A Mediated Amperometric Enzyme Electrode Based on Nucleoside Oxidase and its Applications

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ヌクレオシドオキシダーゼを固定したベンゾキノン含有グラファイトペースト電極およびその応用

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Abstract

Nucleoside oxidase, which catalyzes the oxidation of nucleoside to the corresponding 5'-carboxylic acid with forming hydrogen peroxide, was purified to homogeneity from cells of *Flavobacterium meningosepticum.* This enzyme was immobilized onto the surface of the graphite paste electrode containing benzoquinone and was covered with a dialysis membrane. This enzyme electrode produced biocatalytic current in buffer solutions of pH 6-10. Responses by adenosine, guanosine and inosine were the same and the magnitudes of responses by uridine, cytidine and thymidine were 4 / 5 as active as those by inosine. This electrode did not respond to bases, sugars and nucleotides. The activity of 5'-nucleotidase in serum was determined easily by this electrode and the content of nucleotides in food also determined by the use of nucleotidase. These methods were based on the measurement of the increase in anodic current by the nucleoside that was released enzymatically, and so were not interfered with by the oxidizable compounds in a sample.

Introduction

Nucleoside oxidase (NOD), which catalyzes the oxidation of nucleoside to the corresponding 5'-carboxylic acid with forming hydrogen peroxide, was obtained from *Flavobacterium* 【1】.

This enzyme is expected to be a reagent for the analysis of nucleosides. An NOD -immobilized graphite paste electrode containing benzoquinone or ferrocene produced the biocatalytic anodic current in the presence of nucleosides. Recently, electrochemical behaviors and applicatons of a mediated amperometric enzyme electrode such as this one have been reported 【2, 3, 4】. This type of an electrode can be prepared very easily and has the advantage that dehydrogenase can be used as well as oxidases as the enzyme to be immobilized. Also, the current resulting from the bioelectrocatalytic oxidation of the substrate in the presence of an electron transfer mediator is independent of oxygen tension in the test solution. The different type of NOD was obtained from *Pseudomonas maltophilia* 【5】 and was immobilized onto the graphite paste electrode containing benzoquinone 【6】. At this electrode NOD has laccase-like property in the presence of nucleoside and benzoquinone was used the different way from the present method.

This paper describes the fundamental properties of an NOD electrode, as well as the applications of this electrode for the assay of nucleotidase in serum, and for the determination of nucleotide in foods.

Experimental

Reagent and apparatus

Nucleoside oxidase (NOD, molecular weight 4.8×10^5, isoelectrolic point 5.1) was purified to homogeneity from the cells of *Flavobacterium meningosepticum* 【1】 The specific activity of the preparation was 2.2 U ml^{-1} (0.23 mg of protein ml^{-1}). Benzoquinone (BQ) was purchased from Nakaraitesque Co. and was purified by sublimation. Graphite powder, paraffin liquid and dialysis membrane (thickness is 20 μm in the dry state) were purchased from Nippon Kokuen Co., Merck Co. and Union Carbide Co., respectively. 5'-Nucleotidase from *crotallus atrox* venom was purchased from Sigma Co.. Bases, nucleosides and nucleotides were purchased from Kojin Co.. A Yanagimoto Polarographic Analyzer P1100 was used to apply the potential to a two -electrode system and currents were measured with a Watanabe X(t)-Y recorder WX4421.

Preparation of an electrode

10 μl of NOD preparation was syringed onto the surface (0.07 cm^2) of the graphite paste electrode containing BQ. The solution was allowed to evaporate in a

refrigerator and the surface of an electrode was covered with a round-cut dialysis membrane. The membrane was covered further with a nylon net (material of women's stockings) and the nylon net was fixed with parafilm at the side of an electrode [7]. An electrode prepared in this way is referred to below as an NOD electrode.

Electrochemical measurement

Tris buffer was used as a base solution. The 2.5 ml of test solution in an H-type cell was stirred at 600 rpm with a magnetic stirrer. Potentials were measured against SCE.

Results and discussion

An NOD-immobilized graphite paste electrode did not produce appreciable electrtolytic current in the presence of nucleoside. This shows that a direct electron exchange between the electrode and the immobilized enzyme did not occur. An NOD electrode containing benzoquinone (BQ) or ferrocene in graphite produced biocatalytic anodic current in the presence of nucleoside (Fig. 1).

The occurence of this current is explained below. Nucleoside moved to the NOD layer through the membrane from the solution and was converted to the oxidized

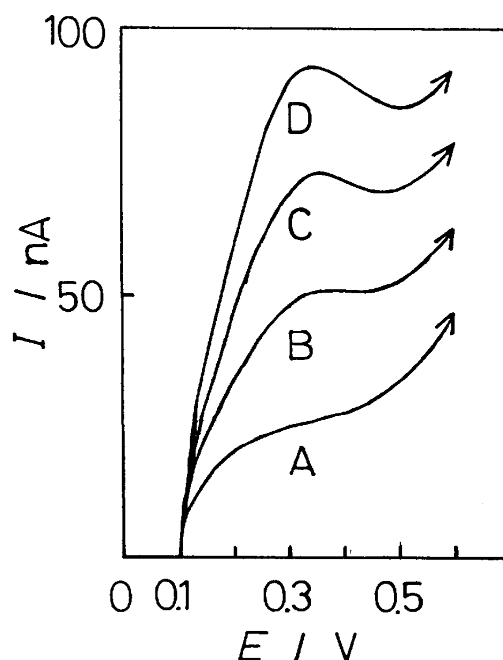

Fig. 1 Voltammograms by tris buffer, pH7.1, (A) and buffer solution containing 10 (B), 20 (C) and 30μM adenosine (D) at 20mV $s^{-1}$and 25°C with an NOD electrode.

product by an enzymatic reaction. At the same time, the oxidized enzyme converted to the reduced form and an oxidized mediator such as BQ functioned as the electron acceptor of reduced enzyme. The resulting reduced mediator might be oxidized at the electrode 【4】.

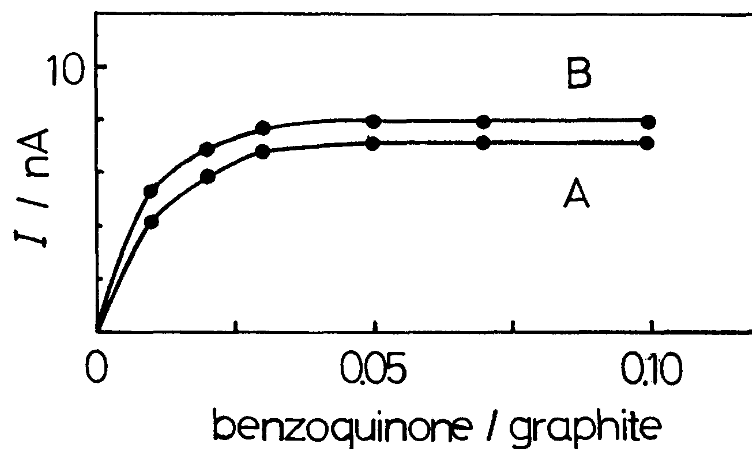

Fig. 2 Dependence of biocatalytic current by 10 μM adenosine at 0.2V (A) and 0.4V (B) on the concentration of benzoquinone with an NOD electrode.

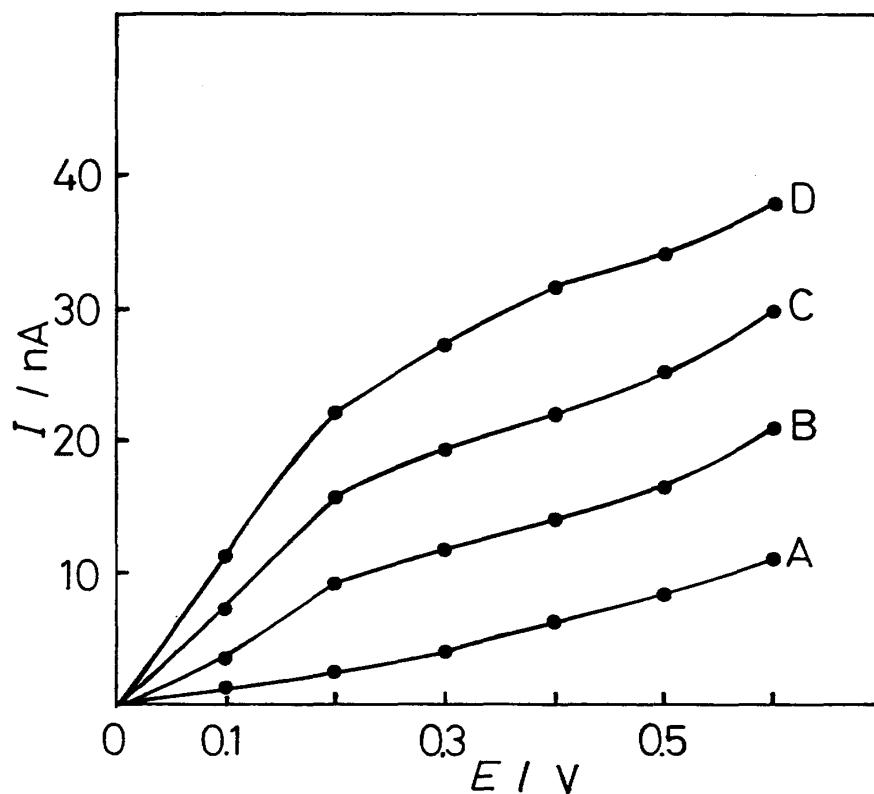

Fig. 3 I—E plots of tris buffer, pH7.1, (A), and buffer solution containing 10(B), 20 (C), and 30μM adenosine (D) at 25°C with an NOD electrode.

With the increase in the amount of BQ in an NOD electrode, the biocatalytic current at a steady-state in the presence of 10 μM adenosine increased and approached a saturation at BQ : graphite = 2.5 : 100 (Fig. 2). The magnitude of base current and the time to reach a steady-state increased with the increase in BQ in the graphite paste. So, an NOD electrode containing 3 % of BQ in graphite was used afterwards.

Figure 3 shows the dependence of the current at a steady-state *(I)* by tris buffer of pH7.1 (A) and buffer solutions containing 10 (B), 20 (C), and 10 μM (D) adenosine on the potential *(E)* with 0.22 unit NOD-immobilized BQ (3 %) electrode. In the potential range more positive than 0.05V, an appreciable biocatalytic current at a steady-state was observed. The net current due to nucleoside approached a saturation near 0.3V at pH7.1.

Figure 4 shows the dependence of the current at 0.4V on the concentration of adenosine. A linear relationship between the current and the concentration of nucleoside was observed ranging from 2 to 30 μM. With the increase in the amount of immobilized NOD, the current due to nucleoside increased. The linear portion between

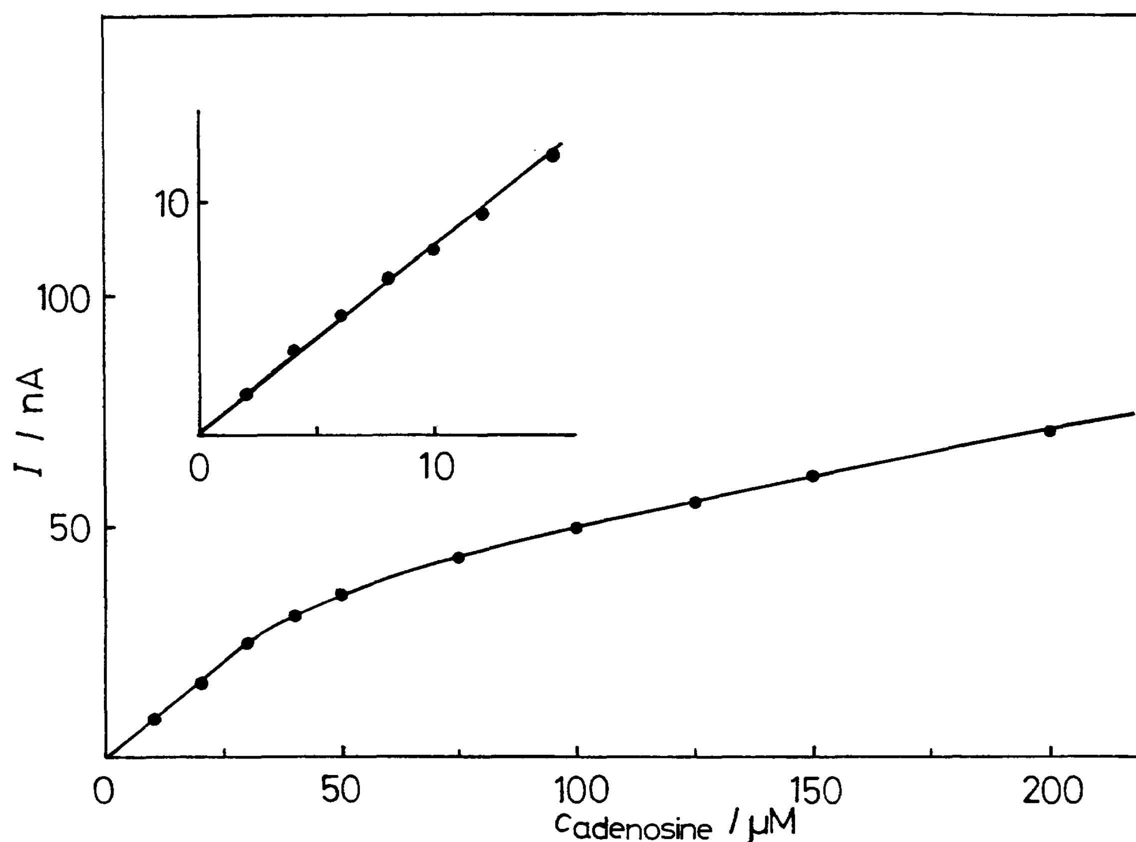

Fig. 4 Dependence of biocatalytic current at 0.4 on the concentration of adenosine in tris buffer, pH7.1, at 25°C with an NOD electrode.

the current and the concentration of nucleoside increased as well. RSD for the magnitude of the current by 10 μM adenosine at 0.4V and pH7.1 was 2.0%. Magnitude of current by deoxygenated solution was almost the same to that by ordinary solution.

The current response by various nucleosides was examined. The ratio of current value at a steady-state by 10 μM substrate solution to that by 10 μM inosine solution is shown in Table 1. Responses by inosine, adenosine and guanosine which contain purine base are the same. Responses by uridine, thymidine and cytosine are the same and the magnitude in response was four fifths as active as that by inosine. Responses by bases, nucleotides, ribose and deoxyribose were not observed. Substrate specificities of NOD for these compounds measured by a photometric method are also shown in Table 1. The two methods nearly agreed.

Table 1 Substrate speclficity of an nucleosidase oxidase-immobilized qraphite paste electrode containing benzoquinone.

Substrate	Relative activity / %	
	By present method*	By photometric method
Inosine	100	100
Adenosine	98	109
Guanosine	95	103
Uridine	81	88
Cytidine	82	50
Thymidine	80	98
Adenosine 2'-phosphate	0	0
Adenosine 3'-phosphate	0	0
Adenosine 5'-phosphate	0	0
Inosine 5'-phosphate	0	0
Adenine	0	0
Guanine	0	0
Uracil	0	0
Cytosine	0	0
Thymine	0	0
D-Ribose	0	0
2-Deoxy-D-ribose	0	0

* current was measured at 0.4V and 10 μM substrate in pH 7.1.

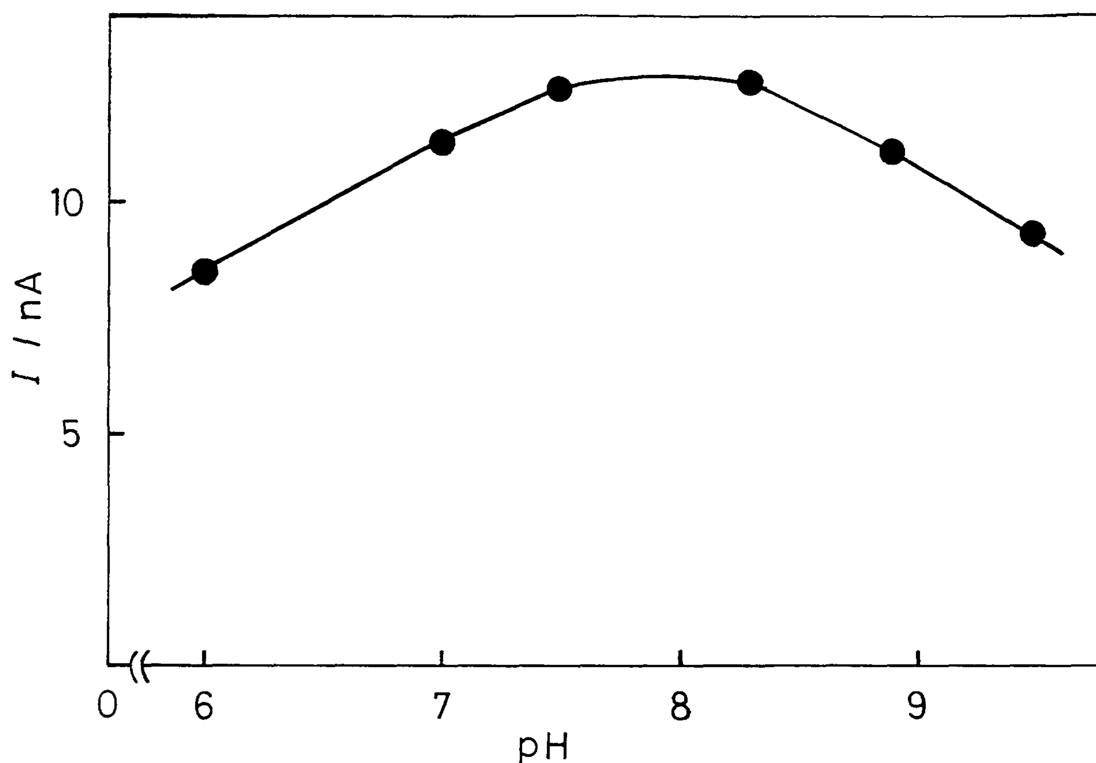

Fig. 5 Dependence of biocatalytic current at 0.3V on pH of solution containing 18 μM inosine at 25°C with an NOD electrode.

Figure 5 shows the dependence of biocatalytic current by 18 μM inosine at 0.3 V and 25°C on pH of the test solution with an NOD electrode. Optimum response was observed in the solution of pH8. Appreciable currents were observed in the pH range from 6 to 10.

The effect of temperature on the biocatalytic current by 20 μM adenosine solution of pH 7.1 was examined (Fig. 6). Figure 7 shows the Arrhenius plots of the result shown in Fig. 6 , where the magnitudes of currents are expressed as the relative value to that at 25°C. Plots give a straight line, showing an apparent overall activation energy of 29 KJ mol^{-1}.

Figure 8 shows the $I-t$ curves for successive additions of 60 μM inosine 5' -monophosphate and nucleotidase from crotalus atrox venom. Current increase in first two min is proportional to the amount of enzyme.

Figure 9 shows the $I-t$ curve for successive additions of serum and inosine 5' -monophosohate at pH 7.5 (A) and 8.3 (B). At first the anodic current by uric acid, ascorbic acid, and so on was observed and reached a limiting value in 3 min. This current decreased with the decrease of pH and almost disappeared in the pH range

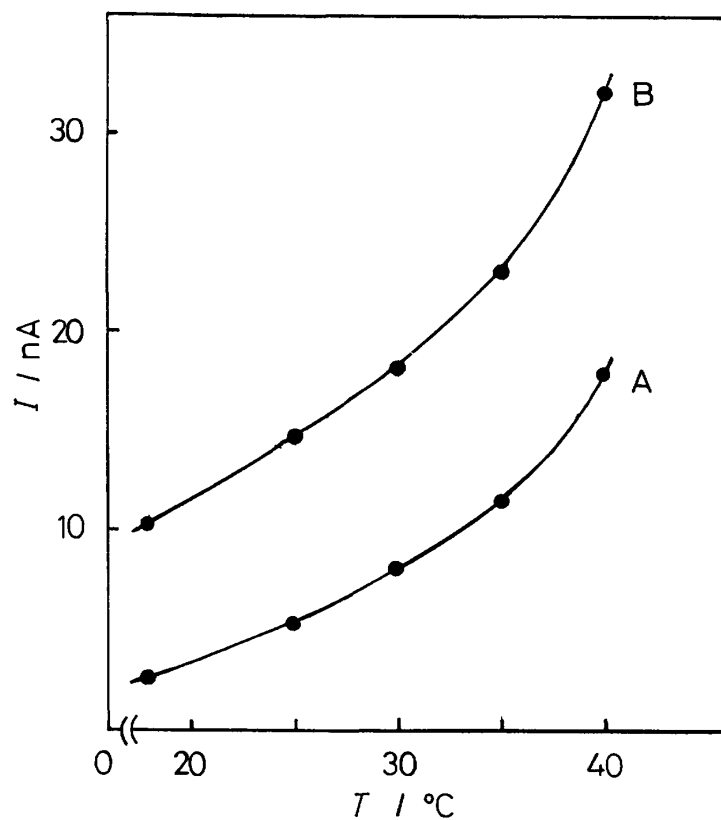

Fig. 6 Dependence of magnitude in current at 0.4V by tris buffer, pH 7.1, (A) and buffer solution containing 20 μM adenosine (B) on the temperature with an NOD-BQ electrode.

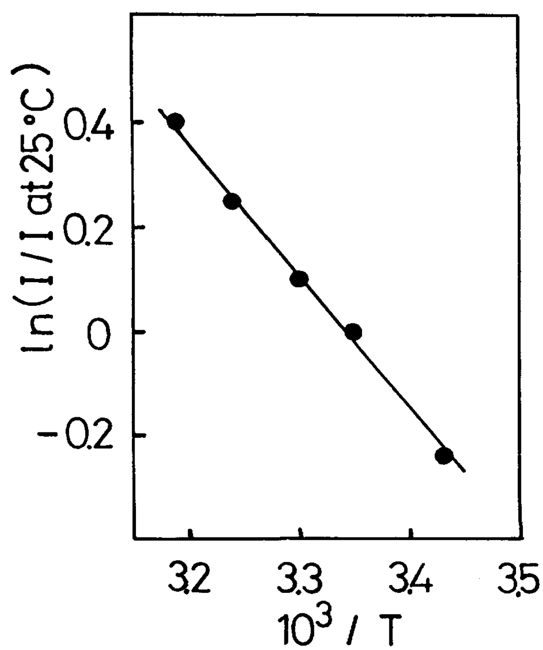

Fig. 7 Arrhenius plots of the biocatalytic current of 20μM adenosine in pH7.1 with an NOD electrode.

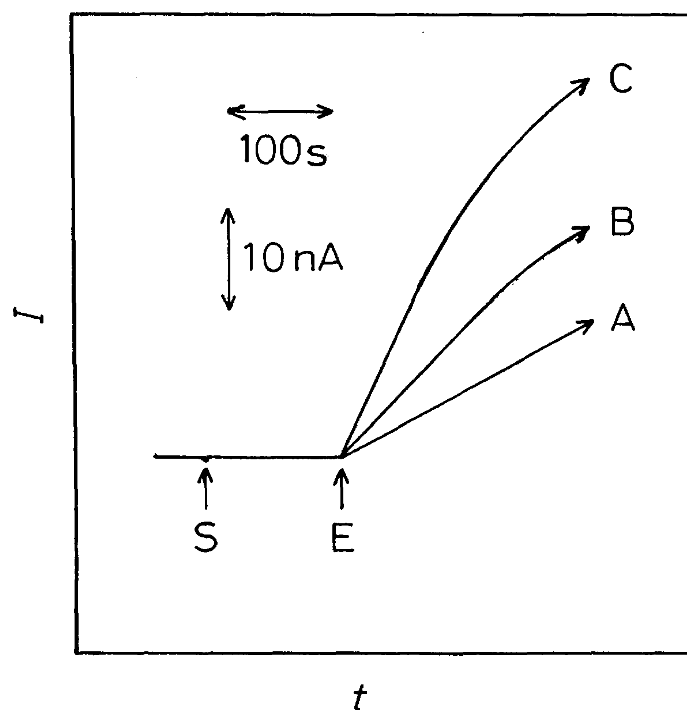

Fig. 8 Anodic current at 0.2V for successive additions of 60 μM inosine 5'-phosphate (S) and nucleotidase (E, A: 1.25, B: 2.50 and C: 5.00 $\times 10^{-2}$ U ml^{-1}) in pH7.5 at 33°C with an NOD electrode.

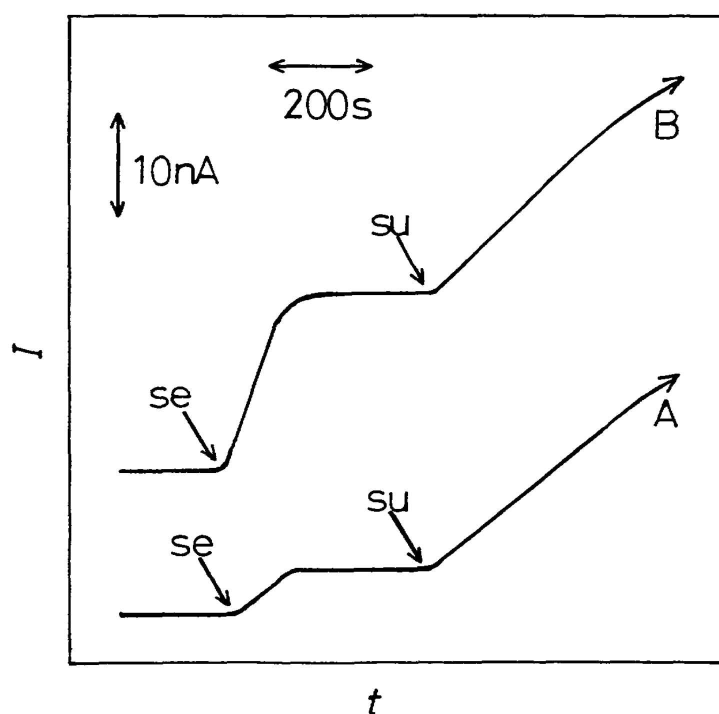

Fig. 9 Current at 0.3V for successive additions of 150μl serum (se) and 20μl of 9 mM inosine 5'-phosphate (su) in 3 ml tris buffer, pH7.5 (A) and pH8.3 (B).

less than 5. After the addition of substrate, the current due to nucleoside which was released by nucleotidase gradually increased. Based on the result as shown in Fig. 8, the activity of nucleotidase can be obtained easily from the slope of $I-t$ curve in first two min.

When nucleotidase was added into the buffer solution containing noodle soup, the increase in anodic current was observed. This increase in current was proportional to the amount of chemical seasoning which was added into the soup. Instead of expensive nucleotidase, abolished serum could be used for this analysis. These two methods are based on the measurement of the increase in current which is produced by the enzymatic reaction of nucleotide in a certain period. Therefore, the oxidizable compounds in a biological sample did not interfere with this method.

Figure 10 shows the long term stability of an NOD electrode. The activity of this electrode did not change for two months when it was used for 8 hr a week.

The work described in this paper was presented before the 37th annual meeting of Polarographic Society of Japan in Kyoto, November 25-26, 1991.

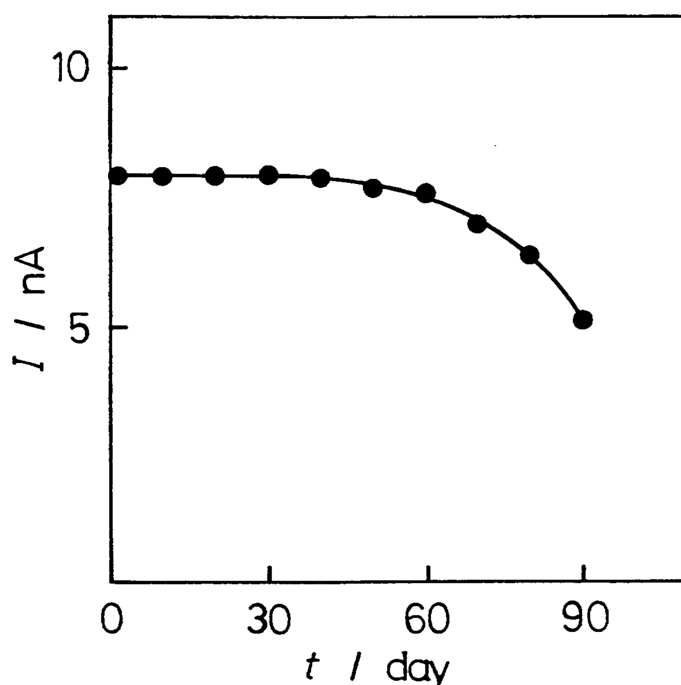

Fig. 10 Long term stability of an NOD electrode.
Current was measured in the presence of 10μM adenosine in pH7.1. An electrode was used for 8 hr a week.

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