The Flow-injection Analysis of D-Glucose Using a Flow-cell with Immobilized Peroxidase and Its Application to Serum

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The D-glucose concentration in a serum sample of less than 0.01 cm^3 could be continuously determined at the rate of about 20 samples/h by the use of a chemiluminescent reaction between luminol and hydrogen peroxide, a flow-cell with immobilized peroxidase, and a column with immobilized glucose oxidase. By immobilizing peroxidase as a chemiluminescent catalyst inside the flow-cell, simplification could be achieved with regard to apparatus, reagents and operation. The detection limit of hydrogen peroxide was about 5×10^{-6} mol dm^{-3}, and the calibration curves for both hydrogen peroxide and D-glucose were approximately linear in the range of 5×10^{-5}—1×10^{-3} mol dm^{-3}, a range which corresponded to the glucose concentration, 0.1—18 mg cm^{-3}, obtained by diluting serum to 50 times its initial volume.

The present study was carried out with the object of establishing a simple, rapid, and economical method by which D-glucose in a sample could be determined continuously by measuring the chemiluminescence (CL) emitted on a flow-cell with immobilized peroxidase (EC 1.2.1.7)(PO).

Generally speaking,[1] CL analysis is superior to fluorometric or absorptiometric analysis in the following points: 1) High sensitivity, 2) a wide dynamic range, 3) a simple apparatus (needs no spectrometer), and 4) an easy establishment of a continuous analysis system.

Several reports[2,3] on the chemiluminescent determination of glucose have, therefore, been published; in them, the glucose in a sample is first converted to hydrogen peroxide (H_2O_2) with fixed glucose oxidase (EC 1.1.3.4) (GO) and then determined by measuring the intensity of the CL emitted by the reaction between the H_2O_2 and 5-amino-2,3-dihydro-1,4-phthalazinedione(luminol) in the presence of potassium hexacyanoferrate(III) ($K_3[Fe(CN)_6]$) as a catalyst.

However, this method requires a considerable amount of $K_3[Fe(CN)_6]$, an environmental pollutant; moreover, it makes it imposible to recycle the used $K_3[Fe(CN)_6]$. These problems can, however, be overcome by the use of a flow-cell with immobilized PO, for PO acts on the reaction between H_2O_2 and luminol as a catalyst.[4] Though Freeman *et al.*[5] developed a CL fiber-optic probe for a batch-by-batch measurement of H_2O_2 by immobilizing the PO in a polyacrylamide gel on the end of a fiber-optic, the probe can not be used for a continuous measurement of H_2O_2 because the enzyme phase prepared by immobilizing PO and luminol by physical entrapment in a polyacrylamide gel has to be kept with a definite concentration of the luminol solution. With the use of the flow-cell with immobilized PO which has previously been proposed by the present authors, the following advantages can be expected: 1) A simple system for a how-injection analysis can be easily constructed; 2) the reagents necessary for an analysis can be minimized; 3) waste treatment becomes easier than in conventional methods in which $K_3[Fe(CN)_6]$, cupper(II), cobalt(II), and so on are used as catalysts, and 4) an improvement is made in the selective measurement of glucose because of the PO specificity for H_2O_2.

In the present paper, a flow-cell with immobilized PO has been successfully applied to the determination of glucose and then extended to serum.

Experimental

Immobilization of Glucose Oxidase. GO (Toyobo, grade II, 100 U/mg) was immobilized on 80—120 mesh of porous glass beads (CPG-10, pore size 7500 nm) in accordance with the diazotization method of Weetall[6] after the introduction of an aminoalkyl group[7] onto the porous glass beads.

Immobilization of Peroxidase. Five procedures were tried for the immobilization of PO, and the following procedure was found to be most satisfactory: A glass disk 12 mm in diameter and 1.8 mm thick, with one ground plane, was refluxed for 6 h in a 2% (3-aminopropyl)ethoxysilane solution in toluene. The glass-disk was dried at 60 °C after washing with toluene, and then refluxed for 6 h in a chloroform solution containing 5% triethylamine and 10% *p*-nitrobenzoyl chloride. The glass-disk was washed with chloroform and dried. The nitro group on the glass-disk was reduced by refluxing for 1 h with a 5% sodium dithionite solution. After the glass-disk has been washed with water, it was dipped in a 1 mol dm^{-3} hydrochloric acid solution, 3 cm^3 of a 0.5 mol dm^{-3} sodium nitrite solution was added at 0 °C over 15 min, and the mixture was reacted for an additional 15 min. The glass-disk was picked up, washed with a 5% sulfamic acid solution and water in that order, and immediately dipped in a 5 cm^3 solution of PO (Toyobo, grade III, 102 U/mg) at the concentration of 1 mg cm^{-3}, the PO have previously been made to react with

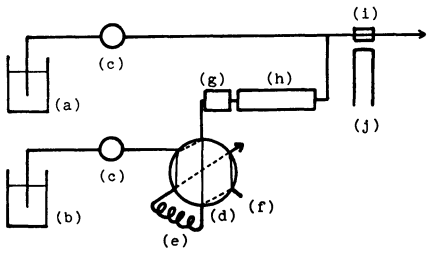

Fig. 1. Apparatus.
a: Luminol, b: buffer solution, c: pump, d: six-way cock, e: sampling loop, f: sample inlet, g: DEAE-cellulose column, h: immobilized glucose oxidase column, i: flow-cell with immobilized peroxidase, and j: photomultiplier.

the glass-disk for 48 h at 3 °C. Thus, the glass-disk with immobilized PO was obtained.

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

Procedure. The determination of glucose was done with the apparatus shown in Fig. 1. A Teflon-made tube (1 mm i. d.) and Teflon-made joints (GU-2F and GT-2F, Gasukuro Kogyo) are used for piping. The flow path between (d) and (i) is about 80 cm long. Figure 1(a) is filled with a 1.0×10^{-3} mol dm^{-3} luminol solution (pH 8.6) containing a 10^{-4} mol dm^{-3} EDTA solution and a boric acid–potassium chloride–sodium hydroxide buffer solution, and (b), with a potassium dihydrogenphosphate–sodium hydroxide buffer solution (pH 7.1). These solutions are both fed in at the rate of 0.99 cm^3 min^{-1} by means of a pump (c) (Tokyo Rikakikai, MP-12, Micro Tube Pump). By the use of the present pump, two flows can be driven simultaneously, and pulsation in a flow after mixing is minimized, thus resulting in the stabilization of a base line on a recorder chart. The capacity of the sampling loop (e) is about 0.2 cm^3, and each definite volume of the sample solution is injected into a column (g) by rotating a six-way cock (d). After being passed through a 100 mm × 3 mm i.d. column (g) charged with DEAE-cellulose (Nakarai Chemicals, 0.89 mequiv. g^{-1}), the sample solution is introduced into a 250 mm × 3 mm i.d. column (h) charged with the glass beads with immobilized GO; in this column, the β-D-glucose in a sample is converted to gluconic acid and H_2O_2 by means of an enzyme reaction. The H_2O_2 evolved is mixed with a luminol solution immediately before it enters the flow-cell (i) with immobilized PO, and the resulting solution is brought to pH 8.4 by means of the previous calibration. The CL emitted by the chemiluminescent reaction on the flow-cell with immobilized PO is converted to the photocurrent with a photomultiplier (j) (Hamamatsu TV, R-374), and the photocurrent is amplified with a Horiba OPE-402 amplifier, followed by recording with a recorder (Matsushita Communication Industrial, VP6521A). The determination of glucose in a sample is done from the peak height on a recorder chart.

The flow-cell with immobilized PO is shown in Fig. 2. The internal volume of the flow-cell is about 0.2 cm^3, and the glass-disk (d) with immobilized PO is installed as in Fig. 2.

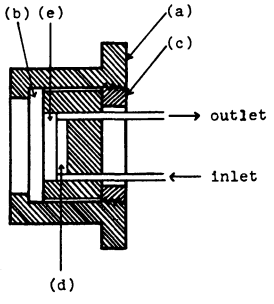

Fig. 2. Flow-cell.
a: Cell holder, d: window-glass, c: pressure screw, d: glass-disk with immobilized peroxidase, and e: flow-cell.

Results and Discussion

Immobilization of Peroxidase. In order to immobilize PO onto the flow-cell (Fig. 1(i)), an investigation was carried out with regard to the following five procedures: 1) Fixation onto the porous glass beads (CPG-10; 80—120 mesh; pore size, 7500 nm) by the glutaraldehyde method[8] after the introduction of an aminoalkyl group; 2) fixation onto the same aminoalkylated porous glass beads as 1) by the diazotization method;[6] 3) fixa tion onto the 6-Nylon disk by the glutaraldehyde method[9] after the partial hydrolysis of 6-Nylon; 4) fixation by physical entrapment[10] into a gel made of alubumin and glutaraldehyde, and 5) fixation onto the glass-disk by the diazotization method.

The glass beads with PO immobilized by Procedures 1) and 2) were charged respectively in Tygon tubes (2 mm i.d.); the tubes were subsequently coiled spirally and used as flow-cells. However, they were not sensitive enough for the present purpose, for the light generated by the chemiluminescent reaction was scattered to all directions. Both the 6-Nylon disk and the gel immobilized with PO by Procedures 3) and 4) were set as in Fig. 2(d) and used as flow-cells. They were first senstive enough, but rapidly became low in sensitivity with the lapse of time because of the lowering of the enzyme activity. The glass-disk with PO immobilized by procedure 5) was best in both the intensity of CL and its life, a and it still showed stable activity as a chemiluminescent catalyst even after analyses of up to 300 samples.

Effect of Luminol Concentration. The relationship between the luminol concentration and the CL intensity is shown in Fig. 3. As can be seen there, the maximum intensity of CL was obtained by the use of a 1.0×10^{-3} mol dm^{-3} luminol solution.

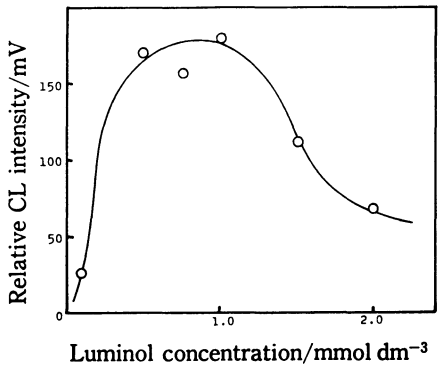

Fig. 3. Effect of luminol concentration on CL intensity.
Experimental conditions: flow rate 1.98 cm^3 min^{-1}; pH 7.8. Sample: 1.03×10^{-4} mol dm^{-3} H_2O_2.

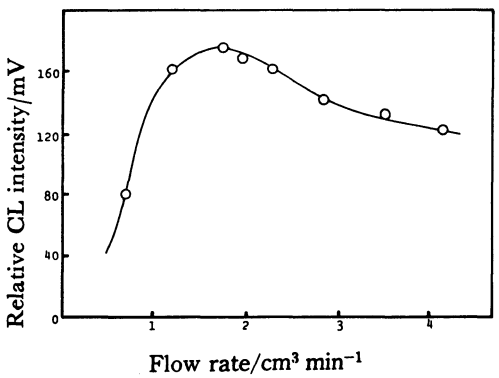

Fig. 4. Effect of flow rate on CL intensity.
Experimental conditions: 1.0×10^{-3} mol dm^{-3} luminol; pH 7.8. Sample: 1.03×10^{-4} mol dm^{-3} H_2O_2.

Effect of Flow Rate. The flow rate is an important parameter and has a close relation with the sensitivity, the precision, and the speed in flow-injection analysis. The relationship between the flow rate of a sample solution and the intensity of CL is shown in Fig. 4. The maximum in Fig. 4 is characteristic for the present flow-cell with immobilized PO, while the maximum intensity of CL appeared around a flow rate of 1.8 $cm^3\ min^{-1}$. This phenomenon is explained as follows: 1) The intensity of CL at a flow rate less than about 1.8 $cm^3\ min^{-1}$ increases with an increase in the flow rate because the amounts of luminol and H_2O_2 fed into the flow-cell within a definite period increase with an increase in the flow rate, and the flow rate does not seriously affect the diffusion amounts of luminol and H_2O_2 to the enzyme phase, and 2) the intensity of CL at a flow rate of more than about 1.8 $cm^3\ min^{-1}$ decreases with an increase in the flow rate because the amounts of luminol and H_2O_2 fed into the flow-cell within a definite period increase with an increase in the flow rate, but the diffusion amounts of luminol and H_2O_2 into the enzyme phase are considerably lowered with an increase in the flow rate. The reaction rate between luminol and H_2O_2 on the present flow-cell with immobilized PO can be expected to be very large, since the reaction between luminol and H_2O_2 in the presence of free PO has been reported by Cormier *et al.*[4] to proceed to completion within 0.2 s. This confirms the usefulness of the present flow-cell with immobilized PO for the determination of glucose.

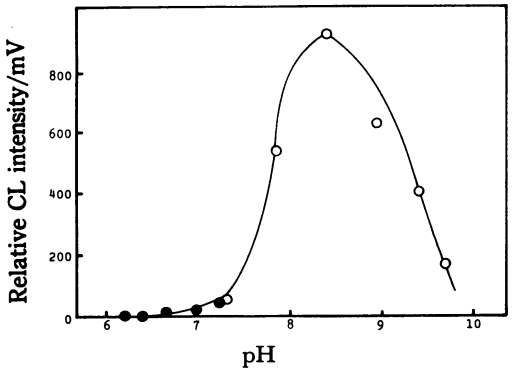

Fig. 5. Effect of pH on CL intensity.
Experimental conditions: 1.0×10^{-3} mol dm^{-3} luminol; flow rate 1.98 $cm^3\ min^{-1}$; ●: KH_2PO_4–NaOH buffer solution, ○: H_3BO_3–KCl–NaOH buffer solution. Sample: 1.03×10^{-4} mol dm^{-3} H_2O_2.

Effect of pH. The catalytic activity of free PO shows its maximum around pH 6, whereas the quantum yield of CL between luminol and H_2O_2 shows its maximum around pH 11.[11] Therefore, the optimum pH for the maximum intensity of CL should be expected in the reaction between luminol and H_2O_2 in the presence of PO. The relationship between the pH of a solution and the intensity of CL is shown in Fig. 5. As can be seen from Fig. 5, the maximum intensity was obtained around pH 8.4.

On the basis of these experimental results, the experimental conditions for the maximum intensety of CL were chosen as follows: Flow rate, 1.98 $cm^3\ min^{-1}$; luminol concentration, 1.0×10^{-3} mol dm^{-3}, and pH, 8.4.

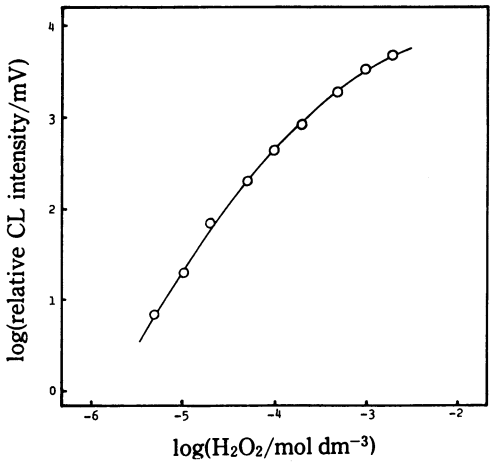

Fig. 6. Calibration curve for H_2O_2.

Calibration Curve of H_2O_2. The calibration curve of H_2O_2 as a sample is shown in Fig. 6. The time necessary for an analysis by means of the present apparatus with a DEAE-cellulose column and an immobilized GO column was about 3 min, while it was 30 s when an apparatus without the above columns was used. Since the band broadening of a sample was observed when it was passed through those columns, a sample was injected every 3 min to avoid overlapping of peaks. The detection limit for H_2O_2 was about 5×10^{-6} mol dm^{-3}, more or less inferior to that obtained by the use of $K_3[Fe(CN)_6]$ as a catalyst. The calibration curve was approximately linear between 5×10^{-5} mol dm^{-3} and 1×10^{-3} mol dm^{-3} H_2O_2. The intensity of CL at a H_2O_2 concentration of more than 1×10^{-3} mol dm^{-3} deviated from the straight line, but this was due to the fact that the experiment was carried out at a definite concentration of the luminol solution, while the chemiluminescent reaction gradually depended on the concentration of the luminol solution. At a H_2O_2 concentration of less than 5×10^{-5} mol dm^{-3}, the slope of the calibration curve shown by the log-log plot approaches 2, but this phenomenon is similar to that obtained by the CL fiber optic probe proposed by Freeman *et al.*[5]

Calibration Curve of D-*Glucose.* The calibration curve of D-glucose as a sample is shown in Fig. 7. The intensity of CL in Fig. 7 was smaller than the corresponding intensity in Fig. 6. This is mainly attributed to the fact that D-glucose is composed of about 66% of β-D-glucose, 34% of α-D-glucose, and a trace amount of aldehyde-type D-glucose, while only the β-D-glucose is made to react with GO. The large deviation from the straight line at a concentration of D-glucose of more than 1×10^{-3} mol dm^{-3} may be attributed to the treatment capacity of the column with immobilized GO. According to the present apparatus, an approximately linear calibration curve is obtained in the concentration range of 5×10^{-5}—1×10^{-3} mol dm^{-3} glucose.

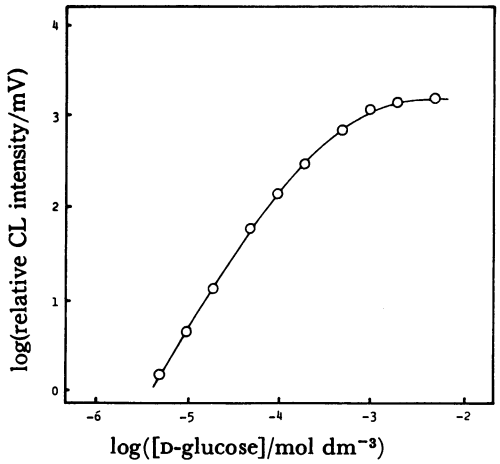

Fig. 7. Calibration curve for glucose.

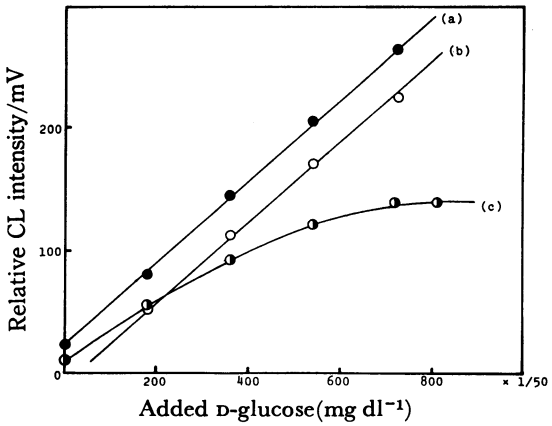

Fig. 8. Calibration curve for glucose added to serum. a: Serum+D-glucose, b: D-glucose, c: serum+D-glucose (without using a DEAE-cellulose column).

Determination of D-*Glucose in Serum.* About 1 mg cm^{-3} of glucose is contained in a normal human serum. Therefore, the concentration of glucose in a serum is brought to around 10^{-4} mol dm^{-3} by diluting it to 50 times its initial volume; it can be expected to be determined by the use of the linear part of the calibration curve in Fig. 7. Then, the relationship between the amount of glucose added and the intensity of the CL was examined after Hyland Control Serum I had been diluted to 50 times its initial volume. The results obtained are shown in Fig. 8. When an experiment was done without using a column(g) (Fig. 1), serum proteins were captured on the glass-disk with immobilized PO. This resulted in markedly lowering the intensity of CL (Fig. 8(c)), and no correlation was found between, the amount of glucose added and the intensity of the CL after analyses of 20 samples.

In order to avoid the capture of serum proteins on the above-mentioned glass-disk, a 100 mm × 3 mm i.d. column charged with various capturing agents was placed between a six-way cock (Fig. 1(d)) for sampling and a column (Fig. 1(h)) with immobilized GO. The efficiency of the capturing agents for the capture of serum proteins was examined by the use of: 1) Glass beads (80—120 mesh), 2) active charcoal (35—60 mesh), 3) silica gel (60—80 mesh), 4) activated alumina (about 200 mesh), and 5) DEAE-cellulose. Glass beads were not effective for the capture of serum proteins. Active charcoal captured serum proteins as well as glucose, and no serum proteins were observed on the glass-disk with immobilized PO. Here, the peak on a recorder chart showed tailing phenomenon and the intensity of the CL was considerably smaller than the expected value. Silica gel, activated alumina, and DEAE-cellulose were more or less effective for the removal of the interference of serum proteins, but DEAE-cellulose was most excellent in peak height and reproducibility (Fig. 8(a)).

The CL observed without adding glucose to serum in Fig. 8(a) is attributed to the glucose contained in Hyland Control Serum I. There was a parallel relationship between the calibration curve (Fig. 8(a)) obtained by adding glucose to Hyland Control Serum I and the calibration curve (Fig. 8(b)) obtained by glucose alone, where the difference between the intensities of the two lines corresponded approximately to a 1 mg cm^{-3} of glucose concentration in serum.

Judging from the complexity of serum composition, the standard addition method in which, for example, two serum samples are diluted to 50 times their initial volume with water and a definite concentration of a glucose solution respectively, seems to be most recommendable for the rapid and exact determination of glucose in serum by the present method.

It is concluded from the above results that a glucose concentration in serum of less than 0.01 cm^3 can be continuously determined at the rate of about 3 min/sample by the present method, in which a flow-cell with immobilized PO is used.

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