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**Characterization of Glucose Microsensors Small Enough
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by

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CHARACTERIZATION OF GLUCOSE MICROSENSORS SMALL ENOUGH FOR INTRACELLULAR MEASUREMENTS

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UPCOMING RESEARCH

Glucose sensors with 2- μm structural tip diameter have been developed by immobilization of glucose oxidase on platinized carbon ring electrodes. These sensors have sub-second response times and have been used to monitor glucose transients in single cells.

ABSTRACT

Ultrasmall glucose sensors have been constructed by using platinum deposited carbon ring microelectrodes with glucose oxidase. Response times as low as 270 ms have been obtained with these sensors. Moreover, there is a linear relationship between sensor tip diameter and response times. The use of these sensors has been demonstrated in the detection of glucose in single cell cytoplasm of the large dopamine cell of the pond snail, *Planorbis corneus*. Current responses obtained at these sensors implanted into a cell increase following injection of 2 pL glucose solution (3 M) into the cell. Results obtained from these experiments show that these sensors are suitable for glucose monitoring in ultrasmall environments. In addition, characterizations of these sensors have been investigated under different O₂ concentrations. At atmospheric oxygen concentrations, glucose levels in the submillimolar range can be measured without oxygen interference; however, oxygen interference can be substantial at low oxygen concentrations.

INTRODUCTION

In vivo voltammetry continues to be very important tool in the study of neurochemical dynamics. Since the early work of Adams and co-workers¹, a great deal of work has been carried out to amperometrically and voltammetrically monitor easily oxidized neurotransmitters and metabolites^{2,3}. Perhaps the area that has been most widely studied is that of dopamine dynamics in the extracellular fluid of the brain⁴, although several other neurotransmitters as well as oxygen⁵ have been measured by voltammetry in the brain. In all this work, an emphasis has been placed on both small size of the electrode used to minimize tissue damage and on selectivity in measuring the easily oxidized neurotransmitters and metabolites. Some recent work has been aimed at amperometrically or voltammetrically measuring substances at^{6,7} or inside⁸⁻¹⁰ single cells. To further develop our ability to characterize the neurochemical microenvironment, it is clearly apparent that the electrochemical methods available must be expanded to include selective determination of solutes not as easily oxidized. One avenue for these developments appears to be the use of amperometric enzyme-based electrodes.

A considerable amount of effort has been expended in the search for, and study of, amperometric glucose sensors¹¹. Recently, several efforts have been aimed at producing enzyme electrodes with small size and rapid response time. These efforts include the use of platinized microelectrodes with immobilized glucose oxidase to detect glucose with response times of only a few seconds¹². Horseradish peroxidase has also been covalently linked to carbon fiber microelectrodes via a biotin/avidin/biotin tether to produce a structurally small enzyme-based sensor¹³. Finally, we have recently immobilized glucose oxidase on platinized carbon ring electrodes with total tip diameters of only 2 μm ¹⁴. These electrodes have been used to monitor glucose transients in the cytoplasm of single large invertebrate cells.

Glucose electrodes small enough to be used inside single cells should provide a means to monitor single cell respiration and metabolism during specific chemical events including nerve cell stimulation and pharmacological manipulation. In this paper, we detail the procedure to construct glucose sensors based on glucose oxidase immobilization on 2 μm diameter platinized carbon ring electrodes. These sensors have been characterized to determine response time, the effect of oxygen concentration and selectivity for glucose relative to other glycols. Finally, the response time of these sensors has been examined and shows a linear dependence on the structural diameter of the electrode tip.

EXPERIMENTAL SECTION

Chemicals. Glucose oxidase (EC 1.1.3.4 type VII-S, from *Aspergillus niger*, 132,000 units/g), D-glucose, D-galactose, D-maltose, D-fructose, albumin, and glutaraldehyde were obtained from Sigma Chemical Co. (St. Louis, MO). Hexachloroplatinate and lead acetate were purchased from Aldrich Chemical Co. (Milwaukee, WI). 0.5 M phosphate buffer (pH 7.00) was prepared with double distilled water (Corning Mega-Pure MP-3A). This solution was used to prepare fresh glucose and other sugar solutions.

Construction of Microglucose Enzyme Sensors. Carbon ring electrodes were fabricated as described previously¹⁵. The general procedure for constructing glucose sensors is similar to that described by Ikariyama et al.¹⁶. Briefly, this procedure involved platinization on carbon ring microelectrodes by reducing 10 mM hexachloroplatinate in the presence of lead acetate. Platinized carbon electrodes were oxidized in a phosphate buffer solution at 1.1 V vs a sodium chloride saturated calomel electrode (SSCE) for 15 min. This process minimizes the direct oxidation of glucose on platinum¹⁷. Electrodes were then immersed in glucose oxidase (100 mg/mL) in a phosphate buffer for 3 min and subsequently immersed in buffer solution for 3 hours, immersed in a bovine serum albumin solution, and then in a glutaraldehyde solution to coat the electrode surface with a thin albumin film.

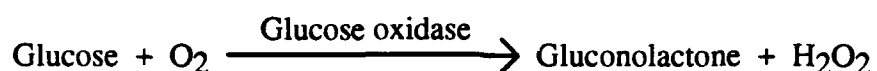
Flow Injection Experiments. The flow injection analysis system consisted of two stainless steel valves and a 250 μm i.d. fused-silica capillary obtained from Polymicro Technologies (Phoenix, AZ). A piece of capillary was held in the electrochemical cell with a stainless steel fitting. Phosphate buffer served as supporting electrolyte. The tip of the microglucose sensor was manipulated (Newport manipulator, model 422-1S) into the end of the capillary while viewed under a microscope (Reichert, Stereo Star Zoom).

Electrochemical Measurements. Amperometric measurements were carried out in the two-electrode mode with a SSCE reference electrode and a Keithley model 427 current to voltage amplifier to monitor current. Current-time curves were recorded on a strip chart recorder (Soltec, Model 1241). Gas atmosphere in solution was controlled by using a two-way gas flow meter (Matheson, Model FM-1050-VDA). In vitro experiments were carried out in a phosphate buffer solution thermostated at 25 °C.

Intracellular Measurements. Preparations for intracellular voltammetry were similar to those described previously¹⁸. *Planorbis corneus* were obtained from NASCO (Fort Atkinson, WI) and were maintained in aquaria at room temperature until used. The snails were pinned in a wax-filled petri dish and dissected under snail Ringer's solution (39 mM NaCl, 1.3 mM KCl, 4.5 mM CaCl₂, 1.5 mM MgCl₂, 6.9 mM NaHCO₃, pH 7.4)¹⁹ to reveal the left and right pedal ganglia. Micromanipulators (Narishige, No 8418) were used to place the electrode and a glass micropipette into the identified dopamine neuron²⁰. Intracellular injections of glucose and control solutions were carried out using an Eppendorf Model 5242 microinjector. Injection volumes were calibrated by injection of aqueous solution into oil under a microscope and calculating the volume of the spherical droplet. All experiments were aided by a stereomicroscope (Zeiss, Stemi SV8 microscope) and shielded with a copper mesh Faraday cage.

RESULTS AND DISCUSSION

Characterization of Microglucose Sensors. A schematic of the small glucose sensors described here is shown in Figure 1. These electrodes have been fabricated by depositing glucose oxidase onto platinum-coated carbon ring electrodes. Reduction of hexachloroplatinate in lead acetate solution appears to result in a porous platinum coating on the carbon ring electrode. This is evidenced by an increased residual current following platinum deposition. The larger surface area of the porous platinum maximizes the amount of enzyme attached to the electrode surface. Bovine serum albumin, cross linked with glutaraldehyde, is used to immobilize the enzyme to the porous platinum surface. Glucose is oxidized enzymatically by the reaction,



and the H_2O_2 is detected amperometrically at the electrode surface. This procedure results in glucose sensors with total tip diameter as small as 2 μm . In addition, the carbon ring electrodes used have ring thicknesses on the order of approximately 100 nm ²¹. Hence, these sensors are extremely small in this dimension.

The small size of these glucose sensors should lead to an extremely fast response time. The response time for the determination of glucose has been measured using flow injection analysis. Figure 2 compares the response time of the glucose microsensor to injections of dopamine (control) and glucose. Response times are determined between 10 % to 90 % of full response. The average rise and fall times for dopamine at sensors having tip diameter less than 5 μm are 125 ± 35 ms ($n=5$) and 140 ± 30 ms ($n=5$), respectively. The average rise and fall times of glucose electrodes having tip diameter less than 3 μm are 460 ± 190 ms and 430 ± 220 ms ($n=4$), respectively.

Figure 3 shows the relationship between electrode tip diameter and rise time for an array of sensors having tip between 2 and 10 μm diameter. The correlation between electrode diameter and rise time appears to be linear with a great deal of scatter in the data. A least squares fit of this data has a slope of 194 ms/ μm , an intercept of 0.022 s and a correlation of 0.902. The linear relationship between electrode tip diameter and rise time suggests that enzyme/albumin films placed on larger microelectrode are thicker and/or restrictive to mass transport of glucose or H_2O_2 in the film. This is, at present, clearly speculative as it is very difficult to control or determine membrane thickness on these very small sensors.

Calibration of Microglucose Electrodes. Calibration of glucose microelectrodes has been carried out in static solutions following standard additions of glucose solutions. Figure 4 shows a typical calibration curve obtained under atmospheric conditions. The response of the sensor appears to be linear (correlation coefficient = 0.989) between concentrations of 50 μM and 5 mM. At higher glucose concentrations (5 mM and above) deviation from linearity is apparent. This appears to be the result of the ambient oxygen concentration limiting the reaction rate. Hence, high concentrations of glucose appear to be more easily affected by oxygen concentration and could be difficult to monitor with this scheme.

Effect of Oxygen on Electrode Response. The amperometric response of a glucose oxidase sensor is expected to be stoichiometrically dependent on oxygen concentration. This dependence has been studied in a stationary electrochemical cell with varied oxygen concentration. Figure 5 shows the relative response to glucose over a range of oxygen concentration from 100 % oxygen atmosphere (1.2 mM) down to a thoroughly deoxygenated solution. In solutions of 0.5 and 5 mM glucose, a strong dependence between response and oxygen concentration is observed below 0.2 mM. At oxygen concentrations above this level, very little dependence is observed. At low oxygen concentration in the solution, oxygen is clearly a limiting reagent for H_2O_2 production and demonstrates that the sensor enzymatically measures both glucose and oxygen levels. This dependence is reduced at low glucose concentrations. When the electrode response is examined in 0.05 mM glucose, virtually no dependence on oxygen concentration is observed, even at 50 μM oxygen. These results indicate that the enzyme electrodes developed should be useful when the oxygen concentration is high or the glucose concentration is low. Hence, glucose levels in the submillimolar range can be measured without severe interference from oxygen.

Interference from pH and Neurotransmitters. The current response of glucose oxidase electrodes is expected to be dependent on solution pH²². We have examined the electrode response over a pH range spanning the physiological region (pH 6.0 to 8.0) and no change in response is observed.

The influence of easily oxidized neurotransmitters such as dopamine has been examined by comparing the responses of 0.5 mM glucose and 0.5 mM dopamine. Currents for dopamine at 0.6 V vs SSCE are approximately one order of magnitude higher than those for glucose. This result indicates that care must be used in using these sensors for measuring glucose in the presence of easily oxidized neurotransmitters.

Stability of Glucose Oxidase Microelectrodes. The stability of glucose oxidase microelectrodes has been examined in solutions of 0.5 and 3 mM glucose for 20 hours while continuously monitoring the amperometric current. The loss of response under these conditions is $23.5 \pm 1.5 \%$ ($n=3$) and $25.5 \pm 2.5 \%$ ($n=3$), respectively. The loss in response observed is most likely the result of a loss of glucose oxidase activity, although this could also result, in part, from electrode fouling. However, one would expect electrode fouling due to the H_2O_2 and glucose oxidation products to be dependent on concentration and this is not observed. The end result is that these electrodes are stable enough to provide reliable and accurate measurement of glucose on the time scale of a few hours.

Intracellular monitoring of Glucose. One goal in developing ultrasmall glucose electrodes is to provide methodology to dynamically determine glucose levels in the cytoplasm of single cells. To evaluate the applicability of the glucose sensors developed here, they have been placed into the single large dopamine cell of the pond snail, *Planorbis corneus*, and used to detect transient concentrations of glucose.

Figure 6-A shows a typical intracellular response for a glucose oxidase electrode implanted in the giant dopamine cell of *Planorbis corneus* following intracellular injection of 2 μ L of 3 M glucose solution. The volume of glucose solution injected is approximately 0.1 % of the cell volume. The oxidation current increases immediately following the injection of glucose. This is followed by a gradual decrease returning to baseline after about 2 min. A similar response is obtained for a repeated injection of glucose. One control experiment carried out involves examining the response obtained by injecting only phosphate buffer into a cell and monitoring with a glucose electrode. This is shown in Figure 6-B where no response is observed after several injections of buffer solution into the cell. Hence, it appears that the response observed is not the result of cell volume change or ionic strength. Another control experiment has been carried out to examine the possibility of glucose oxidation at the underlying platinum electrode. Platinum-coated carbon ring electrodes without the glucose oxidase/albumin coating have been placed in dopamine cells and the current monitored following intracellular injection of glucose. The results are shown in Figure 6-C and no definitive response is observed. This result also indicates that compounds electrochemically oxidized at 0.6 V vs SSCE., such as dopamine, do not increase inside the cell following glucose injection. The results described above strongly suggest that the responses obtained in Figure 6-A are based on the oxidation of H_2O_2 generated by the catalytic reaction of glucose with glucose oxidase. It appears that the current response observed in Figure 6-A represents a

rapid increase in glucose concentration in the cell cytoplasm following glucose injection. This is followed by a more gradual decline due to metabolism and/or storage of glucose in the cell.

These electrodes are found to lose response during the intracellular measurement. Comparison of pre- and post-calibrations shows the loss in response as $36.5 \pm 4.5 \%$ ($n=4$). These losses are modest in comparison to naked platinum electrodes used for intracellular voltammetry where the loss in response can be as high as 60 %¹⁸. On the basis of post-calibration data, the peak change in glucose concentration observed in the cytoplasm following injection of glucose is 0.8 mM. We have recently measured the oxygen concentration in the cytoplasm of this cell and found it to be 0.032 ± 0.004 mM²³. At these low oxygen levels, the quantitative determination of glucose in the submillimolar range could be in error by as much as one-third. The present capability to monitor glucose dynamics with approximate quantitative ability at the single cell level is, however, extremely promising.

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FIGURE CAPTIONS

- Figure 1. Schematic representation of a glucose microsensor. (A) Ultrasmall carbon ring electrode: a, drawn quartz capillary; b, thin carbon film; c, epoxy resin; d, mercury. (B) Platinum coating. (C) Incorporated glucose oxidase. (D) Albumin film coating.
- Figure 2. Current responses of glucose microsensors for (A) 0.5 mM dopamine solution, (B) 0.5 mM glucose solution in a flow injection system. Flow rate of solutions is 0.215 mL/min.
- Figure 3. Comparison of response time to glucose as a function of electrode total tip diameter for 18 different electrodes.
- Figure 4. Current vs concentration curves for a typical glucose microsensor (tip diameter; 4 μm). Solution conditions: static solution; pH 7.0 phosphate buffer; 0.6 V vs SSCE electrode potential.
- Figure 5. Effect of oxygen concentration on current response obtained with glucose microsensors in ($\square$) 0.05 mM, ($\circ$) 0.5 mM, and ($\triangle$) 5mM glucose solution. All responses have been normalized to the current observed when the solution was purged with air (100 % response). Solution conditions: static solution; pH 7.0 phosphate buffer; 0.6 V vs SSCE electrode potential.
- Figure 6. Current observed at electrodes placed in the large dopamine neuron of *Planorbis corneus*. (A) Response to a 2 pL, 3 M intracellular glucose injection. (B) Response to a 8 pL intracellular injection of only pH 7.4 buffer. (C) Response of an electrode not coated with glucose oxidase to a 8 pL, 3 M intracellular glucose injection. The electrode potential was 0.6 V vs SSCE in all cases.

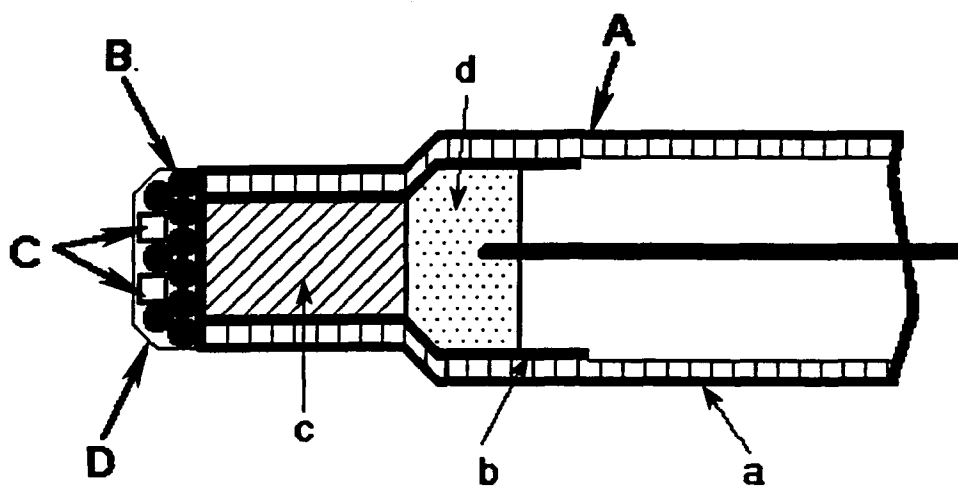


Fig. 1

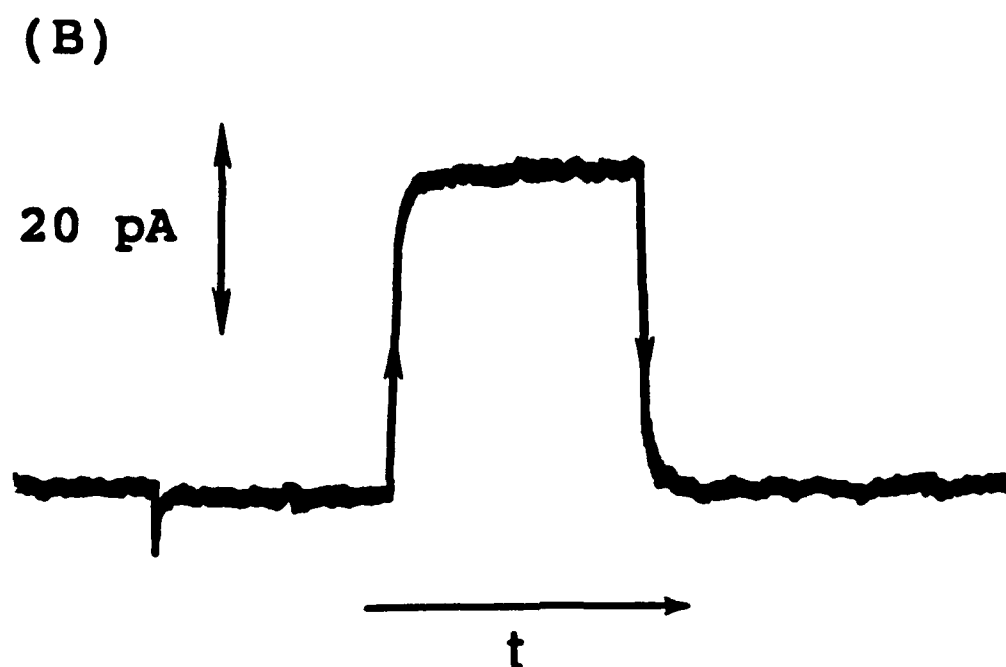
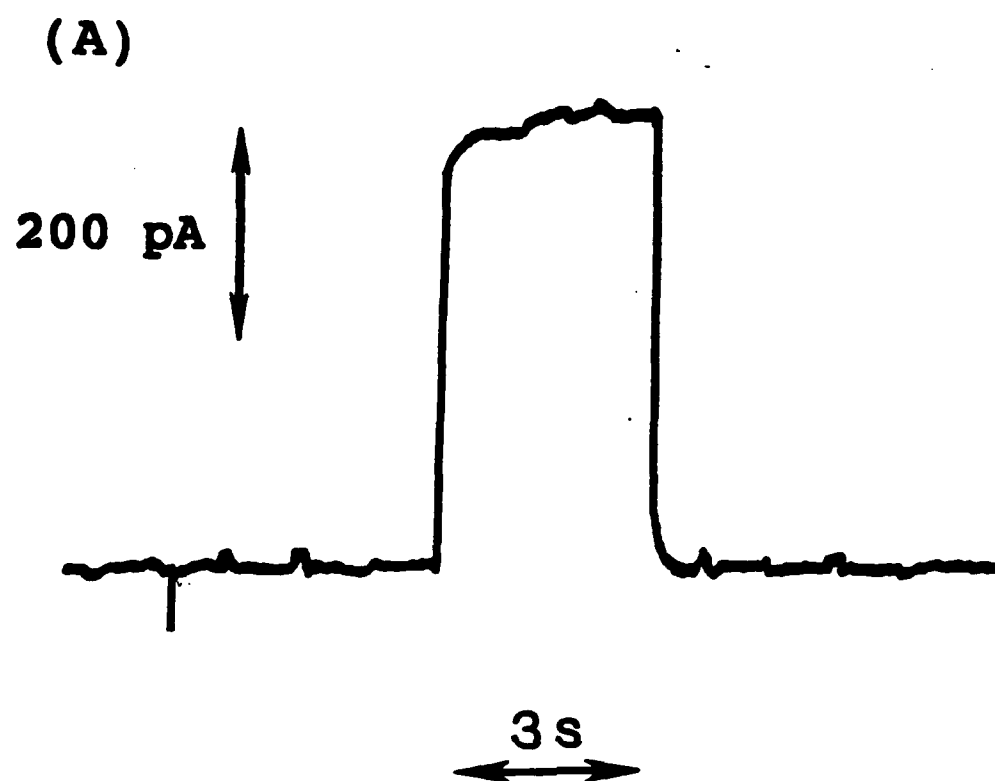


Fig. 2

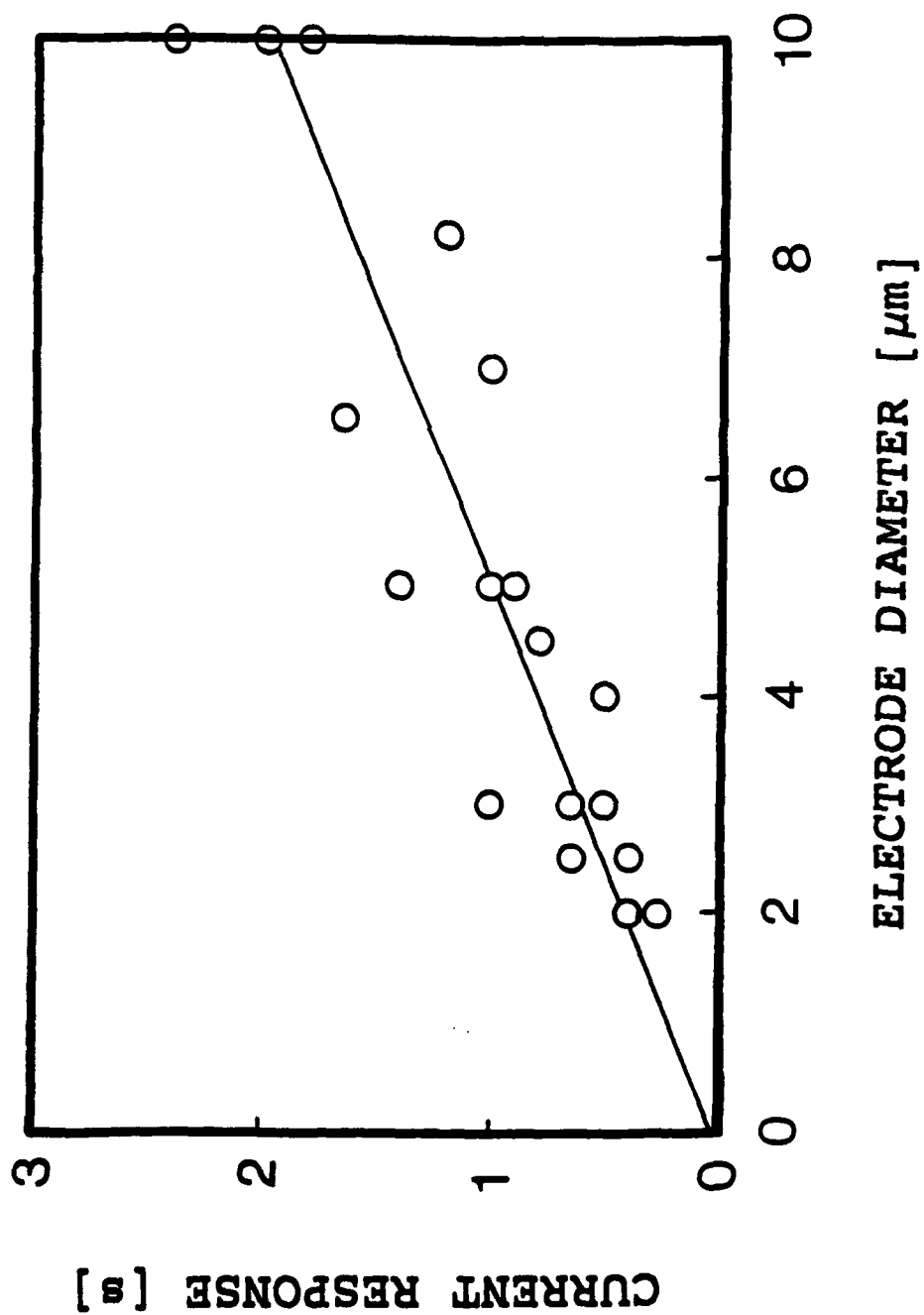


Fig. 3

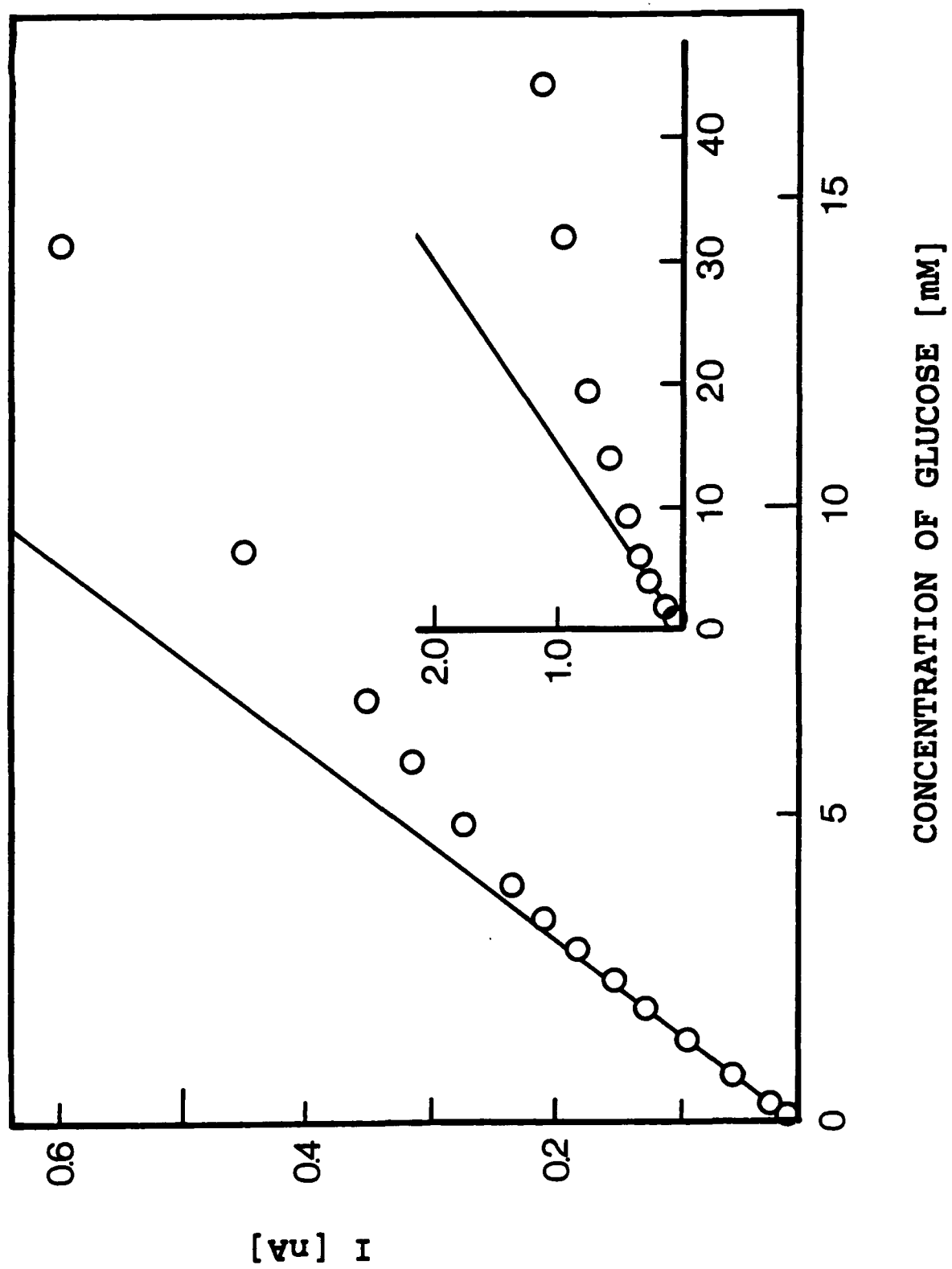


Fig. 4

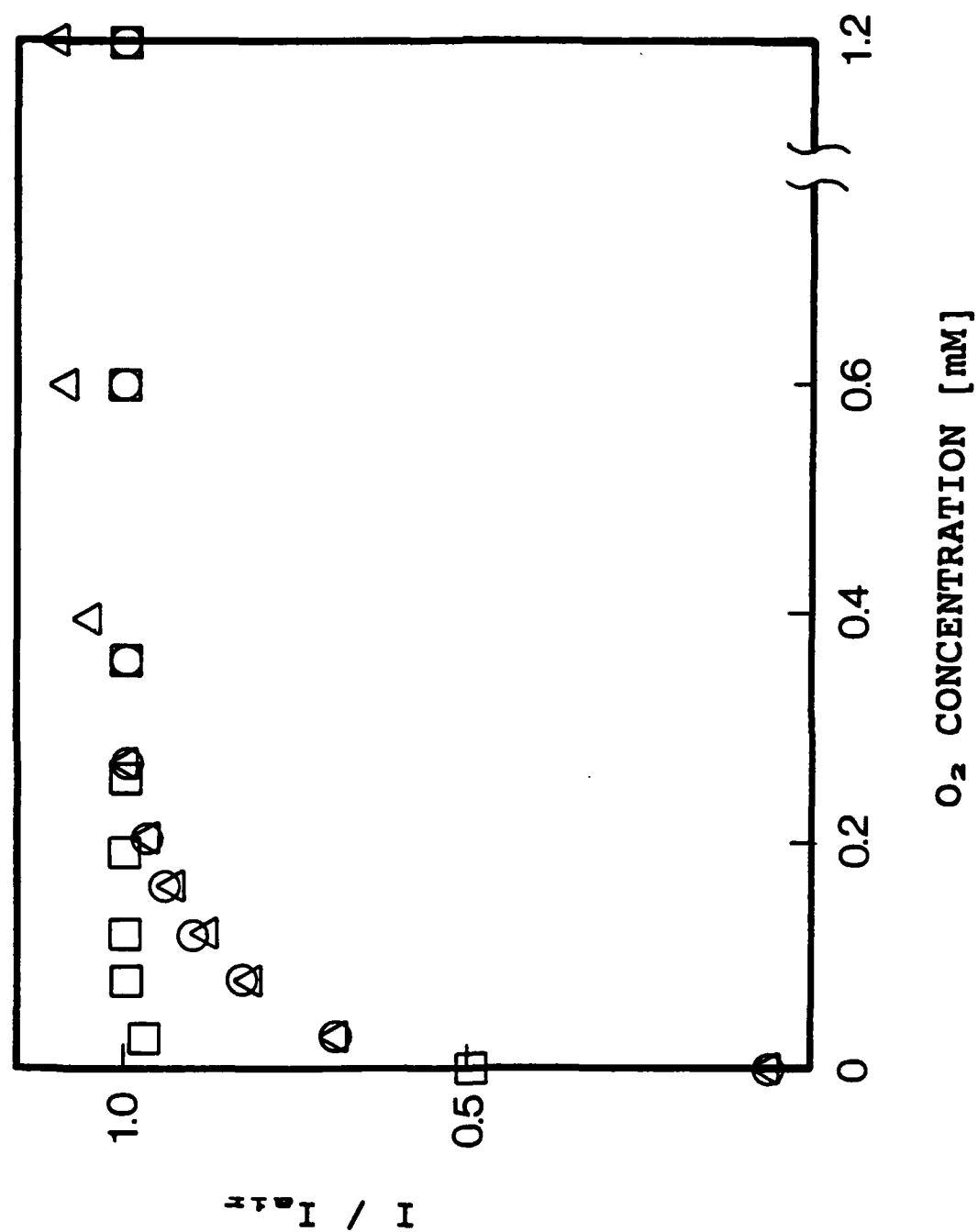


Fig. 5

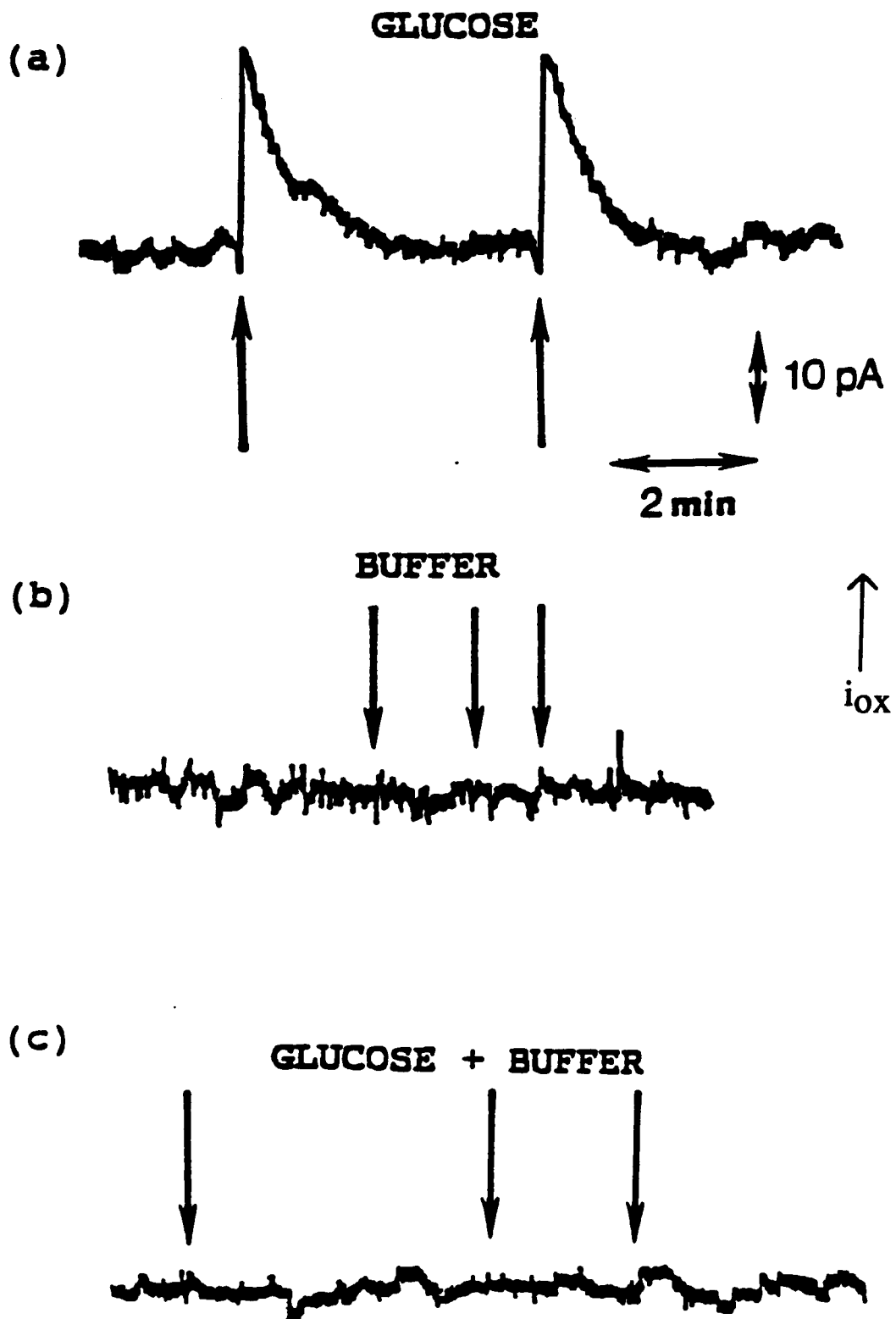


Fig. 6