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Potentiometric Studies of Selective Reactions of Bioactive Substances on the Surface-Modified Tantalum Electrodes

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ABSTRACT

The method of highly selective potentiometric detection of bioactive substances developed by us has been much improved by using tantalum metal as a electrode material, by employment of a new method of modification (using γ-aminopropyltriethoxysilane and cyanogen bromide), and by the pretreatment with inert protein before the measurement. As the consequence, the response in the electrode potential shift has been increased and the reproducibility of the data reached almost 100%.

Index Entries: Potentiometry; trypsin; aprotinin; biosensor; surface potential; surface modification; electrode potential; immunoassay; enzyme assay.

INTRODUCTION

In several of our previous reports, we have described a new method for the detection of biologial substances that are capable of selectively reacting with other biological substances, e.g., antigen, antibody, enzyme, enzyme inhibitor, and so on. The method comprises the measurement of the change of the electric potential between a metal electrode surface-modified with a chemically bound bioactive substance and a reference electrode in an aq. solution, induced by the reaction of the bioactive substance with

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the reactive counterpart added in the solution (*1–6*). For example, a titanium electrode whose surface was modified with anti-hCG (antibody to human chorionic gonadotropin) and immersed in a buffer solution caused a selective response on the addition of hCG. The electric potential against the reference electrode changed gradually until it reached a constant value. The potential changed with time following the kinetic curve, from which the concentration of the added substance could be determined. Thus, the method turned out to be useful for the quantitative detection of biological substances with a fairly high sensitivity. A similar change was also studied for trypsin and its inhibitor (aprotinin). The properties of some other metal electrodes and other modification techniques were also studied.

Although this is a unique method that appears to be highly promising, a number of improvements were needed for its employment in clinical technology. The most important point seemed to be that one should minimize the erroneous responses of nonselective adsorption of various substances other than the substance to be detected on the working electrode as well as on the reference electrode. In the present paper, we have studied the methods to eliminate errors arising from this effect. This and some other refinements have led to a notable improvement of this potentiometric method of analyzing biological substances in the selectivity and reproducibility.

EXPERIMENTAL

Trypsin (Type III, 11,680 SU mg^{-1}) and aprotinin (10 TIU mg^{-1}) from Sigma Chemical Co., bovine serum albumin (BSA) from Teikoku Hormone Mfg. Co., cyanogen bromide (95%) from Wako Pure Chemicals Co., and γ-aminopropyltriethoxysilane (APTES) from Shin-etsu Silicon Co. were used. Reagent grade chemicals were used for all other materials.

The experimental setup for the potentiometric measurements is shown schematically in Fig. 1. Tantalum purchased from Japan Lamp Industrial Co. was used as electrode materials. The tip of the electrode (d in Fig. 1) is a tantalum cylinder 4 mm in diameter into which a tantalum rod 2 mm in diameter (a) was screwed in. The rod was put into a poly-chlorotetrafluoroethylene pipe (c) and tightened with a stainless steel nut (b) as shown in the figure.

The surface modification of the electrode was carried out as follows: The tantalum tip was polished with 1 μm alumina abrasive (Furuuchi Chemical Co.), washed by ultrasonification in water for 15 min, etched with 48% hydrofluoric acid for 30 s, and then immersed in 1*M* (mol dm^{-3}) sodium hydroxide solution for 1 h in order to make a thin oxide layer. The electrode thus treated was then immersed in a 10 vol % aq. solution of APTES adjusted to pH 7 with hydrochloric acid at 50°C for 2 h. The electrode was then chemically activated in a stirred mixture of a 60 mL, 2*M* aqueous solution of sodium carbonate with 5 mL of a 0.5*M* acetonitrile

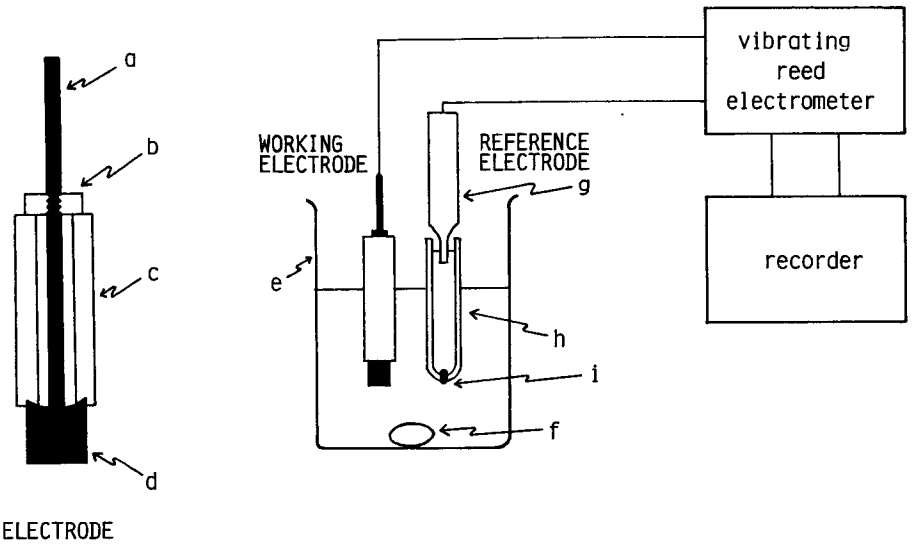

Fig. 1. Schematic illustration of the experimental setup for the potentiometric measurement.

solution of cyanogen bromide for 90 s (5). After washing in a 0.02*M* sodium bicarbonate solution, the enzyme immobilization was carried out immediately by gently stirring for 1 h in a pH 8.6 barbital buffer solution containing 2.5 mg/mL trypsin and 0.02*M* calcium chloride. The electrode was then rinsed in a pH 8.0 phosphate buffer solution and stored in a box at 4°C. We will hereafter call such an electrode a "new" electrode.

For the purpose of comparison, trypsin modification was carried out by the method employed in our previous work as follows: The tantalum electrode whose surface was oxidized by the similar method as above was placed in a stirred aq. solution of cyanogen bromide for 20 min, then in a pH 8.6 barbital buffer solution containing 0.1 mg/mL trypsin for 1 h, and stored at 4°C. We will call such an electrode an "old" electrode.

The trypsin-modified electrode and the reference electrode were placed in a small glass container shown in Fig. 1 as (e) having a 30 mL, 0.01*M*, pH 8.0 phosphate buffer solution, kept at room temperature, and stirred with a magnetic stirrer (f). The reference electrode consists of an Ag/AgCl electrode (g) connected to a salt bridge tube (h) filled with the same buffer solution that is in contact with the cell solution through a piece of porous alumina ceramic (i) (Multiporon AVD from Mitsui Kensaku Toishi Co.) having an average pore size of 1 μm fused by heating with a burner to the bottom of the salt bridge tube.

The static potential between the electrodes were measured with a TR-8411 vibrating reed electrometer of Takeda Riken Industry Co. The Auger electron spectroscopic measurements were made by use of a PHI Model 10-155 cylindrical mirror analyzer, together with a lock-in amplifier at frequency 2 kHz. A 3 keV electron beam at 2 μA and with the spot size of 100 μm was used as excitation.

RESULTS

The Improved Reference Electrode

As already mentioned, the effect of nonselective adsorption on the reference electrode surface was a confusing factor. In our previous work, we used a reference electrode made of the same metal as employed for the working electrode, and in order to avoid the effect of nonselective adsorption, we modified its surface with urea or bovine serum albumin (BSA). However, the annoying effect of the nonselective adsorption owing to the added protein was not completely avoided. In the present work, we therefore adopted an Ag/AgCl reference electrode and a salt bridge as shown in Fig. 1. With this setup, the adsorption of proteins on the electrode during the measurement can be avoided. However, the question still remained whether the protein adsorption in the ceramic pore might have caused a membrane potential affecting the potential measured. In order to check this, we made two identical sets of reference electrodes each connected to salt bridges, measured the potential between them in a 0.01*M* phosphate buffer at pH 8, then immersed one into a protein solution for a while, washed it, put it back into the buffer solution, and immediately measured the potential against the other electrode. In this way we confirmed that the protein adsorption caused negligible effect on the potential of the reference electrode.

The Examination of the Effect of Nonselective Adsorption on the Working Electrode

By use of this reference electrode, we are now in a position to elucidate the factors affecting the interfacial potential of the working electrode. Table 1 shows the equilibrium potentials of tantalum electrodes against the reference electrode in a phosphate buffer solution and the change observed by adding aprotinin into the solution. Bare tantalum electrodes showed equilibrium potentials ranging between −300 and −350 mV in

Table 1
The Equilibrium Potentials of Various Tantalum Electrodes Against the "New" Reference Electrode in a Phosphate Buffer Solution of 0.01*M*, pH 8.0 and Their Changes Caused by the Addition of Aprotinin, △U

Electrode	Equilibrium potential, mV vs Ag/AgCl	△U, mV
Bare	−300∿−350	35∿44
Modified with APTES	−220∿−260	3∿5
Modified with BSA[a]	−370∿−400	3∿5
Modified with trypsin[a]	−400∿−500	11∿15

[a]The tantalum electrode modified with BSA or trypsin by the method used for the "new" electrode.

the buffer solution, and a shift of 35 to 44 mV by addition of aprotinin in the solution. This shift, presumably caused by the adsorption of aprotinin, is indeed large, showing that it is substantial to keep the bare spots on the electrode surface minimal.

As Table 1 also shows, the tantalum electrode modified with APTES or the electrode modified with APTES and BSA, caused much smaller shifts in potential by the addition of aprotinin. This, however, indicates that the potential of a tantalum electrode modified by these substances are still affected by the nonselective adsorption of a protein. Finally, Table 1 shows that the electrode modified with APTES and trypsin caused shifts of 11 to 15 mV by the addition of aprotinin, indicating the order of magnitude of the shift induced by the specific reaction between trypsin and aprotinin. It should be added that this shift is much higher than those observed for the trypsin electrodes studied in our previous work. This result indicates that the trypsin coverage on the electrode surface has been much enlarged in the "new" electrode than in the "old" ones.

Auger Studies of the Modified Electrode Surfaces

Figure 2 shows a typical Auger spectrum of a "new" trypsin-modified tantalum electrode. It gives two strong signals assigned to C and O atoms, and small signals assigned to Si, Ta, and N, indicating that the tantalum

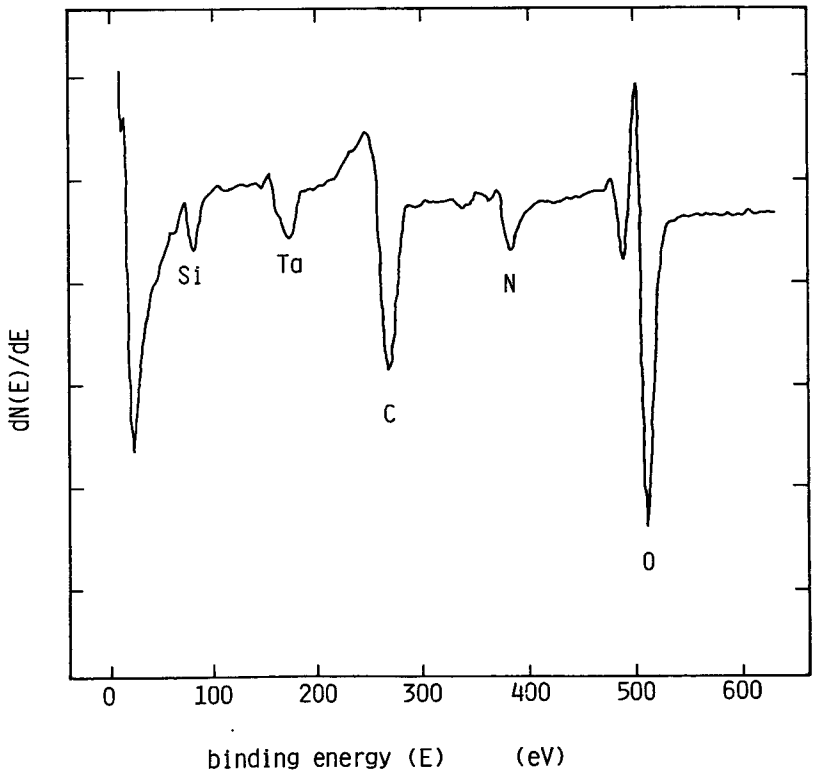

Fig. 2. The Auger spectrum of a "new" electrode.

surface is mostly covered by APTES and the protein. The tantalum signal here is about one-third that of a bare Ta as seen from Table 2. As the escape depth of an electron is 0.5–0.75 nm (*7*) and the mean diameter of trypsin is about 4.0 nm, it is concluded that there are some bare parts still existing on the metal surface. When the trypsin-modified electrode was treated in a solution of BSA, washed, and dried, the Ta signal was further weakened and the N signal was strengthened as shown in the third row of Table 2, indicating that BSA was adsorbed to some extent. By treating the trypsin-modified electrode with aprotinin, a further decrease of the Ta signal and an increase of the N signal were observed as seen in the fourth row, indicating that the bare part was more thoroughly covered by the complexing of aprotinin with trypsin.

The Measurement of Electrode Potential

The above results have shown that BSA treatment of the trypsin-modfied electrode is effective in reducing the bare part of the Ta surface. Now let us see how this affects the potential. Figure 3 shows, for a typical case, the change of the electric potential between the working electrode and the reference electrode. Although Curve A for the "old" electrode makes a large response to the addition of BSA and a small response to the addition of aprotinin, Curve B for the "new" trypsin electrode shows a less response to BSA and more of a response to aprotinin. This is, of course, a good result indicating that the new electrode is more profoundly covered with trypsin.

Figure 3 also shows that the "new" trypsin electrode treated with BSA gives no response to inactive materials, for example, by addition of glycine to the final concentration of $1 \times 10^{-5} M$, addition of IgG to $2.2 \times 10^{-8} M$, and so on, or further addition of BSA, but it shows a very large response to aprotinin. Thus, the "new" electrode with the pretreatment with BSA have shown much improved behavior as an aprotinin detector.

It is to be noted also that the "new" electrodes have shown ca. 90% reproducibility, a value much higher than those for the "old" electrodes. Moreover, it was shown empirically that the trypsin electrodes that showed

Table 2
The Relative Atomic Concentrations of Tantalum Electrodes Normalized with Respect to the Trypsin-Modified Electrodes

Electrode	Ta	C	N
Ta, bare	2.88	0.23	0
Ta-Try[a]	1	1	1
Ta-Try-BSA[b]	0.66	1.04	1.10
Ta-Try-Apro[c]	0.52	1.27	1.27

[a]A "new" trypsin-modified Ta electrode.
[b]The same as [a] treated with a BSA solution.
[c]The same as [a] treated with an aprotinin solution.

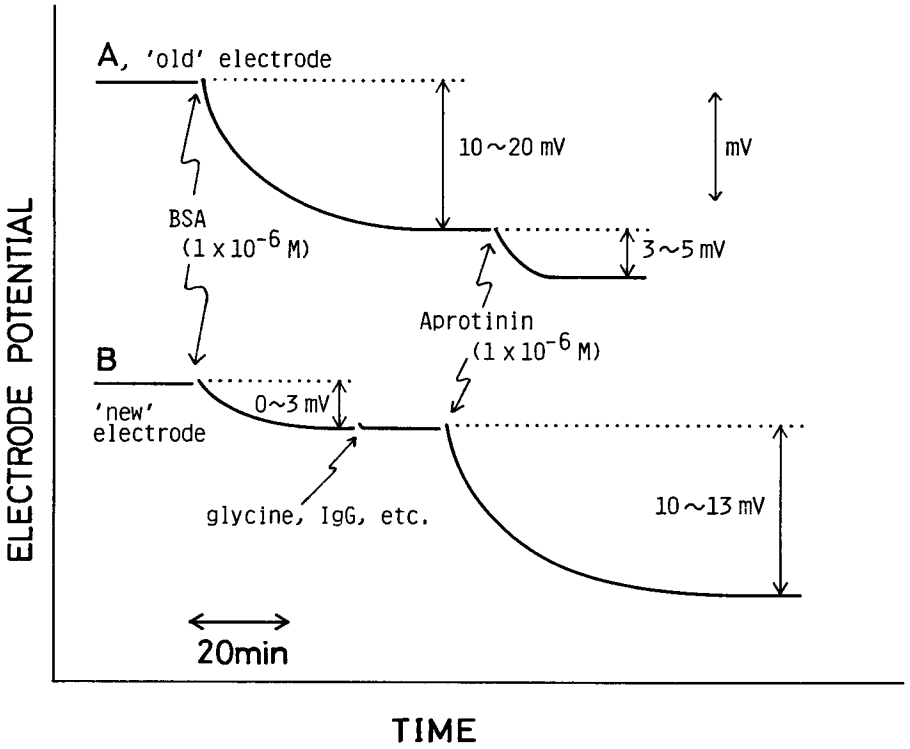

Fig. 3. The change in potential by addition of BSA, aprotinin, and so on to the "old" (A) and "new" trypsin-modified tantalum electrodes.

an equilibrium potential against the reference electrode in the range of −400 to −500 mV behaved properly on addition of aprotinin. In other words, if we exclude the trypsin electrodes having potentials against the reference electrode outside the above mentioned range, the reproducibility became much better. Sets of electrodes (each 30–40 in number) in most cases behaved correctly without exception.

The Kinetic Analysis of the Electric Response

It was reported that the dissociation constant (K_d) for the trypsin-aprotinin complex in solution was extremely small, ca. $3\times10^{-11}M$ at pH 7.8 (*8*). This means that the reaction is almost irreversible. Hence, we can express the reaction by the equation below

$$A + B \xrightarrow{k} C \qquad (1)$$

where A denotes trypsin chemically bound on Ta surface, B is aprotinin in the solution, C is the complex fixed on the surface, and k is the rate constant. By assuming that the potential shift is proportional to the surface concentration of the complex on the electrode surface, the change $U(t)$ at time t after addition of aprotinin is expressed as follows (*1,3*)

$$U(t) = U_{max}[1 - \exp(-k[B]_0 t)] \qquad (2)$$

where $[B]_0$ is the initial concentration of aprotinin, and U_{max} is the maximum, or the limiting, value of the potential change. In Fig. 4, the $\ln[U_{max} - U(t)]$ values are plotted vs time for a typical run, giving a good straight line in accordance with Eq. 2. By use of the least square method, k was determined to be $1.36 \times 10^3 M^{-1}s^{-1}$. This is a value much smaller than the reaction of trypsin and aprotinin in aq. solutions $3.05 \times 10^5 M^{-1}s^{-1}$ (*8*).

From Eq. 2, it is understood and empirically confirmed that the response becomes weak and dull as the reactant concn. gets lower. But the presence of aprotinin was detectable at concentrations as low as $1 \times 10^{-8} M$ or even lower, though the quantitative analysis at such a low range is difficult.

The Regeneration of the Electrode

The activity of the trypsin electrode, once used for the aprotinin detection, can be recovered to some extent. Table 3 shows some of the results. In the first row, the responses of a "new" trypsin electrode to BSA, and then to aprotinin are given. After the measurement, the electrode was immersed in a 1*M* hydrochloric acid solution for 90 s. The elec-

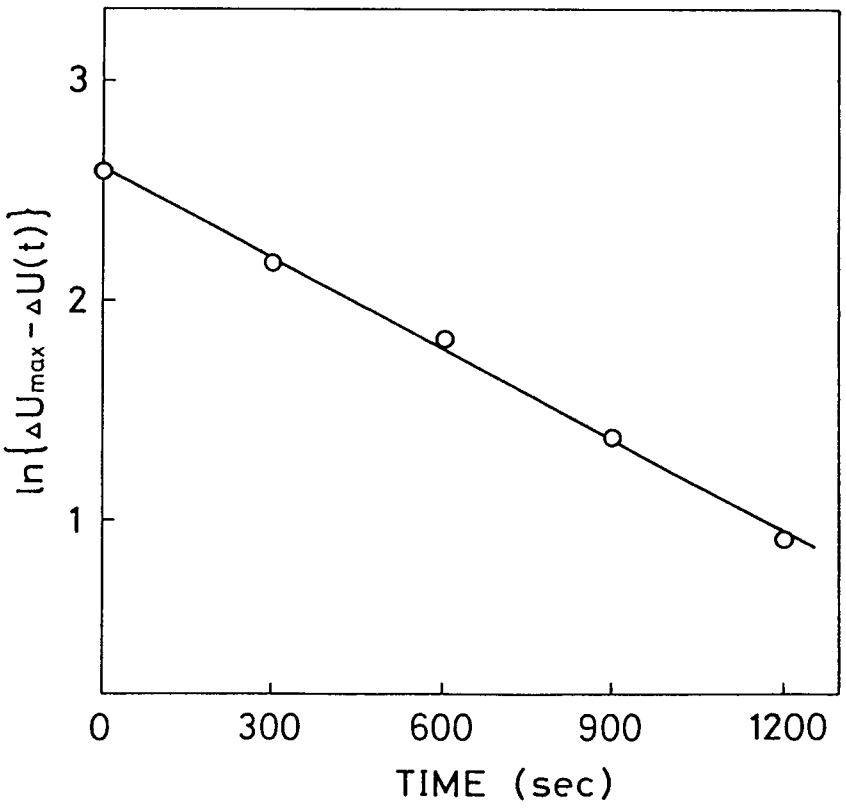

Fig. 4. The relation between the change of potential of the "new" trypsin electrode with time.

Table 3
The Limiting Value of the Shift of Potential in the "New" Trypsin Electrode and Those Reactivated by Hydrochloric Acid Solution

response, mV		
to BSA	then to aprotinin	rate constant ($10^3 M^{-1}sec^{-1}$)
3.2[a]	13.2[a]	1.36
1.5[b]	10.2[b]	1.35
6.5[c]	3.4[c]	1.24

[a] A "new" trypsin electrode.
[b] After measurement of [a], the electrode was treated with HCl.
[c] After measurement of [b], the electrode was treated with HCl again.

trode activity was then recovered, showing a small response to BSA and a response to aprotinin slightly less than that of the original electrode as indicated in the second row. However, as shown in the last row, when this electrode was again treated with hydrochloric acid, the response to aprotinin became much less and the response to BSA was larger. This indicates that a large portion of the fixed trypsin was removed from the surface by the second treatment. In all cases, the rate constants (K_a) obtained were nearly the same, indicating that the trypsin was not much denatured by the present method of treatment with hydrochloric acid.

DISCUSSION

It is known that the surface of tantalum is covered by a thin oxide layer that is very stable in a wide range of pH. Lerner et al. (*9*) reports that tantalum electrodes used in vivo are stable and give reproducible electrode potentials. In our work, we have found also that tantalum electrodes are more stable and give more reproducible results than the metals previously used by us.

As already discussed in our previous papers, the electric potential response of the working electrode, by the addition of the substance to be detected, is most probably brought about by the surface potential owing to the electrical double layer formed by the reaction between the bioactive substance attached on the working electrode surface and the substance to be detected dissolved in the solution. Figure 5 schematically shows the structure of the surface of the electrode modified with substance A and its complexing reaction with another substance B dissolved in the solution. The bonding between A and B will, in general, cause the charge transfer between the two substances or the dipole alignment leading to the forma-

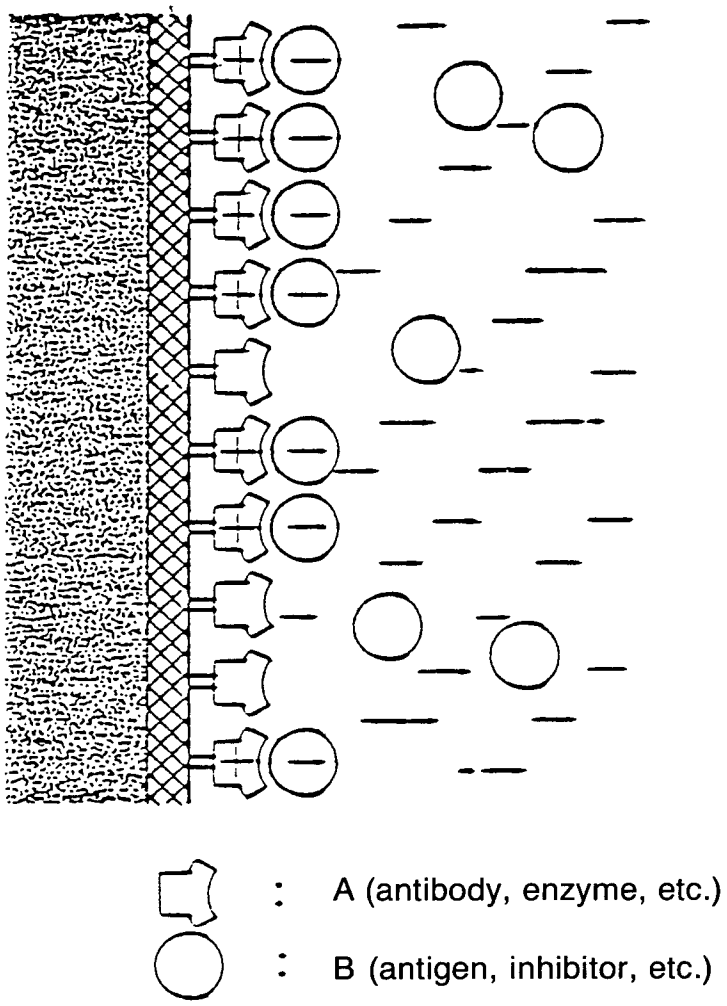

Fig. 5. Schematic illustration of the electrode modified with bioactive substance A and its complexation with B dissolved in the solution.

tion of an electrical double layer that in turn causes a change in the electrode potential.

From this model, it is deduced that the surface potential change must be proportional to the number of the complex in a unit area of the surface, N_p, which changes with time by the second order reaction kinetics as follows

$$dN_p/dt = k(N_{Ao} - N_p)[B]_0 \quad (3)$$

where N_{Ao} and $[B]_0$ designate the initial number of free A in a unit area of the surface and the initial concentration of B in the solution, respectively, and k is the rate constant. It is easy to prove that this equation leads to Eq. 2 for the potential change with time if the concentration of B is assumed to be unchanged within the reaction time.

The above model also predicts that, if there are naked spots on the electrode surface, proteins or other substances existing in the sample solution as impurities will readily be adsorbed and cause undesirable contribution to the surface potential. One can understand, therefore, that it is important to cover the whole electrode surface with the active substance and make the naked parts as small as possible.

Regarding the method of modification of the metal surface, we had employed cyanogen bromide for activation in most cases of our previous work. In this method, a cyanogen bromide molecule needs two functional groups (OH, NH_2, and so on) adjacent to each other. Therefore, a high concentration of these functional groups are necessary for covering a large part of the metal surface. For this reason, we had tried various methods to increase the functional groups, namely thermal oxidation or radiofrequency plasma treatments, and so on (*6*). However, all these trials were found to be not fully satisfactory. In the present work, we employed the method in which the tantalum electrode was once modified by APTES, which reacted not only with the OH groups on the metal surface but also with the OH groups of other APTES molecules, forming a condensation polymerization products. This caused more extensive coating of the metal surface as well as a more abundant supply of functional groups (OH and NH_2), leading to thicker coating of the metal surface with the working substance, trypsin. The effect of this is evident from the smaller electric response of the trypsin-modified electrode to an inert protein, BSA, and a higher response to aprotinin.

The results of electrode potential and Auger spectroscopic measurements, however, have shown that the Ta electrode, modified by the present method, still has naked spots and is incomplete for avoiding the erroneous electric response arising from the nonspecific interaction of substances other than that to be detected. Then we discovered that pretreatment of the working electrode and the reference electrode with a solution of an inert protein, BSA for example, is effective. BSA is then adsorbed on the naked part of the electrode surface and gives a small shift of the potential. After this treatment, aprotinin to be detected is selectively attached to the reacting points of trypsin and consequently causes a specific change in the electrode potential. This pretreatment has been very successful in reducing the error arising from nonspecific adsorption of proteins and other substances on the naked part of the working electrode as well as the reference electrode.

As the result, the reproducibility of the measurements with the "new" electrodes was much improved, approaching near 100%.

It has also been found that the electrode has to some extent a regenerating capability by using hydrochloric acid, although it is deteriorated by repeated use. For a practical purpose, the discovery of a better reagent for the dissociation of the protein complexes is desirable.

The above model, as well as Eq. 3, also indicates that the change of the potential becomes slower, the lower the concn. of the protein to be detected, until the potential change becomes hardly detectable. This limit-

ing concn. defines the sensitivity of this method. As the noise of the potential curve is very small, the detectable limiting concn. is fairly small, as already described in the results section.

In conclusion, the new techniques described here have been successful in improving the stability and reproducibility of the potentiostatic method of detecting bioactive substances. Although the present result was obtained for the trypsin–aprotinin reaction, this method will certainly be expanded to other substances, like antigens, antibodies, hormones, and so on.

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