinsic dissociation constants of S.BPN'-PS complex at pH 7.8 to 10.2. Fig.6-14 shows the pH dependence of the intrinsic dissociation constants or $pK'$ ($= -\log K'$) vs. pH plots (Dixon plots$^{75}$) of the first and second binding of S.BPN' to PS. The broken lines in Fig.6-14 are so-called guide lines of Dixon plots, whose slopes are zero and -1, and show two inflection points at about pH 8.1 and 9.4. These results suggest that one ionizable group in the reactant protein species and another ionizable group in the product protein species participate in the first and/or second binding of S.BPN' with PS.

Here we consider simple bimolecular interaction system between enzyme and inhibitor, where enzyme and complex exist in two states of ionization, as follows:

\[
\begin{align*}
E^n + I^m & \leftrightarrow K^a E^{n-1} + I^m + H^+ \\
E^{-}I^{1+n} & \leftrightarrow K^b E^{-}I^{1+n-1} + H^+
\end{align*}
\]

(6-15)
In this scheme, superscripts represent the number of ionizable protons in protein species and we will assume that the form of $E^n$ of the enzyme combines with inhibitor $I^m$ with pH-independent dissociation constant, $K_d$, defined by

$$\frac{[E^n][I^m]}{[EI^{n+m}]}$$  \hspace{1cm} (6-16)

$k^a$ and $k^b$ refer to the ionization state of the enzyme and the enzyme-inhibitor complex, respectively, that is

$$K^a = \frac{[E^n][H^+]}{[E^n]}$$  \hspace{1cm} (6-17)

$$K^b = \frac{[EI^{n+m-1}][H^+]}{[EI^{n+m}]}$$  \hspace{1cm} (6-18)

We use for convenient the subscript $t$ to denote the sum of the concentrations of all different forms of the enzyme and the complex; thus for this system we have

$$[E]_t = [E^n] + [E^{n-1}]$$  \hspace{1cm} (6-19)

$$[EI]_t = [EI^{n+m}] + [EI^{n+m-1}]$$  \hspace{1cm} (6-20)

Apparent dissociation constant $K_d$ can be expressed by

$$K_d = \frac{[E]_t[I^n]}{[EI]_t}$$  \hspace{1cm} (6-21)

Upon substituting Eqs. (6-16) to (6-20) into Eq. (6-21), we can get

$$pK_d = pK_d^* + \log(1 + 10^{PH-pK^b}) - \log(1 + 10^{PH-pK^a})$$  \hspace{1cm} (6-22)
Eq. (6-22) can be applied to the system where inhibitor exists in two states of ionization, I\textsuperscript{m} \rightleftharpoons I\textsuperscript{m-1} + H\textsuperscript{+}, instead of E. Then $K^a$ and $K^b$ can generally be considered to refer to the ionization state of the reactant (the enzyme or the inhibitor in this scheme) and the product (the enzyme-inhibitor complex), respectively. Also above discussion is available for the microscopic dissociation equilibrium of E and I\textsubscript{2}. Thus, we get, for the first stage of microscopic dissociation of EI\textsubscript{2} (or I\textsubscript{2}E),

$$pK_1' = \frac{pK_1 - \log(1 + 10^{PH-pK^b_1})}{1 + 10^{PH-pK^a_1}}$$  \hspace{1cm} (6-23)$$

and for the second stage of microscopic dissociation of EI\textsubscript{2}E,

$$pK_2' = \frac{pK_2 - \log(1 + 10^{PH-pK^b_2})}{1 + 10^{PH-pK^a_2}}$$  \hspace{1cm} (6-24)$$

We tried to fit the curve represented by Eqs. (6-23) and (6-24) to the $pK_1'$ and $pK_2'$ vs. pH plots, by adjusting three parameters $K_1'$, $K_1^a$, and $K_1^b$, and $K_2'$, $K_2^a$, and $K_2^b$, respectively. The results of the least-squares analysis using SALS program\textsuperscript{28} were $pK_1' = 8.74 \pm 0.17$, $pK_1^a = 8.10 \pm 0.23$ and $pK_1^b = 9.38 \pm 0.22$ for the first binding step, and $pK_2' = 8.31 \pm 0.17$, $pK_2^a = 8.17 \pm 0.28$ and $pK_2^b = 9.40 \pm 0.26$ for the second binding step.

The difference in $pK'$, $pK_1' - pK_2'$, implies that the pH-independent intrinsic free energy change of the first binding of the enzyme to the inhibitor is 0.6 kcal mol\textsuperscript{-1} larger than that of the second binding. The $pK_1^a$ value and $pK_1^b$ value do not significantly differ from $pK_2^a$ and $pK_2^b$, respectively, and $pK_1^a$ or $pK_2^a$ is smaller by about one unit than $pK_1^b$ or $pK_2^b$.

The $pK_1^a$ or $pK_2^a$ value obtained here (8.1 ~ 8.2) is slightly higher than those so far reported $pK = 5 ~ 6$, if it is assumed to represent the $pK$ of histidine at the active site of S.BPN' (serine-enzyme),\textsuperscript{76} as suggested by Uehara et al.\textsuperscript{63} who reported the involvement of an ionizable group of $pK$ 8.5 in the association of S.BPN' and SSI. A contribution of ionizable group of lysin in PS or tyrosine in S.BPN' may not be excluded.
Although whether these ionizable groups of $pK_1$, $pK_2$, $pK_1^b$, and $pK_2^b$ exist in S.BPN' or PS is not clear, these results may be interpreted as that $pK$ value of a ionizable group of S.BPN' or PS in the free state ($pK = 8.1 \sim 8.2$) increases by about one unit ($pK = 9.4$) due to the complex formation between S.BPN' and PS.

The determination of the dissociation constant of the S.BPN'-PS complex described above is based on the assumption of dimeric S.BPN'-PS complex formation. Then, the results may be considered as tentative, since according to Kakinuma et al.\textsuperscript{61} trypsin-PS complex exists in a monomeric form in tris buffer of pH 7.5. Further study is continued.

**SUMMARY**

Brdicka currents of SSI and PS have been studied by d.c., t-a-t, and differential pulse polarographic techniques and potential sweep voltammetric technique. The Brdicka current of SSI or PS decreased with addition of S.BPN', which was attributed to the formation of proteinase-inhibitor complexes. Analysis of the dependences of Brdicka currents of SSI and S.BPN'-SSI complex on cobalt ion concentration revealed that one of two SS bonds of SSI became hardly accessible to Brdicka reaction by the complex formation. SSI and PS could be directly titrated by polarographic technique based on the Brdicka current in the concentration level of $10^{-7}$ to $10^{-9}$ mol dm$^{-3}$.

The first and second dissociation constants of S.BPN'-SSI and S.BPN'-PS complexes as low as $10^{-8}$ to $10^{-10}$ mol dm$^{-3}$ were determined by fitting to the titration data theoretical curves, in which the multiple equilibrium involving microscopically distinct forms of S.BPN'-inhibitor complex was taken into account. The intrinsic free energy change in the first binding of S.BPN' to dimeric SSI or PS was larger than that of the second binding.

The effect of pH on the intrinsic dissociation constants of S.BPN'-PS complex has suggested the participation of ionizable groups with
pK = 8.1 and 9.4 in the first step and of ionizable groups with pK = 8.2 and 9.4 in the second step of binding.
Chapter 7. POLAROGRAPHIC STUDY ON INTERACTION BETWEEN HUMAN IgG AND SHEEP ANTI-HUMAN IgG ANTISERUM AND ITS ANALYTICAL APPLICATION

Immunoglobin is a protein containing disulfide bonds and hence produces Brdicka current. In this chapter the author has applied d.c. and differential pulse polarographic techniques for the study of the complex formation of human immunoglobin G (IgG) with sheep antihuman IgG antiserum (anti-IgG) at the concentration level as low as $10^{-10}$ mol dm$^{-3}$. The (average) intrinsic dissociation constant of the IgG - anti-IgG complex has been determined. The results also suggest a possible analytical application of Brdicka current coupled with the immunochemical reaction.

EXPERIMENTAL

Materials:

Lyophilized preparation of human immunoglobin G (IgG) was a gift of Wako Pure Chemical Industries, Ltd. and used as received. The concentration of IgG was determined spectrophotometrically using $E_{1\text{cm}}^{1\%}(280\text{ nm}) = 15.0$. Affinity chromatographic liquid preparation of sheep antihuman IgG antiserum (anti-IgG) was also a gift of Wako Pure Chemical Industries, Ltd. and used as received. The titer of the anti-IgG preparation was 2.0 mg antibody per cm$^3$, as determined by the quantitative precipitation reaction. The molecular weights of IgG and anti-IgG were estimated at 160,000. The optimum proportion of anti-IgG to IgG in the precipitation reaction was 5:1. All other chemicals were of reagent grade quality.

Apparatus:

D.c. polarograms were recorded with a Yanagimoto polarograph P-8,
equipped with a Yanagimoto drop controller P-8-RT, and differential pulse polarograms were recorded with a Yanagimoto voltammetric analyzer P-1000, equipped with a Watanabe recorder WX-4401. The characteristics of the dropping mercury electrode were $m = 0.852 \text{ mg s}^{-1}$ and $\tau = 11.08 \text{ s}$ at $h = 50.0 \text{ cm Hg}$ and $E = -1.50 \text{ V}$ in an ammoniacal buffer. In recording polarograms the drop time was controlled at $\tau = 2.0 \text{ s}$, unless otherwise stated.

**Electrochemical Measurements:**

As the base solution, $0.1 \text{ mol dm}^{-3} \text{ NH}_3 - 0.1 \text{ mol dm}^{-3} \text{ NH}_4\text{Cl} - 0.1 \text{ mol dm}^{-3} \text{ KCl}$ buffer solution ($\text{pH} 9.5$ and the ionic strength $I = 0.2 \text{ mol dm}^{-3}$) containing $2 \times 10^{-4} \text{ mol dm}^{-3} \text{ Co(NH}_3)_6\text{Cl}_3$ was used. All measurements were made under nitrogen atmosphere at $25.0 \pm 0.5 \degree \text{C}$ in a thermostat. Other details of the electrochemical measurements have been described in previous chapters.

**RESULTS AND DISCUSSION**

**D.c. Polarograms of IgG, anti-IgG and their Mixture:**

In d.c. polarography, IgG and anti-IgG gave a well-defined protein wave due to Brdicka current, as shown by polarograms (1) and (2), respectively, in Fig.7-1. The Brdicka current, $i_B$, usually measured at $-1.35 \text{ V}$ from the limiting current of cobalt ion, increased linearly with the concentration of IgG or anti-IgG up to about $6 \times 10^{-8} \text{ mol dm}^{-3}$. As described in chapter 6, Brdicka current is additive of each contribution. Actually, when a small amount of bovine pancreas ribonuclease-A (RNase), as indifferent protein to anti-IgG, was added to $2.5 \times 10^{-8} \text{ mol dm}^{-3}$ anti-IgG in the base solution, the Brdicka current increased additively with addition of RNase up to $2.2 \times 10^{-7} \text{ mol dm}^{-3}$. On the contrary, when IgG was added to $2.5 \times 10^{-8} \text{ mol dm}^{-3}$ anti-IgG in the base solution, the Brdicka current did not additively increase but the Brdicka current of
the mixture of IgG and anti-IgG was always smaller than the sum of the Brdicka currents of IgG and anti-IgG (see Fig.1, curve 3). This result may be explained by that with addition of IgG to an anti-IgG solution IgG – anti-IgG complexes are formed and that the SS bonds in the proteins become inaccessible to Brdicka reaction by the complex formation, as described in chapter 6.

\[ \Delta i_B = i_B([Ag]_0) + i_B([Ab]_0) - i_B([Ag]_0 + [Ab]_0) \quad (7-1) \]

where \( i_B([Ag]_0) \), \( i_B([Ab]_0) \) and \( i_B([Ag]_0 + [Ab]_0) \) are the Brdicka currents produced by antigen (IgG in our case) at the concentration of \([Ag]_0\)’, antibody (anti-IgG) at the concentration of \([Ab]_0\)’, and their mixture at the concentrations of \([Ag]_0\) and \([Ab]_0\) in the base solution, respectively. Broken line in Fig.7-1 shows a \( \Delta i_B \) vs. potential curve at \([Ag]_0 = 2.44 \times 10^{-8} \text{ mol dm}^{-3} \) and \([Ab]_0 = 2.50 \times 10^{-8} \text{ mol dm}^{-3} \).
As seen in Fig. 7-2, the $\Delta i_B$ vs. potential curve has two humps at -1.35 V and -1.60 V, respectively. Subsequent addition of IgG results in incremental increase in the difference Brdicka current, as shown in Fig. 7-2. Fig. 7-3 shows three plots of the relative difference Brdicka currents, $\Delta i_B$, defined by

\[ \Delta i_B = i_B - i_{B0} \]

Fig. 7-3. D.c. polarographic titration of anti-IgG with IgG (calibration curves for IgG) at the concentrations of a) $1.25 \times 10^{-8}$ mol dm$^{-3}$, b) $2.50 \times 10^{-8}$ mol dm$^{-3}$, and c) $3.75 \times 10^{-8}$ mol dm$^{-3}$. The solid lines are regression curves calculated on values of $K = 1.3 \times 10^{-7}$ mol dm$^{-3}$ and $\Delta K = 0.48$. 

Fig. 7-2. $\Delta i_B$ vs. potential curves observed with mixtures of IgG and anti-IgG at the concentrations of 2.50 x $10^{-8}$ mol dm$^{-3}$ anti-IgG with a) $6.1 \times 10^{-8}$ mol dm$^{-3}$, b) $1.22 \times 10^{-8}$ mol dm$^{-3}$, c) $1.88 \times 10^{-8}$ mol dm$^{-3}$, and d) $2.44 \times 10^{-8}$ mol dm$^{-3}$ IgG.
\[
\Delta I_B = \frac{\Delta I_B}{i_B([\text{Ab}])_0}
\]  

(7-2)

against the added amount of IgG represented by \([\text{Ag}]_0/\text{[Ab]}_0\) at \([\text{Ab}])_0 = 1.25, 2.50, \text{ and } 3.75 \times 10^{-8} \text{ mol dm}^{-3}. \text{ The } \Delta I_B \text{ value increases with } [\text{Ag}]_0/[\text{Ab}]_0 \text{ and tends to approach a certain limiting value.}

**Determination of Dissociation Constants of IgG - anti-IgG Complexes:**

Mathematical formulation of the antigen-antibody complex formation is very complicated, since generally both antigen and antibody are multivalent. In the following, we limit our discussion to the primary interaction of antigen and antibody and adopt following assumptions for simplification. We assume first that all the binding sites of the n-valent antigen are of equal affinity with the antibody and behave independently from each other. We also assume that the antibody behaves as monovalent ligand in the complex formation. Then the formation of the antigen (Ag) - antibody (Ab) complex is expressed by,

\[
\text{Ag} + \text{Ab} = \text{Ag(Ab)}_1
\]

\[
\text{Ag(Ab)}_1 + \text{Ab} = \text{Ag(Ab)}_2
\]

\[
\ldots
\]

\[
\text{Ag(Ab)}_{j-1} + \text{Ab} = \text{Ag(Ab)}_j
\]

\[
\ldots
\]

\[
\text{Ag(Ab)}_{n-1} + \text{Ab} = \text{Ag(Ab)}_n
\]

(7-3)

In these equations, the protein species represented by \(\text{Ag(Ab)}_j\) involves \(\binom{n}{j}\) species of microscopically distinct \(\text{Ag(Ab)}_j\) complex. We also have

\[
K = \frac{[\text{Ag}][\text{Ab}]}{[\text{Ag(Ab)}_1]} = \frac{[\text{Ag(Ab)}_{j-1}][\text{Ab}]}{[\text{Ag(Ab)}_j]} \quad (j = 2, 3, \ldots, n)
\]

(7-4)

where \(K\) is the (average) intrinsic dissociation constant of the binding
site of antigen with antibody, and \([\text{Ag}], [\text{Ab}]\) and \([\text{Ag}(\text{Ab})_j]\) are the concentrations of free antigen, free antibody and individual microscopic form of \([\text{Ag}(\text{Ab})_j]\), respectively. We further have

\[
[\text{Ag}]_0 = [\text{Ag}] + \sum_{j=1}^{n} C_j [\text{Ag}(\text{Ab})_j] \quad (7-5)
\]

\[
[\text{Ab}]_0 = [\text{Ab}] + \sum_{j=1}^{n} j C_j [\text{Ag}(\text{Ab})_j] \quad (7-6)
\]

The Brdicka current intensities, \(i_B([\text{Ag}]_0), i_B([\text{Ab}]_0)\) and \(i_B([\text{Ag}]_0 + [\text{Ab}]_0)\) in Eq.(1) can be expressed by

\[
i_B([\text{Ag}]_0) = \kappa_{\text{Ag}} [\text{Ag}]_0 \quad (7-7)
\]

\[
i_B([\text{Ab}]_0) = \kappa_{\text{Ab}} [\text{Ab}]_0 \quad (7-8)
\]

\[
i_B([\text{Ag}]_0 + [\text{Ab}]_0) = \kappa_{\text{Ag}} [\text{Ag}] + \kappa_{\text{Ab}} [\text{Ab}] + \sum_{j=1}^{n} \kappa_{\text{Ag}(\text{Ab})_j} j C_j [\text{Ag}(\text{Ab})_j] \quad (7-9)
\]

where \(\kappa_{\text{Ag}}\) and \(\kappa_{\text{Ab}}\) are the proportional constants converting the concentrations of antigen and antibody to their Brdicka currents, respectively, and \(\kappa_{\text{Ag}(\text{Ab})_j}\) is the proportional constant for the microscopic forms of \([\text{Ag}(\text{Ab})_j]\) assumed to have the same proportional constant for a given \(j\). Note that \(\kappa_p's\) are characteristic of the proteins, \(P\), for a given polarographic technique with a given electrode system. It is reasonable to assume that \(\kappa_{\text{Ag}(\text{Ab})_j}\) changes with \(j\) in an arithmetical series,

\[
\kappa_{\text{Ag}(\text{Ab})_j} = j \kappa_{\text{Ag}(\text{Ab})_1} - (j - 1) \kappa_{\text{Ag}} \quad (j = 1, 2, \ldots, n) \quad (7-10)
\]

Upon substituting Eqs.(5) to (10) into Eq.(1) we get
\[ \Delta I_B = ((\kappa_{Ag} + \kappa_{Ab} - \kappa_{Ag(\text{Ab})}) \sum_{j=1}^{n} J_n C_j [\text{Ag(\text{Ab})}_j]) \]

\[ = ((\kappa_{Ag} + \kappa_{Ab} - \kappa_{Ag(\text{Ab})}) ([\text{Ab}]_0 - [\text{Ab}]) \]

Also upon substituting Eqs. (4), (5), (6) and (11) into Eq.(2), we get

\[ \Delta I_B = (\Delta \kappa/2[\text{Ab}]_0)(n[\text{Ag}]_0+[\text{Ab}]_0+K_0 \sqrt{(n[\text{Ag}]_0-[\text{Ab}]_0+K_0)^2+4K[\text{Ag}]_0}) \] (7-12)

where

\[ \Delta \kappa = (\kappa_{Ag} + \kappa_{Ab} - \kappa_{Ag(\text{Ab})}) / \kappa_{Ab} \] (7-13)

We fitted the curve represented by Eq.(12) with \( n = 5 \) (see EXPERIMENTAL) to the \( \Delta I_B \) vs. \( [\text{Ag}]_0/[\text{Ab}]_0 \) plots in Fig.7-3 by adjusting two parameters \( K \) and \( \Delta \kappa \) using a Facom M-200 computer in Data Processing Center of Kyoto University. The results of the least-squares analysis are \( K = (1.3 \pm 1.0) \times 10^{-7} \text{ mol dm}^{-3} \) and \( \Delta \kappa = 0.48 \pm 0.20 \) at pH 9.5, \( I = 0.2 \text{ mol dm}^{-3} \) and 25°C. Solid lines in Fig.7-3 are regression curves calculated on values of \( K = 1.3 \times 10^{-7} \text{ mol dm}^{-3} \) and \( \Delta \kappa = 0.48 \). This \( K \) value \( 1.3 \times 10^{-7} \text{ mol dm}^{-3} \) seems a reasonable one, though this value should be considered in views of the assumptions adopted for simplification as an average value representing the affinity of binding between IgG and anti-IgG.

**Analytical Application:**

The \( \Delta I_B \) vs. \( [\text{Ag}]_0/[\text{Ab}]_0 \) curves in Fig.3 can be used as the calibration curves for the determination of IgG at the concentration level of \( 2 \sim 40 \times 10^{-9} \text{ mol dm}^{-3} \).

Differential pulse (DP) polarographic technique is more sensitive than d.c. polarographic one to detect proteins using Brdicka current as described in chapter 5. Fig.7-4 shows DP polarographic Brdicka currents.
Fig. 7-4. Differential pulse polarographic Brdicka currents of 1) \(5.5 \times 10^{-9}\ \text{mol dm}^{-3}\) IgG, 2) \(2.5 \times 10^{-9}\ \text{mol dm}^{-3}\) anti-IgG and 3) \(\Delta i_B\) vs. potential curve observed with a mixture of \(5.5 \times 10^{-9}\ \text{mol dm}^{-3}\) IgG and \(2.5 \times 10^{-9}\ \text{mol dm}^{-3}\) anti-IgG. \(h = 56.0\ \text{cm Hg}, \tau = 3.0\ \text{s}\).

Fig. 7-5. A calibration curve for \((5 \sim 5.5) \times 10^{-10}\ \text{mol dm}^{-3}\) IgG obtained at the anti-IgG concentration of \(2.5 \times 10^{-9}\ \text{mol dm}^{-3}\) by the differential pulse polarographic method.
potential curve in DP polarography has a peak at -1.28 V. Direct titration of anti-IgG with IgG was performed amperometrically at the fixed potential of -1.28 V. Fig. 7-5 shows a $\Delta I_B$ vs. $[\text{Ag}]_0/[\text{Ab}]_0$ curve or a calibration curve at $[\text{Ab}]_0 = 2.5 \times 10^{-9}$ mol dm$^{-3}$. These results reveal that the DP polarographic method based on Brdicka current can be used for detection of IgG as low as $10^{-10}$ mol dm$^{-3}$.

Calibration curves for the determination of antibody, i.e. $\Delta I$ vs. $[\text{Ag}]_0/[\text{Ab}]_0$ plots, can similarly be constructed. Application of this method for detection of hapten may be possible by replacing antigen with hapten in the above calibration curves (Figs. 7-3 and 7-5).

The present polarographic method of analysis based on Brdicka current coupled with antigen-antibody complex formation can be characterized by its considerably high sensitivity. Also this method does not require the labeling of the reagents (antibody or antigen) with radio active isotopes, enzyme or fluorescent molecules, as employed in conventional immunoassay techniques. Co-existing substances, so far as they are Brdicka-inactive, do not interfere with the determination. However, the co-existence of proteins which are indifferent to the interest immunoreaction but Brdicka-active, if present in a large excess, should interfere with the determination.

**SUMMARY**

Brdicka currents of human immunoglobulin G (IgG) and sheep antihuman IgG antiserum (anti-IgG) were studied by d.c. and differential pulse polarographic techniques. The Brdicka current of a mixture of IgG and anti-IgG was smaller than the sum of the currents of IgG and anti-IgG. This difference in current is attributed to the primary complex formation between IgG and anti-IgG. Anti-IgG could be directly titrated with IgG (or *vice versa*) by the polarographic technique based on the difference in Brdicka current. The average intrinsic dissociation constant was
estimated from the titration curve. The polarographic method based on Brdicka current coupled with the immunoreaction can be used to determine trace amounts of antigen (or antibody) as low as $10^{-10}$ mol dm$^{-3}$ using the differential pulse polarographic technique.
In this study, the polarographic catalytic hydrogen current or Brdicka current of proteins was investigated with intent to light on fundamentals and applications of Brdicka current.

In PART I, the author has studied fundamental of Brdicka current. First, basic equation of Brdicka current is presented, in which Brdicka current is expressed by a function of the surface concentration of protein, the bulk concentration of cobalt ion and two parameters, \( n_c \) and \( k_f/k_d \), where \( n_c \) is the number of the total sites, on which the complex can be formed, in a protein molecule, and \( k_c \) and \( k_f/k_d \) are the (average) constants representing the intrinsic catalytic activity and the life time, respectively, of the complex. The theoretical equation has been experimentally verified with respect to the dependence of Brdicka current on the conditions of electrode system as well as the concentrations of protein, cobalt salts. The method to determine the two parameters \( n_c \) and \( k_f/k_d \) has been established.

Brdicka current activities \( k_B = n_c k_c (k_f/k_d) \) or \( n_c k_c \) and \( k_f/k_d \) of SS (or SH) group, heme c group and S-(ethylsuccinimide) group were determined. The difference in their activities between these three groups has been found as mainly due to the difference in the stability \( (k_f/k_d) \).

Effects of ammonia buffer concentration, \( C_{\text{amm}} \), pH and temperature on \( n_c k_c \) and \( k_f/k_d \) values have been investigated. The dependence of \( n_c k_c \) on \( C_{\text{amm}} \) indicated that water molecule as well as ammonium ion participates in the catalytic reaction as a proton donor. The \( k_f/k_d \) value slightly decreases with \( C_{\text{amm}} \), suggesting participation of ammonia molecule in the protein-Co(0) complex formation. The sigmoidal pH-dependence has been found for \( n_c k_c \) and \( k_f/k_d \) values corrected for the effect of buffer salts. This has been interpreted as due to the ionization of functional group (probably sulfhydryl group) in adsorbed protein. With increasing temperature, the \( n_c k_c \) value increases, but \( k_f/k_d \) value decreases. The decrement of \( k_f/k_d \) is larger than the increment of \( n_c k_c \), resulting in the decrease in overall catalytic activity with temperature.
The apparent activation energy of the electrochemical hydrogen evolution reaction has been established.

On the basis of the basic equation of Brdicka current, theoretical expressions of normal pulse (NP) and differential pulse (DP) polarographic Brdicka currents have been derived. These equations well explain the experimental results of NP and DP polarographic Brdicka current.

Pulse polarographic study on Brdicka current has revealed that at the less negative potential than -1.40 V the protein SSI (Streptomyces subtilisin inhibitor) is adsorbed strongly and irreversibly on mercury electrode and the surface concentration is controlled solely by diffusion. At more negative potential than -1.50 V the adsorption of SSI becomes weak with increasing negative potential and the surface concentration is controlled by both diffusion and adsorption. The latter is dependent on the electrode potential.

In PART II, the author has studied applications of Brdicka current. First, DP polarographic technique based on Brdicka current has been developed. Amperometry has been performed by applying a fixed potential of $E_{\text{max}}$ ($= -1.28$ V), which corresponds with the potential of the first maximum of the DP polarographic Brdicka current of the protein. The Brdicka current at $E_{\text{max}}$ is linearly depends on the protein concentration in the range between $3 \mu g \text{ cm}^{-3}$ and $5 \text{ ng cm}^{-3}$. Amperometry under non-deaerated conditions has also been studied, in which protein as low as $20 \text{ ng cm}^{-3}$ can be detected.

Second, Brdicka currents of SSI and PS (plasminostreptin) have been studied by d.c., tast, and DP polarographic techniques and potential sweep voltammetric technique. The Brdicka current of SSI or PS decreased with addition of subtilisin BPN' (S.BPN'), which is attributed to the formation of proteinase-inhibitor complexes. Analysis of the dependence of Brdicka currents of SSI and S.BPN'-SSI complex on cobalt ion concentration revealed that one of two SS bonds of SSI became hardly accessible to Brdicka reaction by the complex formation. SSI and PS could be directly titrated by polarographic technique based on Brdicka current in the concentration level of $10^{-7}$ to $10^{-9}$ mol dm$^{-3}$. 
The first and second dissociation constants of S.BPN'-SSI and S.BPN'-PS complexes as low as $10^{-8}$ to $10^{-10}$ mol dm$^{-3}$ were determined by fitting to the titration data theoretical curves, in which the multiple equilibrium involving microscopically distinct forms of S.BPN'-inhibitor complex was taken into account. The intrinsic free energy change in the first binding of S.BPN' to dimeric SSI or PS was larger than that of the second binding.

The effect of pH on the intrinsic dissociation constants of S.BPN'-PS complex has suggested the participation of ionizable groups with $pK = 8.1$ and 9.4 in the first step and of ionizable groups with $pK = 8.2$ and 9.4 in the second step of binding.

In the last chapter, Brdicka currents of human immunogloblin G (IgG) and sheep antihuman IgG antiserum (anti-IgG) were studied by d.c. and DP polarographic techniques. The Brdicka current of a mixture of IgG and anti-IgG was smaller than the sum of the currents of IgG and anti-IgG. This difference in current is attributed to the primary complex formation between IgG and anti-IgG. Anti-IgG could be directly titrated with IgG (or vice versa) by the polarographic technique based on the difference in Brdicka current. The average intrinsic dissociation constant was estimated from the titration curve. The polarographic method based on Brdicka current coupled with the immunoreaction can be used to determine trace amounts of antigen (or antibody) as low as $10^{-10}$ mol dm$^{-3}$ using the DP polarographic technique.
ACKNOWLEDGEMENTS

The author wishes to express his deep gratitude to Dr. Mitsugi Senda, Professor of Kyoto University. His scientific insight, patience and personal concern have been invaluable contributions to this research. The author is also grateful to Dr. Tokuji Ikeda, Associate Professor of Kyoto University for his continuous guidance and valuable advice in carrying out this work.

It is a great pleasure to acknowledge the interest and discussion on S.BPN'-SSI interaction of Dr. Keitaro Hiromi, Professor of Kyoto University and Dr. Ben'ichiro Tonomura, Associate Professor of Kyoto University, and the encouragement of Dr. Tanekazu Kubota, Professor of Gifu College of Pharmacy.

The author is grateful to Dr. Sawao Murao, Professor of Osaka Prefecture University and Dr. Keitaro Hiromi for their kind gift of SSI preparation, to Dr. Atsushi Kakinuma, Takeda Chemical Industries, Ltd., for his kind gift of PS preparation, and to Osaka Research Laboratory of Wako Pure Chemical Industries, Ltd. for the kind gift of IgG and anti-IgG preparations.

Thanks are due to Mr. Ichiro Tokimitsu and Miss Sachiko Ibe for their many helpful collaborations. The kind suggestions and the continuous encouragements by Dr. Tadaaki Kakutani, Dr. Takashi Kakiuchi, Dr. Hiromichi Morikawa and Dr. Junko Takeda, instructors of Kyoto University, and Dr. Hideaki Kinoshita, Professor of Kwassui Women's College, during this work are gratefully acknowledged.

The author is indebted to members of the Laboratory of Chemistry and Physics of Biopolymer, Department of Agricultural Chemistry, Kyoto University for their helpful suggestions and discussion.
REFERENCES

10) M.Senda and T.Ikeda, Bunseki, 1977, 780.
64) H.Sugino, N.Morira, S.Nakagawa, A.Kakinuma, M.Isono, S.Iwanaga,
69) Y.Mitsui, Y.Watanabe and S.Hirono, Tanpakushitsu Kakusan Koso, 24, 96 (1979).
i) K.Kano, S.Ibe, T.Ikeda and M.Senda, to be submitted for publication.