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Low-level electrochemical detection of glucose oxidase and a glucose oxidase conjugate

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Abstract: An amperometric sensor for glucose oxidase is described. Partial optimization of the working electrode potential at 0·4 V *vs.* Ag/AgCl, 0·1 M KCl at a glucose concentration of 30 mM allows a low detection limit of 50 pM glucose oxidase within 25 min. The sensor exhibits a high degree of precision and reproducibility, and the same electrode may be re-used for many successive quantitative determinations. A mechanism for the sensor is proposed, in which hydrogen peroxide generated by glucose oxidase in bulk solution is oxidized at the working electrode, generating a current response that can be related to the glucose oxidase concentration. Conjugation of glucose oxidase with a coumarin-based pesticide analogue does not affect the amperometric response.

Keywords: glucose oxidase, platinised carbon paper electrode, amperometry, hydrogen peroxide, glucose oxidase conjugate, enzyme label.

INTRODUCTION

Amperometric glucose oxidase electrodes

Glucose oxidase (GOD) has been employed as the biological recognition component in a wide variety of biosensors for glucose (Wilson & Turner, 1992). It has the practical advantages of high stability, high substrate specificity, good solubility in aqueous media and low unit cost. Also, because the mechanism of the enzymic reaction is a redox process, it naturally lends itself to electrochemical signal generation, and consequently, sensors employing glucose oxidase for the analysis of glucose are amperometric or coulometric. In contrast, relatively little attention has been given to the electrochemical measurement of glucose oxidase (Aubree-Lecat *et al.*, 1989).

The enzyme assay presented here is based on glucose sensor studies (Bennetto *et al.*, 1987, 1988) which showed that immobilization of glucose oxidase into the three-dimensional matrix of platinised carbon paper electrodes (PCP), confers a high degree of stability on the enzyme, and allows rapid transduction of charge from

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enzymatic oxidation of glucose. The GOD-PCP electrode is easily prepared, requires no added mediator, has a negligible requirement for oxygen and is highly reproducible. The response to glucose concentration covers a wide range, and the unusually large amperometric signal allows sufficient sensitivity for accurate measurements at μM levels.

Application of platinised carbon paper electrodes to amperometric determination of glucose oxidase

The present study was prompted by the observation that, when the glucose concentration is maintained at a constant level, the build-up of the amperometric signal from a PCP electrode is related to the amount of GOD added to the experimental cell. This suggested that PCP could be employed in an alternative sensor mode, *i.e.* as a basis for a sensitive electrochemical assay of GOD itself. The low-level detection of GOD is of interest for ELISA applications in which the free enzyme is released as the final stage in an analytical reaction sequence, or for related applications, in which an enzyme conjugate is employed.

An additional aim of the present study was towards a possible clarification of the mechanism of interaction of GOD with the PCP electrode. Although the many advantages of the electrode have been clearly demonstrated, uncertainties remain as to whether electron transfer to the electrode occurs *via* hydrogen peroxide as a carrier or, more directly, from some intermediate in the enzyme reaction. An understanding of how this may be related to the concentration of bulk (or adsorbed) hydrogen peroxide, could in principle aid the elucidation of this interesting problem. To this effect, some modelling of the electrode system is also presented.

Amperometric detection of labelled glucose oxidase or glucose oxidase conjugates

An additional objective of the present study was to devise an assay for particular glucose oxidase conjugates to be used in an assay for low-levels of pesticides (Allen *et al.*, 1994). The conjugate of GOD with N-(7-amino-4-methyl-coumarin-3-propanoyloxy)-succinimide was selected for study.

THEORY

The oxidation of glucose by glucose oxidase in the presence of dissolved oxygen occurs with the following mechanism:

$$\text{glucose} + \text{GOD(FAD)} \rightarrow \text{gluconolactone} + \text{GOD(FADH}_2\text{)}$$

$$\text{GOD(FADH}_2\text{)} + \text{O}_2 \rightarrow \text{GOD(FAD)} + \text{H}_2\text{O}_2$$

A flavin moiety at the active site of the enzyme accepts two electrons and two protons from a glucose molecule, which are subsequently passed onto molecular oxygen yielding hydrogen peroxide. The hydrogen peroxide can be electrochemically oxidized to oxygen at a sufficiently positive electrode potential, releasing the electrons and protons that were originally on glucose. Alternatively, the natural co-substrate of the enzyme, oxygen, can be replaced with a mediator such as ferrocene or ferricyanide (Cass *et al.*, 1984). The current from the oxidation of the hydrogen peroxide or the reduced mediator can be related to the glucose concentration, and forms the basis of many sensors for glucose.

$$\text{H}_2\text{O}_2 \rightarrow \text{O}_2 + 2\text{H}^+ + 2\text{e}^-$$

Under certain conditions, the relationship between the bulk hydrogen peroxide concentration in the cell electrolyte and the steady state oxidation current, i_s, approaches linearity with a proportionality constant k_1:

$$i_s = k_1 \cdot [\text{H}_2\text{O}_2] \quad (1)$$

Thus k_1 is the slope of a plot of the hydrogen peroxide concentration against i_s at each hydrogen peroxide concentration, and it can be determined by the addition of known quantities of hydrogen peroxide to the cell and measurement of i_s over an appropriate range of concentration. If the response of the cell to changes in the hydrogen peroxide concentration is significantly faster than the rate of production of hydrogen peroxide by the enzyme, then the current, i, due to the oxidation of hydrogen peroxide from the enzyme reaction will obey Eq. (1). Substituting i for i_s in Eq. 1 and differentiating yields an expression for the rate of current increase:

$$\frac{di}{dt} = k_1 \cdot \frac{d[\text{H}_2\text{O}_2]}{dt} \quad (2)$$

For a fixed quantity of enzyme the relationship between the initial rate of substrate utilization and the substrate concentration is given by the

Michaelis–Menten equation. When enzyme is added to the cell, hydrogen peroxide will be generated by the enzyme at the same rate as glucose is used up, and destroyed at a rate proportional to the current. Combining the Michaelis–Menten equation with Faraday's law, the initial rate of change of hydrogen peroxide with time is given by:

$$\frac{d[H_2O_2]}{dt} = \frac{\left(\frac{d[H_2O_2]}{dt}\right)_{max} \cdot [glucose]}{k_m + [glucose]} - \frac{i}{n \cdot F \cdot v} \quad (3)$$

where

$\left(\frac{d[H_2O_2]}{dt}\right)_{max}$ = rate of substrate utilization at enzyme saturation (mol dm^{-3} s^{-1});

k_m = the Michaelis–Menten constant (mol dm^{-3});

n = No. of e^- transferred per molecule of H_2O_2 oxidized (2);

F = Faraday constant (96500 C mol^{-1});

v = cell volume (dm^3).

The initial rate of substrate utilization when the enzyme is saturated can be calculated from the enzyme concentration [GOD] (mol dm^{-3}), and its specific molar activity, A, in units of (mol substrate) (mol enzyme)$^{-1}$ s^{-1}, where

$$\left(\frac{d[H_2O_2]}{dt}\right)_{max} = A \cdot [GOD] \quad (4)$$

A short time after adding enzyme to the cell, when i is small, the Michaelis–Menten equation is valid, the second term in Eq. 3 can be ignored, and on combining Eq. 2, 3 and 4, it can be written that

$$\frac{di}{dt}(i \rightarrow 0) = \frac{k_1 \cdot A \cdot [GOD] \cdot [glucose]}{k_m + [glucose]} \quad (5)$$

Equation 5 predicts that when enzyme is added to the cell, the current arising from the oxidation of hydrogen peroxide from the bulk electrolyte will initially increase linearly with a slope proportional to the enzyme concentration. This property of the system allows it to be used as an electrochemical sensor for glucose oxidase in free solution. From Eq. 3, it can be seen that after a long period of time the current will pass through a maximum, when the first and second terms balance, and then decrease as the glucose becomes used up. However, equation 3 cannot be applied directly for long periods of time, because the assumptions made in its derivation break down.

EXPERIMENTAL

Materials

The working electrodes were of platinised carbon paper (EFCG-S, E-tek) consisting of pyrolytic carbon particles (XC-72, diam. 30 nm coated with platinum particles (0·35(±0·05) mg cm^{-2}, diam. 1·5–2·5 nm), bound with PTFE and deposited onto a conducting carbon paper support (Toray, TGPH-120, thickness 0·35 mm).

Glucose oxidase from *Aspergillus niger* (Biozyme) was made up as a stock solution (1 ml, 5 mg ml^{-1}, pH 7, 0·1 M phosphate buffer, 0·1 M KCl), stored at 4°C and diluted in buffer as needed. The RMM of glucose oxidase is 160,000 g mol^{-1}. Glucose (1 M, pH 7, 0·1 M phosphate buffer, 0·1 M KCl) was prepared as a stock solution, stored at 4°C and diluted in buffer as needed. The buffer and glucose solutions were filtered (0·2 μm, Minisart) into sterile bottles to prolong their useful life. Glucose oxidase was covalently coupled to N-(7-amino-4-methyl-coumarin-3-propanoyloxy)-succinimide by mixing the enzyme (40 mg) in sodium carbonate buffer (4 ml, pH 8·6, 0·05 M) with a 20 molar excess of the coumarin (1·88 mg) at room temperature for 2 h. Unreacted coumarin was removed by gel filtration, in which the enzyme conjugate (~5 mg ml^{-1}) was eluted with phosphate buffer (pH 7, 0·1 M, 0·5 M in NaCl). The specific activity of the glucose oxidase (138 μmol min^{-1} mg^{-1}, 368 (mol substrate) (mol enzyme)$^{-1}$ s^{-1}, in air saturated solution) was determined using a standard colorimetric technique. The concentration of the conjugate solution was estimated from its activity, assuming no loss in activity during the conjugation reaction.

All the reagents were of Analar grade and were used without further purification.

Apparatus

The apparatus is shown in Fig. 1, where the cell is drawn to scale and the remainder of the apparatus is represented schematically. A conventional three-electrode arrangement was used in which the working electrode was polarized relative to the reference, and the current was

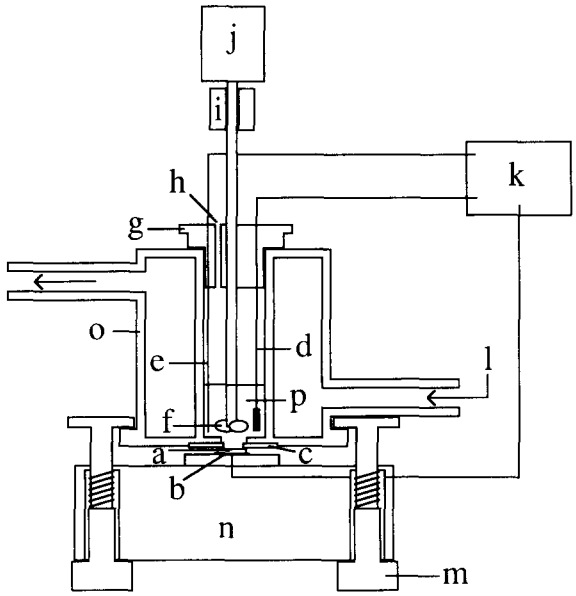

Fig. 1. Schematic diagram of the apparatus with the cell drawn to the scale shown. (a), working electrode; (b), platinum disc contact; (c), silicone rubber gasket; (d), platinum counter electrode; (e), Ag/AgCl reference electrode; (f), glass propeller; (g), PTFE stopper; (h), injection port; (i), PTFE collar; (j), motor; (k), potentiostat; (l), water from constant temperature bath; (m), sprung catch to hold cell onto base; (n), perspex base; (o), glass cell; (p), electrolyte.

measured between the auxiliary and working electrodes.

The cell was a glass cylinder oxygen electrode (Rank) in which the base had been modified to accommodate a disc of platinized carbon paper material held flat between a platinum disc contact on its underside and a silicone rubber gasket on its upper-side. The inner diameter of the gasket (5 mm) determined the geometric area of the electrode (0·20 cm^2) exposed to the solution. The counter electrode was a platinum rod (diameter 1 mm) with a sheet of platinum (7 mm × 7 mm × 0·1 mm) attached to the end. The reference electrode (Ag/AgCl, 0·1 M KCl, E = 0·288 V *vs.* NHE) was a silver rod (diameter 1·5 mm) coated with silver chloride, and all solutions were 0·1 M in potassium chloride. All subsequent voltage values are quoted relative to this reference. The counter and reference electrodes were held in place by a stopper in the top of the cell (PTFE) which fixed effectively the relative positions of the electrodes. The cell was stirred with a glass propeller driven from an overhead motor, in order to minimize electrical noise. The stirrer speed was measured directly using a small magnet at the top of the propeller, a search coil and an oscilloscope (Gould, OS4200). A travelling microscope was used to adjust the propeller height relative to the cell to within 0·01 mm. The temperature of the cell was maintained at 25(±0·1)°C by circulating water from a constant temperature bath (ZD, Grant) through the water jacket. The cell assembly was surrounded by an earthed Faraday cage to eliminate any electrical noise from the overhead stirrer or any other source outside the cell.

The PC based computer-controlled potentiostat system (PSTAT10, Autolab, Utrecht) allowed current, voltage and time data to be recorded and processed digitally. Pre-programmed sets of instructions could be carried out and data files saved automatically using batch files running under the DR DOS 6 operating system. A batch file consisted of a list of procedures that were carried out in order, allowing a high degree of consistency to be achieved between experiments. For current values of less than 100 μA a software filter with a time constant of 1 s was applied to the current data, reducing the scatter by 40%. Values of i were recorded every 1 s and di/dt was calculated using linear regression over the time period of interest. In order to simplify the interpretation of the results, enzyme was not added to the cell until di/dt for the background current was a constant over the time period of interest. The background values of di/dt (which were zero or an insignificantly small value) were subtracted from the initial values recorded after the addition of enzyme.

Methods

Preparation of electrodes

Individual working electrodes (diam 6·5 mm) were cut in batches of 20 from a sheet of the electrode material using a hole borer and then wetted by soaking in propan-2-ol for 10 min. Excess alcohol was removed by boiling the electrodes in 500 ml of water for 2 h. The electrodes were stored in water before use and in buffer (0·1 M phosphate, pH 7, 0·1 M KCl) after any contact with enzyme.

Before assembling the cell, the counter electrode was cleaned by heating to red heat in a bunsen flame and the reference electrode was washed in water.

Cell assembly

The glass cell was cleaned in chromic acid and dried in air. With the cell upside down, the rubber gasket was positioned centrally over the hole in the base and the working electrode was placed on the gasket. The cell was inverted and mounted on the perspex base such that an electrical contact was made with the base and a water-tight seal was formed between the gasket and the cell. The water jacket was connected up to the constant temperature bath. Buffer or glucose solution was added to the cell; the stopper holding the electrodes and stirrer was fitted. The cell was levelled with a spirit level. The stirrer and cell positions were adjusted to enable the stirrer to rotate freely at low speed (~150(±2) rpm). The stirrer propeller was adjusted to within 0·01 mm until the distance between the propeller and the base of the cell was approximately 1 mm. The stirrer speed was increased to 950(±10) rpm and the cell was left for 5 min while the temperature equilibrated at 25°C. Solutions were added to the cell through the injection port using glass syringes (Hamilton).

Amperometric response to additions of glucose oxidase

Dependence on polarizing voltage

The cell was set up containing a working electrode (prepared as described in section 3.3.1.) in contact with stirred buffer (3 ml, pH 7, 0·1 M phosphate buffer, 0·1 M KCl). Glucose solution (30 μl, 1 M, pH 7, 0·1 M phosphate buffer, 0·1 M KCl) was added, making the final concentration 9·9 mM. With the stirrer turned on, the working electrode was polarized at 0·1 V and the current was recorded. After 1 h glucose oxidase solution (500 ng, 0·1 ml, 5 μg ml^{-1}, pH 7, 0·1 M phosphate buffer, 0·1 M KCl) was added to the cell. This procedure was repeated for voltages of 0·2, 0·3, 0·4, 0·5 and 0·6 V.

Dependence on glucose concentration

The cell was set up as described above. Glucose solution (30 μl, 1 M, pH 7, 0·1 M phosphate buffer, 0·1 M KCl) was added to the cell, to give a final glucose concentration of 9·9 mM. The working electrode was polarized at 0·4 V and the current was recorded. After 1 h, glucose oxidase solution (500 ng, 0·1 ml, 5 μg ml^{-1}, pH 7, 0·1 M phosphate buffer, 0·1 M KCl) was added to the cell and the current was recorded for a total of 2·2 h. This procedure was repeated for additions of 100 μl of 100 mM glucose, 15, 30, 50, 93, and 142 μl of 1 M glucose solution, providing a range of glucose concentrations from 3·2 to 45 mM.

Dependence on enzyme concentration

A partially optimized standard procedure based on the results of the previous experiments was adopted and is described below. The cell was set up containing a pretreated working electrode (100 cycles) in contact with stirred glucose solution (2 ml, 30 mM, pH 7, 0·1 M phosphate buffer, 0·1 M KCl). The working electrode was polarized at 0·45 V for 100 s and then left at open-circuit. When the open-circuit working electrode potential had decayed to 0·4 V, the working electrode potential was automatically switched to 0·4 V for a total of 900 s and the current was recorded. After 500 s, a known quantity of enzyme in 100 μl of buffer was injected into the cell. The volume of enzyme solution injected was kept constant at 100 μl to standardize the small change. At the end of the 900 s polarization period the working electrode was set to open-circuit, and the contents of the cell were washed out with fresh glucose solution three times to remove any freely dissolved enzyme. The procedure could then be repeated, from the 0·45 V polarization stage, using the same electrode and without the need to cycle it between runs.

This standard procedure was carried out in duplicate for glucose oxidase additions of 1, 10, 25, 50, 75, 100 and 500 ng. To observe the long-term response of the cell to the addition of glucose oxidase (100 ng) one of the standard procedure runs was continued for a total of 22 h.

Prior to the establishment of the standard procedure, the cell response to glucose oxidase conjugate was determined. The cell was initially set up for the standard procedure but with a working electrode prepared as described earlier (see "Preparation of electrodes"). The working electrode was cycled between +1·4 V and −0·2 V 10 times at 50 mV s^{-1}, and then set to open-circuit. When the open-circuit potential had drifted to 0·4 V the working electrode potential was manually switched to 0·4 V. After polarizing the cell for 30 min, glucose oxidase conjugate (25 ng, 0·1 ml) was added to the cell and the current was recorded for a further 30 min. The contents of the cell were washed out and the

procedure was repeated for a range of conjugate concentrations (0, 33, 50, 75 and 100 ng, in 2·1 ml) using a fresh electrode each time.

Reproducibility of the cell response to glucose oxidase

The standard procedure was repeated automatically from a batch file which included a 5 min pause between runs during which the cell was washed out with fresh glucose solution. A single electrode was used to determine the response to 500 ng (12 runs), 100 ng (18 runs) and 25 ng (15 runs) of glucose oxidase.

Oxidation of hydrogen peroxide at the working electrode

The relationship between the bulk concentration of hydrogen peroxide and the steady state current due to its oxidation at the working electrode was determined. The standard procedure for determining the cell response to enzyme was repeated, using the same electrode and adding hydrogen peroxide in place of enzyme. After polarizing the cell for 500 s at 0·4 V, ten additions of hydrogen peroxide (0·844 mM, 0·1 ml, in 30 mM glucose, pH 7, 0·1 M phosphate buffer, 0·1 M KCl) were made at 600 s intervals. This procedure was subsequently repeated for higher hydrogen peroxide concentrations with five additions of 0·2 ml, 8·44 mM followed by a further five of 0·2 ml, 84·4 mM.

RESULTS AND DISCUSSION

Electrode pretreatment

When freshly prepared working electrodes were polarized at 0·4 V in buffer only, a large initial charging current was observed superposed on top of a small and continuously decaying current. The latter could have been attributed to the oxidation of surface groups on the carbon electrode, or to residual alcohol from the wetting procedure. In the presence of glucose (30 mM) a continuously decaying current arising from the oxidation of glucose was observed. (Unplatinized carbon paper electrodes gave a much smaller glucose oxidation current). This current was approximately 100 μA after 15 min and varied by up to 20% between electrodes. Pretreatment of the electrode by cycling in buffer between +1·4 V and −0·2 V 100 times at 50 mV s^{-1} decreased the background by a factor of 20. During the cycling procedure (in buffer only) a small irreversible shoulder was apparent on the voltammogram at +0·83 V in the positive sweep direction, which decreased on successive cycling. This shoulder could be attributable to the oxidation of platinum on the electrode surface. It is possible that the decrease in the size of the shoulder and the decrease in the rate of background glucose oxidation on successive cycling are linked. Polarization of the working electrode at 0·4 V resulted in a slow decrease in the background current, which stabilized at approximately 1 μA after an electrode had been re-used many times. The equilibrium open-circuit potential in the presence of glucose was +0·16 V. The time taken for the background current to stabilize was decreased significantly by eliminating the charging current. This was achieved by polarizing the working electrode for a short period at a potential more positive than the final polarizing potential, disconnecting the cell, and then allowing the open circuit potential to drift towards its equilibrium value (+0·16 V). When the open circuit potential had reached the final polarizing voltage, the cell was polarized. This did not involve a change in potential at the electrode surface, and no charging current flowed. Typically, the current was zero at zero time and reached a plateau (within the scatter of the data) after 5 min. The drift of the open circuit potential in a negative direction towards its equilibrium value is probably caused by some spontaneous electrochemical process such as surface reformation, resulting in the build up of negative charge at the working electrode surface. If this process still occurs after polarizing the working electrode, electrons must flow from the electrode surface to the potentiostat in order for the potential to remain constant, thereby generating the observed positive current.

Amperometric response to additions of glucose oxidase

Dependence on polarizing voltage

Addition of glucose oxidase (500 ng, 1 nM in cell) to the cell resulted in an immediate increase in the current, which passed through a maximum then decayed over a number of hours. For the first 5 min after the addition of enzyme the

current-time plots were substantially linear, enabling initial values of di/dt to be obtained. The dependence of di/dt on the polarizing voltage is shown in Fig. 2.

Figure 2 shows that changing the polarizing potential from 0·1 to 0·4 V results in an increase in di/dt, but beyond 0·4 V no further significant increase occurs. The increase in di/dt up to 0·4 V is to be expected from the Tafel equation, although this equation is not explicitly obeyed due to limiting kinetic factors. The fact that di/dt no longer increases above 0·4 V is probably because at that voltage the rate of oxidation of hydrogen peroxide at the working electrode surface is equal to the rate of its arrival there from the enzyme in the bulk electrolyte. Thus, in the absence of transport limitation, the di/dt value is limited by the enzyme kinetics, and further increases in the potential do not increase di/dt.

Dependence on glucose concentration

Figure 3 shows the dependence of di/dt (on addition of glucose oxidase to the cell), on glucose concentration. Values of di/dt were determined for the addition of glucose oxidase (500 ng, 1 nM in cell) at a polarizing potential of 0·4 V and at glucose concentrations between 0 and 45 mM. Figure 3 shows that di/dt increases

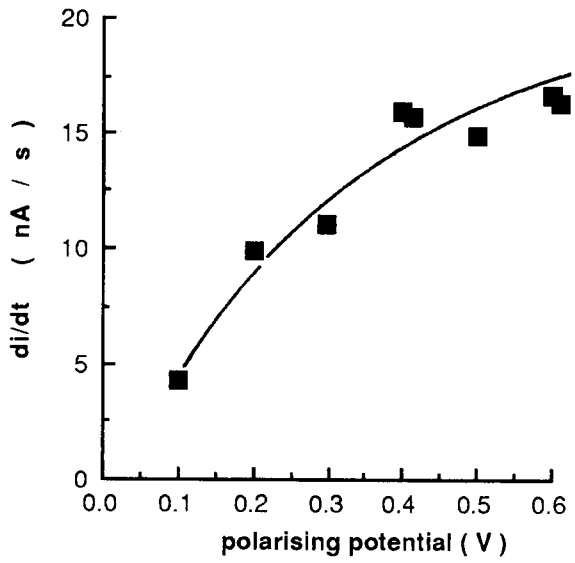

Fig. 2. The dependence of the initial value of di/dt on the potential for the addition of glucose oxidase (500 ng, 1 nM; 3·04 ml cell volume; glucose concentration, 9·9 mM).

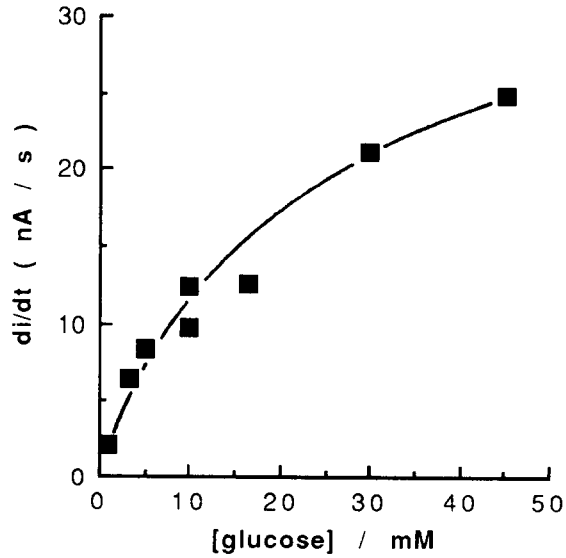

Fig. 3. The dependence of the initial value of di/dt on the glucose concentration for additions of glucose oxidase (500 ng) to the cell at a potential of 0·4 V.

with increasing glucose concentration. This concentration dependence is similar to that for the initial rate of an enzyme as described by the Michaelis–Menten equation. Maximising di/dt at a particular enzyme concentration will maximise the sensitivity of a sensor for glucose oxidase employing di/dt as the transduction signal. From the data presented in Fig. 2 and Fig. 3, a polarizing potential of 0·4 V and a glucose concentration of 30 mM were chosen as near optimal conditions for a glucose oxidase sensor.

Dependence on enzyme concentration

In Fig. 4 an example of the long term response of the cell to the addition of glucose oxidase (100 ng) is presented. The current (corrected for the initial background value of 2 μA) passed through a maximum of 19 μA, 8 h after adding the enzyme, then decayed towards the background level. Over the time scale displayed in Fig. 4, the essentially non-linear nature of the i/t profile is evident. However, as shown in Fig. 5 good linear approximations can be made over the first 5 min (correlation coefficients >0·95). Figure 5 shows a typical result for determining di/dt by the standard procedure. On initial polarization of the cell, no current flowed because the charging current had been eliminated in the prepolarization stage. After a short period a

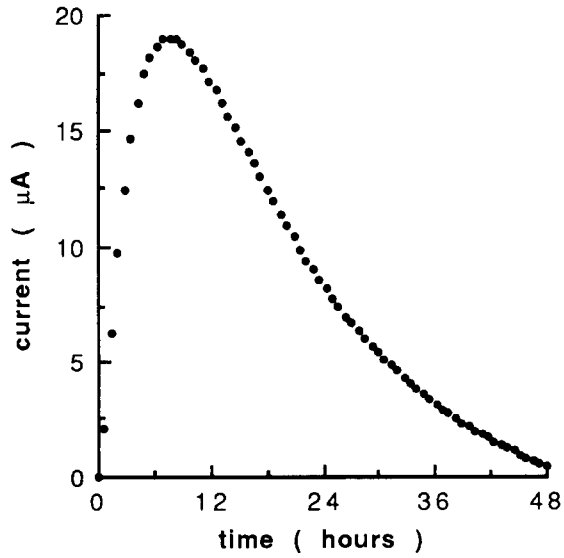

Fig. 4. Amperometric response to addition of glucose oxidase (100 ng) over a long time scale (polarizing potential, 0·4 V; glucose concentration, 30 mM).

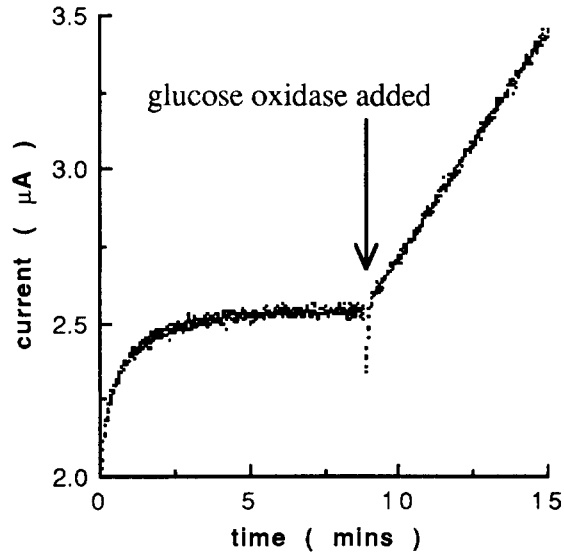

Fig. 5. Amperometric response to addition of glucose oxidase (100 ng) over a short time scale (polarizing potential, 0·4 V; glucose concentration, 30 mM; the final concentration of glucose oxidase in the cell was 0·317 nM).

stable background current with a small value of di/dt was established.

The dependence of di/dt on the glucose oxidase and glucose oxidase conjugate concentrations are shown in Fig. 6. Both data sets are substantially linear giving a calibration slope for the unconjugated enzyme of 8·3(±0·2) A s^{-1} mol^{-1} dm^3 and 9·6(±0·6) A s^{-1} mol^{-1} dm^3 for the conjugate, both with small negative intercepts on the di/dt axis (errors quoted are standard errors in the slopes. The correlation coefficients were 0·991 and 0·959 respectively). The dashed line in Fig. 6 is the least squares fit to the combined data sets and has a slope of 8·8(±0·3) A s^{-1} mol^{-1} dm^3 with a correlation coefficient of 0.969. The data in Fig. 6 show that the system can be used as a sensor for glucose oxidase or its conjugate.

Reproducibility of response to glucose oxidase

The reproducibility of the initial values of di/dt at three different fixed concentrations of glucose oxidase (0·063, 0·317, and 1·59 nM) is indicated in Fig. 7. Each individual run within a series took approximately 25 min. The values of di/dt

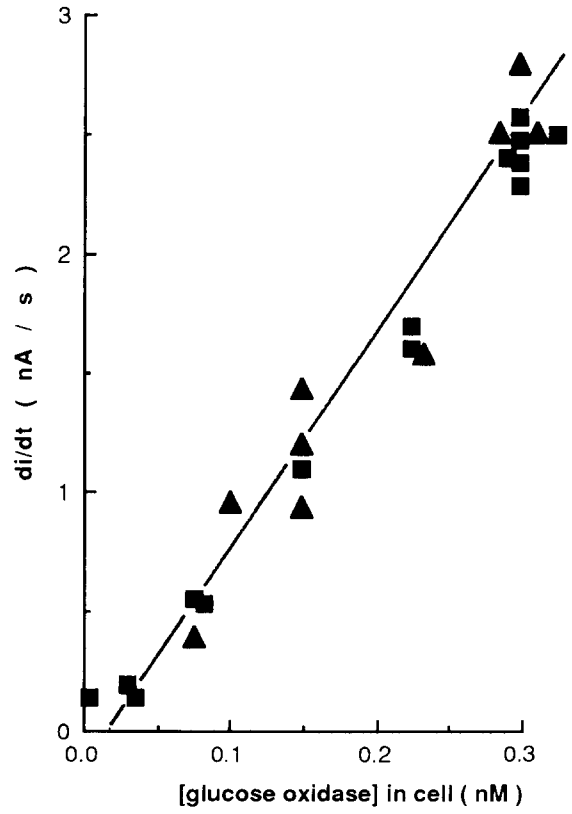

Fig. 6. The dependence of the initial value of di/dt on the concentration of glucose oxidase (■) and glucose oxidase conjugate (▲) (polarizing potential, 0·4 V; glucose concentration, 30 mM).

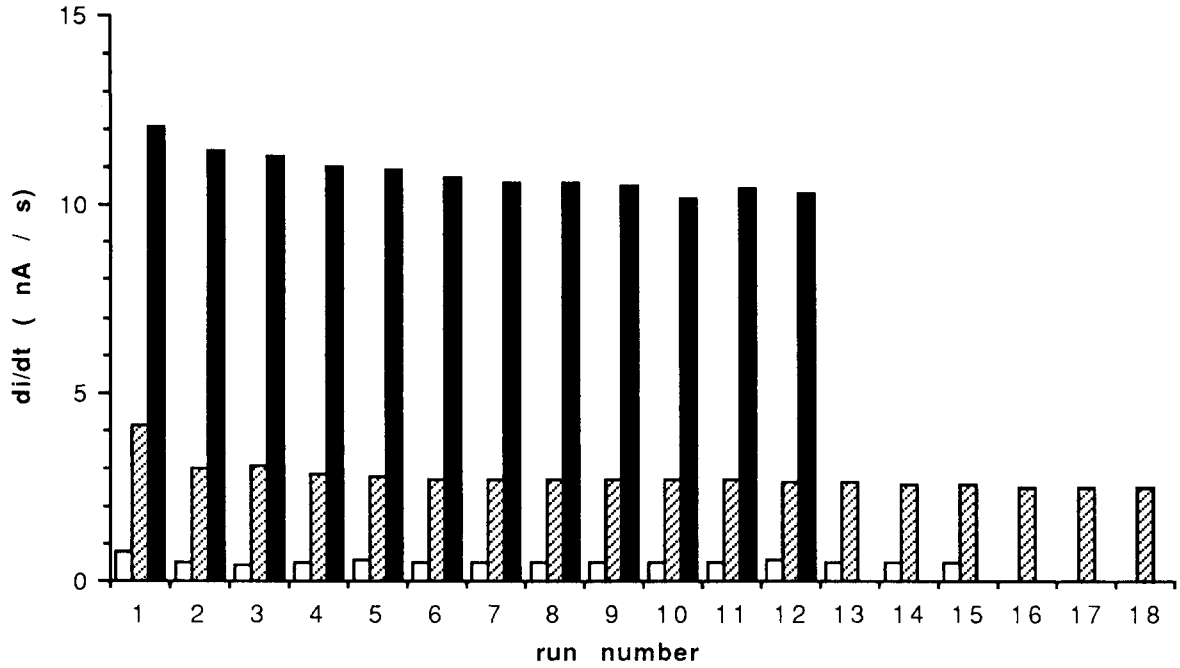

Fig. 7. The reproducibility of the standard procedure used to determine di/dt (glucose oxidase concentration in the cell, 0·063 (□), 0·317 (▨), 1·59 nM (■); polarizing potential, 0·4 V; glucose concentration, 30 mM).

for the runs numbered 1 were each carried out at the beginning of a new day and were larger than the values for subsequent runs. Discounting the first run in a day, the standard deviations over 10 runs were 3% of the mean values for each of the glucose oxidase concentrations in Fig. 7. This data shows how a single electrode can be re-used for many determinations of di/dt with only a slight loss of signal which is more pronounced at higher concentrations. The reason for this decay in response in unknown at present. Figure 7 also suggests that it should be possible to obtain results more reproducible than the essentially preliminary data shown in Fig. 6.

Oxidation of hydrogen peroxide at the working electrode

Values of the steady state current, i_s, for the oxidation of hydrogen peroxide at the working electrode were determined for bulk hydrogen peroxide concentrations up to 40 mM. They increased with hydrogen peroxide concentration to a plateau value of 400 μA at 40 mM. The hydrogen peroxide concentration produced by the highest enzyme concentration shown in Fig. 7 (1·59 nM) over the period of the standard procedure (400 s) was 0·22 mM (specific activity of GOD = 138 μmol min^{-1} mg^{-1}, 500 ng, volume = 2·1 ml). Figure 8 illustrates the dependence of i_s on the hydrogen peroxide concentration over a range that includes all the hydrogen peroxide concentrations produced in the calibration data (Fig. 6). A linear approximation to

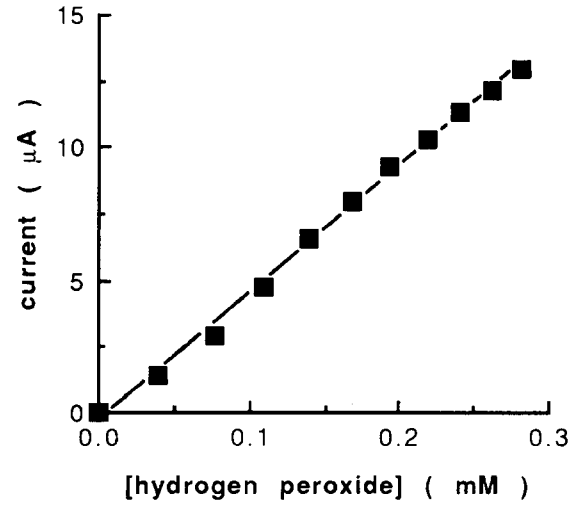

Fig. 8. The dependence of the steady state current for the oxidation of hydrogen peroxide on the bulk hydrogen peroxide concentration (polarizing potential, 0·4 V; glucose concentration, 30 mM).

the data produced a value for k_1 of 0·047(±0·0009) A mol^{-1} dm^3 (cor. coef. = 0·997). Substitution of this value for k_1 into Eq. 5 from the theory predicted a value for the slope of the calibration plot (di/dt *vs.* [GOD]) of 8·6(±0·2) A s^{-1} mol^{-1} dm^3, in agreement with the experimental value for non-conjugated glucose oxidase of 8·3(±0·2) A s^{-1} mol^{-1} dm^3. The close correlation between theory and experiment strongly supports a mechanism for the glucose oxidase sensor in which hydrogen peroxide produced by enzyme in bulk solution is oxidized at the working electrode.

An alternative electron transduction mechanism not involving oxygen/hydrogen peroxide could occur if the platinum particles on the working electrode surface were able to reach into the active site of the enzyme, to allow direct electron transfer from the reduced flavin moiety at the active site to the electrode. However, when catalase was added to the cell electrolyte the current rapidly decreased with pseudo first-order kinetics, indicating that hydrogen peroxide was an essential component of the transduction mechanism.

An alternative mechanism to account for the way in which the current increases on addition of enzyme to the cell is that enzyme adsorbs onto the working electrode from solution. The adsorbed enzyme generates hydrogen peroxide at the surface where it is rapidly oxidized, producing a current proportional to the amount of enzyme at the electrode surface. The rate of increase in the current would be proportional to the rate of the adsorption process, which in turn, is proportional to the enzyme concentration, under the conditions employed. If this mechanism were operating and enzyme bound to the surface was the sole source of electrons for the current signal, then the hydrogen peroxide would be localized at the electrode surface. When catalase is added to the cell it could also adsorb at the electrode surface and decompose the hydrogen peroxide. This alternative mechanism is therefore not ruled out by the data.

If irreversible binding of glucose oxidase to the working electrode occurred, then, on successive re-use of the working electrode, it would be expected that the background current would increase in proportion to the amount of bound enzyme. Because this effect was not observed, it appears that no significant irreversible binding of glucose oxidase occurred. However, reversible binding of glucose oxidase in which the enzyme is removed by the washing procedure has not been ruled out. Previous work has shown that when an electrode is exposed to a much higher concentration of glucose oxidase than was used in the sensor experiments (*e.g.* 5 mg ml^{-1}), the electrode surface becomes saturated with enzyme bound physically and irreversibly. The saturated electrode was shown to be relatively insensitive to the presence of catalase in bulk solution, suggesting that oxygen cycling close to the electrode surface was occurring (Bennetto *et al.*, 1988). Thus it is possible that different mechanisms operate in the case of the GOD-PCP glucose sensor and the PCP-based GOD sensor.

If glucose oxidase were released from an immuno-column at the same concentration as a pesticide analyte, then the detection limit for the glucose oxidase sensor of 56 pM is well within the EC limit of 5 nM for pesticides. However, construction of a practical ELISA for a pesticide would inevitably involve some compromise in sensitivity, for which reason no detection limit for a pesticide can yet be derived from the data presented here.

CONCLUSIONS AND FURTHER WORK

An electrochemical assay for glucose oxidase operating in the batch mode has been devised, having a detection limit of 25 ng (56 pM) and a time requirement of 25–30 min, which compares very favourably with standard colorimetric assays. Similar limits of detection are also established for the glucose oxidase-coumarin conjugate, suggesting that this method could be employed in conjunction with an immuno-sensing technique for assay of very low levels of pesticides. The sensor is based on the fact that when glucose oxidase is added to the cell, the current increases at a rate proportional to the glucose oxidase concentration. Results were obtained that are consistent with a mechanism in which hydrogen peroxide, produced in bulk solution by the glucose oxidase, becomes oxidized at the working electrode to produce the current signal. The slope of the sensor calibration plot was predicted successfully from the proposed mechanism.

The aims of further work include extension of the existing methodology to produce a flow system in which the output of an immuno-column

is passed directly into an electrochemical cell. The cell could be of the flow-through type, in which the analyte is passed across or through the working electrode (Zilkha *et al.*, 1994). Inclusion of a delay loop between the immuno-column and the electrochemical detector would increase the time available for the production of hydrogen peroxide by the enzyme, thereby increasing the sensitivity of the sensor. The detection limit could be lowered even further by decreasing the scatter in the current output of the cell, either by treatment of the electrode or by further signal processing.

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