Micro Enzyme-Sensor with an Osmium Complex and Porous Carbon for Measuring Galactose

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A micro enzyme-sensor, based on galactose oxidase (EC 1.2.3.9) and a tris(2,2′-bipyridine) complex of osmium(II/III) as a redox mediator ($[Os(bpy)_3]^{2+/3+}$), fabricated on a carbon electrode (25 μm diameter), was developed for measuring galactose. To obtain the carbon electrode, a platinum-disk electrode (25 μm diameter) was etched in hot aqua regia to create a cavity (depth of ca. 3—5 μm) at its tip. A porous carbon material was prepared from 90% acetylene black and 10% Teflon emulsion as a binder, and then packed into the cavity of the platinum-disk electrode's tip. The carbon electrode was immersed in the osmium complex with 0.1 mol dm^{-3} $LiClO_4$ in order to adsorb it in the carbon pores, which was monitored based on an increase in the anodic peak current and the cathodic peak current based on the osmium complex redox potential by the cyclic voltammogram. The tip of the carbon electrode was dipped overnight in a buffer solution of pH 7.00 containing galactose oxidase so as to immobilize it on this surface by adsorption. The characteristics of the porous-carbon material surface by X-ray diffraction (XRD) and scanning electron microscopy (SEM), the calibration curve for measuring of galactose, and the effects of the pH, temperature and concomitant compounds were investigated. By the XRD measurement, the porous carbon after treating a Zonyl FSN fluoro-carbon surfactant solution, a 5% Nafion® solution with methanol, and an osmium complex with 0.1 mol dm^{-3} $LiClO_4$ showed good crystallinity compared with carbon powder. The structure of the carbon-electrode surface was visually confirmed using SEM photographs. The carbon surface had many pores, and galactose oxidase existed on it after adsorption. Under the optimum conditions the amperometric response of this sensor was linear over concentration ranges of 0.01—5.00 mmol dm^{-3} galactose; the correlation coefficient was 0.999.

Recently, increasing attention has been paid to enzyme-based micro sensors, since they have clinical, environmental, agricultural, and biotechnological applications.[1—4] An amperometric galactose sensor is one of the most useful enzyme-based biosensors. Galactose is normally present at low concentrations in serum and urine, and measuring its concentration is used in the diagnosis of galactosemia.[5] Although several methods for the assay of galactose are available,[6—14] there have been few reports concerning micro sensors for galactose. A micro-disk electrode has the following characteristics: low current sensitivity, a sensing-point area of micro order radius, and a high current density based on the edge effect.[15—17]

In this work we prepared and tested a micro enzyme-sensor based on galactose oxidase immobilized on a porous carbon material with a redox mediator (osmium complex) for the determination of galactose. This type of micro amperometric disk electrode is sensitive for detecting the current toward galactose concentration. In a previous study conducted to determine galactose, no effort was made concerning detection for the measurement of low concentrations of galactose; four current-response values were obtained for making the liner-calibration curve between 1 and 20 mmol dm^{-3}.[6,11] We now consider the calibration curve for determining low concentrations of galactose, and the effects of the pH, the temperature and a concomitant compound, respectively.

We also investigated the characteristics of the porous carbon surface by X-ray diffraction and scanning electron microscopy in order to obtain each treatment condition and surface roughness. In general, a large number of carbon electrodes having many kinds of surface properties are used for electrochemical investigations. Porous carbon materials comprising acetylene black bound by Teflon® emulsion have many pores on their surfaces. These pores are based on the space adjacent to the acetylene black masses. Hence, the morphology of a porous carbon surface is assumed to be changed by these treatments and the adsorption of an osmium complex.

The micro enzyme-sensor was made by binding the enzyme and immobilizing the redox mediator complex

in an inner porous Nafion®-modified carbon surface. In this way, the micro enzyme-sensor did not require a membrane to retain the enzyme or redox species complex, and measurements were relatively unaffected by the oxygen concentration in the test solution.

The involved enzyme reactions are as follows:

$$\text{D-galactose} + \text{D-galactose oxidase-FAD} \rightarrow \text{D-galacto-hexodialdose} + \text{D-galactose oxidase-FADH}_2. \quad (1)$$

Reaction (1) is followed by

$$\text{D-galactose oxidase-FADH}_2 + 2\text{Os(bpy)}_3^{3+} \rightarrow \text{D-galactose oxidase-FAD} + 2\text{Os(bpy)}_3^{2+} + 2\text{H}^+, \quad (2)$$

where $Os(bpy)_3^{2+}$ is subsequently electrooxidized. The osmium complex mediator is used for a redox electron transfer between galactose oxidase and the electrode. The D-galactose concentration was determined by measuring the anodic current at +650 mV vs. Ag/AgCl.

Experimental

Materials. The following reagents were used: D-galactose (Kanto Chemical Industries, Ltd.), galactose oxidase (E.C.1.2.3.9) (Sigma), human serum (Irvine Scientific), Nafion® solution (5% Nafion 117® solution with methanol, Aldrich) and Zonyl FSN fluoro-carbon surfactant (Du Pont). $Os(bpy)_3(PF_6)_2$ was synthesized from osmium(III) chloride (Aldrich).[18—21] The buffer solution comprised 0.2 mol dm^{-3} disodium hydrogenphosfate and 0.1 mol dm^{-3} citric acid for a pH between 3.48 and 8.36; it was 0.05 mol dm^{-3} sodium carbonate and 0.1 mol dm^{-3} sodium hydrogencarbonate for a pH between 9.44 and 11.0. All of the other chemicals used were standard commercial products.

Preparation of Sensors. The porous carbon material was prepared from 10% Teflon® and 90% acetylene black.[21—24] This porous carbon material was then recycle-extracted in acetonitrile for 48 h. To immobilized the osmium complex on porous carbon materials, several steps were required. The porous carbon material was soaked for 24 h in a 0.25% Zonyl FSN fluoro-carbon surfactant in an aqueous solution in order to make the pores hydrophilic, and was then slowly dried at room temperature for 24 h. For loading the osmium complex onto the carbon surface electrostatically, the carbon material was immersed in a 5% Nafion® solution for 24 h in order to impart a negative charge to the surfaces of the pores. The procedure for preparing the platinum disk electrode was mainly based on that described by Fleishmann et al.[25] The tip of the platinum disk electrode was etched for 1 h in hot aqua regia (80 °C) so as to create a 3—5 μm deep cavity of 25 μm diameter, and packed with porous carbon material by pushing the tip against small lumps of the material on the platinum-electrode surface. The structure of the porous carbon/platinum electrode is shown in Fig. 1.

Immobilized Osmium Complex and Enzyme. The adsorption of a redox mediator, $Os(bpy)_3^{2+/3+}$, to the porous carbon-electrode surface was monitored by cyclic voltammetry over the potential range of 200—900 mV for more than 100 cycles at a sweep rate of 50 mV s^{-1}. The

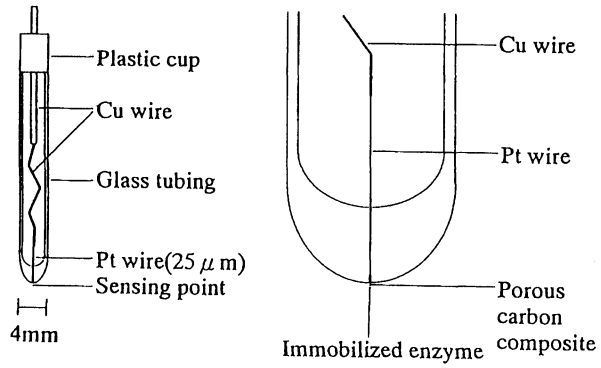

Fig. 1. Diagram of the micro-enzyme sensor.

cyclic voltammgram showed adsorption of the osmium complex to the porous carbon-electrode surface. The osmium complex loading value, calculated form the area increase of the multiple-cycle voltammgram peak, was 1.19×10^{-8} mol cm^{-2} of the geometric electrode surface area. After loading the osmium complex, the porous carbon electrode was dipped overnight in a buffer of pH 7.00 at 4 °C containing galactose oxidase for modifying the galactose. Many kinds of mediators have been used in enzyme sensors.[26—40] For this kind of sensor, the osmium complex is very good for mediating the electron exchange between the sensor and the immobilized enzyme.

Experimental Procedure. Voltammetric experiments were performed using a commercially available Bioanalytical System BAS-100B Electrochemical Analyzer having a preamplifier unit with a three-electrode system. An Ag/AgCl electrode was used as a reference, and a platinum electrode was used as a counter electrode. A cooling circulator CTE-22W Yamato-Komatu thermostat system was used to control the sample temperature in all of the experiments. Experiments concerning the characteristics of the carbon material were performed using an XRD and SEM (Rigaku Denki Co.).

Results and Discussion

Characteristics of Carbon Sheets. The carbon powder, the porous-carbon material and the same one soaked in a Zonyl-FSN fluoro-carbon surfactant (Nafion® solution and osmium complex, successively) were examined with an XRD and SEM. The appearance of a porous carbon-material surface was measured by SEM. The porous carbon surface was confirmed visually, showing many pores, and galactose oxidase existed on the surface. The carbon pores contribute to an enhancement of the electrode surface for a redox reaction field and an immobilization field of the osmium complex. X-Ray diffraction data on the powder were collected using a Rigaku diffractometer with monochromatized Cu $K\alpha$ radiation (30 kV–25 mV). The step scanning technique was applied for measurements within the 2° range from 10° to 70° for a Rietveld analysis. The stepping angle was 2°, and the measurement time was 1 second at each point. The effects of successive treatments were examined using the same carbon sheet; and results are shown in Fig. 2.

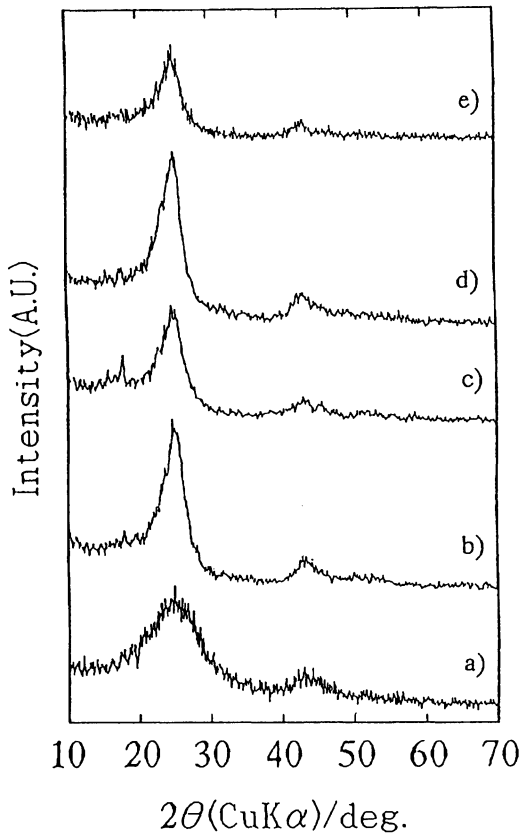

Fig. 2. X-Ray diffraction patterns of carbon powder and porous carbon. a) carbon powder, b) porous carbon, c) porous carbon soaked in Zonyl FSN fluoro-carbon surfactant, d) soaked in Nafion® solution and e) soaked in osmium complex in 0.100 mol dm^{-3} $LiClO_4$ solution.

The porous carbon materials were prepared by Teflon® emulsion and acetylene black; each treatment is described above. The porous carbon surface characteristics include a different typical glassy carbon surface and a carbon powder. All of the XRD spectra peaks in Fig. 2 are due to the carbon included acetylene black. Hence, the osmium complex (Nafion® polymer and Zonyl FSN fluoro-carbon surfactant) shows no chemical reaction on chemical binding with the carbon materials. These substances, which are used for treatments, are just stable sites on the carbon surface or carbon pores. The XRD spectra from Fig. 2b to Fig. 2e show a clear linearity compared with the Fig. 2a spectra. The porous carbon crystallinity was much higher than that of carbon powder. The acetylene black is collected as if it was a particle layer. However, the resolutions of the XRD and SEM photographs were poor for detecting surface differences after a treatment using a Zony FSN fluoro-carbon surfactant solution, a Nafion® solution and osmium complex, and distributing the osmium complex on the porous carbon surface and inner carbon pores.

Sensor Response. The anodic current value, which depended on the galactose concentration, was measured at 650 mV (vs. Ag/AgCl). This potential, obtained by a cyclic voltammogram, is half of the redox potential of the osmium complex in the inner carbon pores. The enzyme-sensor is applied a potential of between 200 and 900 mV, and a scan rate of 2 mV s^{-1} in the sample solution (presented above as reaction schemes (1) and (2)). Galactose is oxidized by immobilized galactose oxidase. Electrons are directly obtained, and then transferred through the osmium redox reaction to the carbon materials. However, the anodic current response of a sensor without the osmium complex is small (Fig. 3a), the current-response curve of which shows the same shape for any concentration of galactose. An enzyme sensor without the osmium complex fails reaction (2), as described in the introduction section; the immobilized galactose oxidase dully exchanges electrons with the electrode when galactose reacts on the electrode surface. Obviously, for an electrode with the osmium complex and galactose oxidase, the increasments of the anodic current depend on the galactose concentrations at the applied potential (650 mV vs. Ag/AgCl). The enzyme-sensor response time was 30 s, which was sufficient to measure the sample solutions.

Effect of pH. The current response from the enzyme sensor at 1 mmol dm^{-3} of galactose was studied as a function of the pH in the surrounding buffer solution. The effect of the pH was examined in the pH 3.48—11.0 range of the buffer solution at 37 °C. As shown in Fig. 4, the pH optimum for immobilized galactose oxidase on the enzyme sensor is pH 10.4. This pH

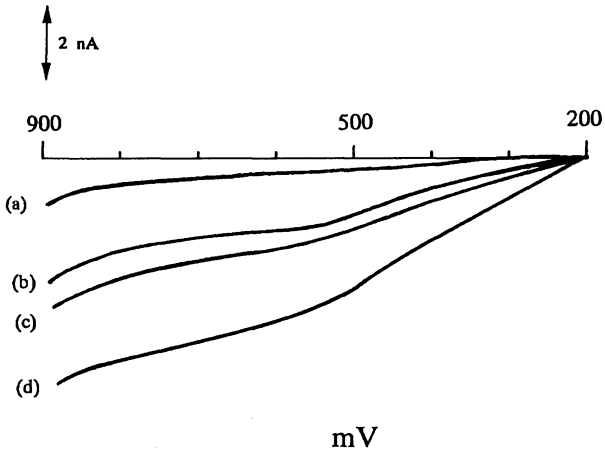

Fig. 3. Voltamograms of micro enzyme-sensor and sensor without galactose oxidase response to different galactose concentration. (a) sensor without osmium complex in 1.00 mmol dm^{-3} galactose. (b) sensor in buffer solution. (c) 1.00 mmol dm^{-3} galactose with buffer solution. (d) 5.00 mmol dm^{-3} galactose with buffer solution. All sample solutions were used pH 10.4 buffer solution at 40 °C. Experiment conditions; Initial E: 200 mV, High E: 900 mV, Low E: 200 mV, Scan rate V: 2 mV s^{-1}.

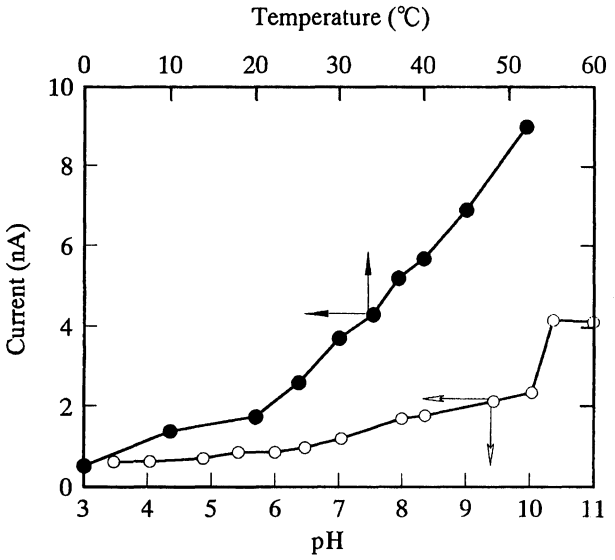

Fig. 4. Effect of pH and temperature on the micro enzyme-sensor. (a) Effect of pH. Sample, 1 mmol dm^{-3} galactose in each buffer solutions at 37 °C. (b) Effect of temperature. Sample, 1 mmol dm^{-3} galactose in pH 10.4 buffer solution.

optimum differs from the optimum pH for galactose oxidase in solution. Soluble galactose oxidase is known to have its pH optimum at ca. pH 7.[5] The optimum pH for galactose oxidase is affected by its immobilization to the electrode surface, and various factors are concerned. This pH shift of the immobilized enzymes can be explained as follows: If the carrier is negatively charged, a high concentration of positively charged ions (H^+) accumulates at its boundary with the surrounding solution and, consequently, the pH at the carrier surface becomes lower than that of the bulk solution.[41,42] In addition, the buffer solution (pH 10.4) can influence the configuration to the immobilized galactose oxidase (Nafion® polymer and osmium complex). Also, the sensor shows a good relationship with galactose oxidase and porous carbon surface-Nafion® polymer with the osmium complex. In previous reports concerning this type of enzyme sensor, a similar pH shift appeared as the result of a pH measurement.[21,22,43] As a result, the electrons obtained from the reaction of galactose were easily transferred from galactose oxidase to the osmium complex, compared with other pH. values. On the basis of these results, all subsequent measurements were carried out in a sample solution of pH 10.4. Although this pH condition seems to produce a large shift (ca. 3.4) from the optimum, the buffer solution had no effect to lose the enzyme-sensor stability for at least one month.

Effect of Temperature. The effect of the temperature on the enzyme-sensor response was examined over the range of 2—55 °C. The sample had a 1 mmol dm^{-3} galactose concentration in a pH 10.4 buffer solution. Figure 4 shows the increase in the anodic current with increasing temperature. The current response depended on the activity of immobilized galactose oxidase, which increased significantly over 20 and 40 °C. Concerning the enzyme activity in general, the enzyme showed maximal apparent activity at over 50—60 °C; denaturation and inactivation of the enzyme occurred then. In addition, sample temperatures above 55 °C can possibly cause a breakage of the sensing point. Therefore, all subsequent measurements were carried out at 40 °C.

Calibration Curve. The relationship between the galactose concentration and the anode current was examined using the enzyme sensor under the optimum conditions (determined as described above). A linear relationship was obtained over the 0.01—5.00 mmol dm^{-3} galactose range (Fig. 5). The calibration curve was represented by the equation $Y=0.0377+0.498X$, where Y is the increase in the anodic current based on measurements using the buffer solution without galactose in nA; X is the galactose concentration in mmol dm^{-3}. The correlation coefficient was 0.999. The enzyme sensor detected in the range of less than 1 mmol dm^{-3} galactose in the buffer solution; the lowest galactose concentration was then determined to be 0.01 mmol dm^{-3}. Concerning the above-mentioned 5.00 mmol dm^{-3} galactose concentration, the anodic current response was nonlinear. However, the limit of the linear range is relevant to the analysis of undiluted normal human serum.

Effect of Concomitant Compounds on the Determination of Galactose. Galactose was determined in the presence of various other compounds. As

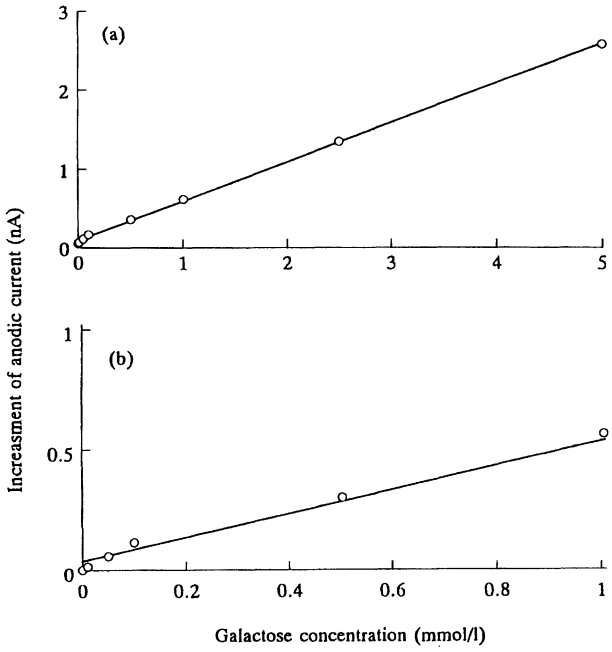

Fig. 5. Calibration curve for galactose. Sample, pH 10.4 buffer solution at 40 °C, the changes of the anodic current at 650 mV (vs. Ag/AgCl). (a) Concentration range between 0.00 to 5.00 mmol dm^{-3}. (b) Concentration range between 0.00 to 1.00 mmol dm^{-3}.

shown in Table 1, it could be determined with a relative error of less than ±8.57% in the presence of molar ratios of 1 of L-ascorbic acid, 1—10 of sodium nitrate, 1—10 of L-phenylalanine, 1 of potassium chloride, 1—10 of potassium hydrogensulfate, 1—10 of sodium dihydrogenphosfate, 1—10 of sodium sulfate, 1—10 of Nicotinamide, 1—10 of sodium L-glutamate, 1—10 of oxalacetic acid, 1—10 of β-alanine, 1—10 of L-cystine, 1—10 of D-tartaric acid, 1—10 of L-cysteine, 1—10 of cyanocobalamine, 1 of L-arginine, 1—10 of L-serine, and 1—10 L-malic acid. The galactose sample solution as well as all concomitant compounds used 10 μl of a 10 mmol dm^{-3} solution in a pH 10.37 buffer solution (5 ml) at 40 °C. The anodic current response of this sensor, based on the galactose oxidase reaction and electron-transfer reaction, showed little inhibition due to the above-mentioned compounds.

Determination of Galactose in Human Serum. The enzyme sensor was applied for determining of galactose in the serum of a healthy human; the results are shown in Table 2. In normal human serum, the galactose level is reported to be less than 20 mg/100 ml (less

Table 1. Effect of Concomitant Compounds on the Determination of Galactose

Concomitant compound	Molar ratio[a)]	Relative error (%)
L-Ascorbic acid	1 : 1	6.14
Sodium nitrate	1 : 1	0.55
	1 : 10	1.26
L-Phenylalanine	1 : 1	−1.12
	1 : 10	−6.89
Potassium chloride	1 : 1	4.66
Potassium hydrogensulfate	1 : 1	−5.39
	1 : 10	−7.82
Sodium dihydrogenphosfate	1 : 1	0.00
	1 : 10	−2.47
Sodium sulfate	1 : 1	−0.90
	1 : 10	−0.90
Oxalacetic acid	1 : 1	0.18
	1 : 10	4.63
Nicotinamide	1 : 1	−1.24
	1 : 10	−1.24
β-Alanine	1 : 1	0.91
	1 : 10	3.54
L-Serine	1 : 1	−2.33
	1 : 10	4.79
L-Malic acid	1 : 1	−5.27
	1 : 10	8.57
L-Cystine	1 : 1	−2.33
	1 : 10	−6.38
D(−)-Tartaric acid	1 : 1	−1.55
	1 : 10	−3.51
L-Cystein	1 : 1	2.66
	1 : 10	7.92
Cyanocobalamin	1 : 1	1.04
	1 : 10	−0.87
Sodium L-glutamate	1 : 1	−3.54
	1 : 10	−4.48
L-Arginine	1 : 1	2.78

a) Galactose: Concomitant compound. Sample; 10 μl of 10 mmol dm^{-3} Galactose and concomitant compounds in pH 10.4 buffer solution, Temp, 40 °C.

Table 2. Determination of Galactose in Human Serum

Sensor	Found[a)]	Found[b)]	C.V.[c)]
	mmol dm^{-3}	mg/100 ml	%
Galactose	0.193[d)]	3.49[e)]	2.49

a) Average of 5 determinations. b) Average of 5 determinations. c) Coefficient of variation. d) Reference value in human serum: less than 1.11 mmol dm^{-3}. e) Reference value in human serum: less than 20 mg/100 ml.

than 1.11 mmol dm^{-3}). With the sensor, however, the value was found to be 3.49 mg/100 ml and/or 0.193 mmol dm^{-3} of galactose (average of 5 determinations). The coefficient of variation was 2.49%. Therefore, this enzyme-sensor shows selectivity for the galactose concentration in serum, and possibility use for its determination.

Stability. The micro enzyme sensor was stable for one month for measurements of the anodic current from 200 to 900 mV. The sensors were stored in a pH 7.0 phosfate buffer solution which included galactose oxidase at 4 °C. In this study, the anodic current response suddenly disappeared after the sensor was used for one month. This behavior is assumed to have been due to carbon masses with the osmium complex and galactose oxidase on the electrode dropping from the tip of the electrode into the test solution. If the decrease in the sensor activity due to immobilized galactose oxidase inactivation was observed, the anodic current response followed the galactose oxidase activity; the anodic current then decreased slowly.

Conclusion

This paper reports on the preparation and properties of a micro enzyme sensor used for measuring galactose. This micro enzyme sensor comprises a platinum wire, copper wire, an osmium complex, carbon compounds, and an immobilized enzyme layer at its tip. No membrane is required on the electrode surface. The diameter of the electrode sensing point is only 25 μm and the sensor diameter is 4 mm. The optimum pH was found to be 10.4. A linear plot of the current was obtained over the range of 0.01—5.00 mmol dm^{-3} galactose; the calibration curve could be represented by the equation $Y=0.0377+0.498X$, where Y is the increasment of the anodic current in nA and X is the galactose concentration in mmol dm^{-3}. The level of galactose in normal human serum determined by this sensor was 3.49 mg/100 ml or 0.193 mmol dm^{-3}. The enzyme-sensor sensitivity was constant for about one month. These results indicate that this sensor should be useful clinically.

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