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A Rapid Polarographic Microdetermination of Glucose with Glucose Oxidase

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A rapid polarographic microdetermination of D-glucose with β-D-glucose oxidase in direct oxygen consumption mode is described. The apparatus composed of polarographic oxygen electrode, recorder (2 mV full scale), voltage prebox for increasing the sensitivity by cancelling unnecessary voltage of the output voltage of oxygen analyzer, and specially designed vial fixed on the magnetic stirrer. A trace amount of sodium azide was used to inhibit catalase contained in the biological sample and crude glucose oxidase. In this method, the relation between the amount of D-glucose and the amount of consumed oxygen gave a straight line at the range of 0.5 to 600 μg of D-glucose. The results obtained by this method on serum glucose with internal standard were enough consistent with those obtained by *o*-toluidine–borate method. The result on blood is obtainable within 5 min after a blood sample (5 μl) was collected from a patient.

Determination of D-glucose with β-D-glucose oxidase (EC 1.1.3.4 β-D-glucose:oxygen oxidoreductase) has recently been developed by many investigators. β-D-Glucose oxidase, one of the flavin enzymes, catalyzes the following reaction;

$$\beta\text{-D-glucose} + H_2O + O_2 \rightarrow \text{D-gluconic acid} + H_2O_2 \qquad (1)$$

It is clear from Eq. (1) that D-glucose in the sample can be measured by determination of formed D-gluconic acid or hydrogen peroxide, and also of consumed oxygen in the presence of the enzyme.

Specific microdetermination of D-gluconic acid is not known, while that of hydrogen peroxide is practiced by using *o*-tolidine,[2] *o*-anisidine,[3] or iodide[4] as the acceptor of oxygen formed from hydrogen peroxide in the presence of peroxidase. Very recently, Guilbault[5] reported on the new fluorometric microdetermination of hydrogen peroxide with homovanillic acid, and applied the method on the determination of D-glucose by β-D-glucose oxidase and peroxidase.

On the other hand, oxygen consumption method originally reported by Keilin and Hartree[6] was simplified by Kadish, *et al.*[7] who employed oxygen electrode and applied on the continuous monitoring of blood D-glucose. In 1966, Hagihara, *et al.*[8] described the determination of D-glucose by his own electrode. In 1967, Konno, *et al.*[9] reported the similar determination of D-glucose in blood in the presence of $K_3Fe(CN)_6$ and KCN for elimination of the influences of hemoglobin and catalase in blood on measurement of oxygen consumption. Updike, *et al.*[10] reported on the glucose oxidase electrode. In 1968, Kadish, *et al.*[11] reported

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2) V. Marks, *Clin. Chim. Acta*, **4**, 395 (1959).
3) E.F. Beach and J.J. Turner, *Clin. Chem.*, **4**, 462 (1958).
4) F.W. Sunderman, Jr. and F.W. Sunderman, *Am. J. Clin. Path.*, **36**, 75 (1961).
5) G.G. Guilbault, P.A. Brignac, Jr. and M. Juneau, *Anal. Chem.*, **40**, 1256 (1968).
6) D. Keilin and E.F. Hartree, *Biochem. J.*, **50**, 331 (1952).
7) A.H. Kadish and D.A. Hall, *Clin. Chem.*, **11**, 869 (1965).
8) T. Kajihara and B. Hagihara, *Jap. J. Clin. Path.*, **14**, 322 (1966).
9) Y. Makino and K. Konno, *Jap. J. Clin. Path.*, **15**, 391 (1967).
10) S.J. Updike and G.P. Hicks, *Nature*, **214**, 986 (1967).
11) A.H. Kadish, R.L. Litle and J.C. Sternberg, *Clin. Chem.*, **14**, 116 (1968).

on the determination of D-glucose in serum using oxygen electrode in rate sensing mode.

Recently, the authors devised a rapid microdetermination of D-glucose by oxygen electrode in direct oxygen consumption mode. In this determination, we used a trace amount of sodium azide to inhibit the catalase contained in the sample and crude β-D-glucose oxidase, and increased the sensitivity by cancelling unnecessary voltage with voltage prebox. A preliminary report had appeared.[12]

Materials and Apparatus

Pure and crude β-D-glucose oxidase used in this study were gifts from Dr. K. Kusai of Nagase & Co.Ltd., Osaka and the crude enzyme was purified by his method.[13] The almost purified enzyme preparation, however, contained a trace amount of catalase. The enzyme was dissolved in distilled water. Reagent grade D-glucose, usually α-D-glucose, was dissolved in distilled water (1%) and stored overnight prior to use, to complete the mutarotation. Sodium azide, a strong inhibitor for catalase, was dissolved in distlled water (0.1%). Acetate buffer (0.2M, pH 5.6) containing sodium chloride (final concentration, 0.4%) was also prepared. All reagent solution must be shaken or aerated prior to use. A kit for the *o*-toluidine–borate method was purchased from Katayama Chemical Industries, Ltd., Osaka.

For measurement of oxygen consumption, a polarographic oxygen analyzer (Model 777, Beckman Instruments Inc., Fullerton, Calif., U.S.A.) connected with recorder (EPR-2TC, Toa Electronics Ltd., Tokyo, Japan) was used. EPR-PREBOX (PB-30A), voltage–current prebox (Toa Electronics Ltd., Tokyo, Japan) which can cancel some unnecessary component of the output voltage of the oxygen analyzer and expand the scale of readings of the recorder, was attached to the recorder when necessary.

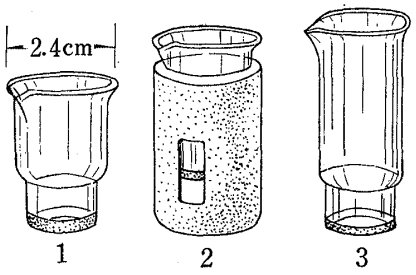

Fig. 1. Two Kinds of Vials for Micro- and Macroestimation of D-Glucose

1: vial-1 (5 ml),
2: vial-1 with plastic holder,
3: vial-2 (10 ml)

A glass vial (5 ml, see Fig. 1-1) specially designed by the authors was prepared and used for the determination of 0.2—100 μg of total D-glucose (α-form+β-form). A circular glass plate was attached to the bottom of the vial for smooth rotation of stirring bar. The vial was inserted in the plastic holder (see Fig. 1–2) fixed on the small magnetic stirrer.

For a microdetermination of D-glucose below 5 μg, a circulating system for controlling temperature of the reaction mixture may be required. A glass vial (10 ml, see Fig. 1–3) was used for the determination of D-glucose up to 600 μg.

A 10 μl microsyringe (Type MS-10, Jintan Terumo Co., Tokyo, Japan) was used for the addition of standard D-glucose. It was found that the microsyringe is better than a glass micropipette in this study, because we can add the standard D-glucose solution to the reaction mixture exactly with the microsyringe without making the bubbles.

Experimental

Principles of the Method——1) Addition of Sodium Azide as Inhibitor for Catalase: As shown in Eq. (1), β-D-glucose oxidase consumes one mole of oxygen for oxidation of one mole of β-D-glucose with forming one mole of hydrogen peroxide. It is a well known fact that catalase, if present in the sample or glucose oxidase preparation, decomposes hydrogen peroxide to one–half mole of oxygen and one mole of water. The oxygen thus formed can be used for oxidation of another molecule of β-D-glucose by Eq. (1). So, the over–all reaction in the presence of catalase may be written as follows:

$$\beta\text{-D-glucose} + 1/2O_2 \rightarrow \text{D-gluconic acid} \qquad (2)$$

The authors added a trace amount of sodium azide to the reaction mixture so that it might inhibit catalase completely, and β-D-glucose oxidase may consume one mole of oxygen for oxidation of one mole of β-D-glucose even in the presence of catalase as written in Eq. (1).

12) J. Okuda and G. Okuda, *Clin. Chim. Acta*, **23**, 365 (1969).
13) K. Kusai, I. Sekuzu, B. Hagihara, K. Okunuki, S. Yamauchi and M. Nakai, *Biochim. Biophys. Acta*, **40**, 555 (1960).

2) Measurable Amount of D-Glucose in the Various Volumes of Reaction Mixture: As it had been reported that pure water contains 8.38 μg of oxygen per one ml at 25°,[14] it is found that this amount of oxygen in 1 ml of pure water is enough for oxidation of at least 47.17 μg of β-D-glucose with β-D-glucose oxidase. Assuming that the reaction mixture is composed of only pure water saturated with air, the measurable amount of D-glucose in the various volume of the reaction mixture is calculated as shown in Table I. Consumption of 8.38 μg (0.262 μmoles) of oxygen corresponding to 74.85 μg of total D-glucose can be recorded by the recorder (EPR-2TC) at 50 mV full scale when 1 ml of the reaction mixture is used. In fact, the reaction mixture contains a fairly large amount of salts, proteins and other biological substances. This implies that the solubility of oxygen in the reaction mixture is smaller than that in pure water, and consequently the measurable amount of D-glucose in the reaction mixture may be a little smaller than theoretically calculated amount of D-glucose as shown in Table I.

Table I. Measurable Amount of D-Glucose in the Various Volumes of Reaction Mixture at 25°

Volume of reaction mixture[a] (ml)	Total amount of dissolved oxygen (μg)	(μmoles)	Total amount of D-glucose (μg)	Total amount of β-D-glucose (μg)
0.5	4.19	0.131	37.42	23.58
1.0	8.38	0.262	74.85	47.17
1.5	12.57	0.393	112.28	70.76
2.0	16.76	0.524	149.70	94.35
3.0	25.14	0.786	224.55	141.53
5.0	41.90	1.310	374.25	235.89
7.0	58.66	1.833	523.95	330.25
10.0	83.80	2.619	748.50	471.79

a) The reaction mixture is assumed to be only pure water saturated with air.

3) Increase of Sensitivity by Voltage Prebox: By cancelling unnecessary component of the output voltage of the oxygen analyzer with EPR–PREBOX (PB-30A) attached to the recorder, we can measure the consumption of 22.4 mμg (0.70 mμmoles) of oxygen corresponding to 0.20 μg of D-glucose at 2 mV full scale.

Procedure——1) Glucose Standards:

(1) D-glucose (below 5 μg)

In a vial-1 with a water circulating system, 1.0 ml of 0.2M sodium acetate–0.4% sodium chloride buffer (pH 5.6) and 10 μl of 0.1% sodium azide were put (The amount of sodium azide should be determined from the preliminary experiment. If pure enzyme is used and the sample solution does not contain any catalase activity, there is no need to add the sodium azide solution). Then, 10 μl of 8 mg/ml of β-D-glucose oxidase was added and the oxygen electrode was immersed into the reaction mixture up to 0.5 cm from the tip of the electrode. The mixture was stirred continuously with a small magnetic stirring bar for 30 sec to equilibrate oxygen content in the reaction mixture. In this microdetermination, EPR–PREBOX (PB-30A) was connected with the recorder (EPR-2TC). After the oxygen meter reading was set at a proper position on the chart, 10 μl of 100 μg/ml of D-glucose (total 1 μg) was added with a microsyringe. A weak oxygen consumption with many steps (A) was recorded in direct oxygen consumption mode within 60 sec (see Fig. 2). After the oxygen consumption reached a plateau, no diffusion of oxygen was observed, because the amount of consumed oxygen is so small that oxygen in the air does not diffuse in the reaction mixture. When another 10 μl of the same D-glucose solution was added, a similar oxygen consumption (B) was observed as shown in the same figure. Then, by adding another 10 μl of the same D-glucose solution, the almost same oxygen consumption (C) as A and B was recorded. These experiments were repeated, and it was found that 1 μg of D-glucose can be estimated within 6% of standard deviation. Further microdetermination below 0.2 μg is possible electrically, but gives the data with large standard deviation.

(2) D-glucose (5—60 μg)

In a vial-1 with plastic holder instead of a water circulating system, 1.0 ml of 0.2M sodium acetate–0.4% sodium chloride buffer (pH 5.6), 10 μl of 0.1% sodium azide and 10 μl of 8 mg/ml of β-D-glucose oxidase were put. It was found that the amount of consumed oxygen was parallel with the amount of D-glucose

14) R. Sawamura, T. Iwato, S. Suenaga, S. Ichikawa, Y. Iwayado, S, Kanno and T. Motoyama, "Standapr Methods of Analysis for Hygienic Chemists," 2nd ed., ed. by S. Akiya, Kanehara Publishing Co., Tokyo, 1965, p. 743.

at the range of 5 to 60 μg of D-glucose (see Fig. 3). At the range of 5 to 20 μg of D-glucose, oxygen consumption should be estimated by the aid of EPR-PREBOX (PB-30A).

(3) D-glucose (60—600 μg)

The procedure was the same as described in the case of (2) except that the volume of the reaction mixture was increased up to 10 ml. A vial-2 (10 ml) is used for determination of this range of D-glucose, because the oxygen amount in 1.0 ml of the reaction mixture is not enough for oxidation of above 74 μg of D-glucose as shown in Table I. The relation between various amount of D-glucose and oxygen consumption gave a straight line (see Fig. 3). It was also found that the formed hydrogen peroxide (even 71.3 μg of hydrogen peroxide formed in the oxidation of 600 μg of D-glucose) did not interfere the enzyme reaction.

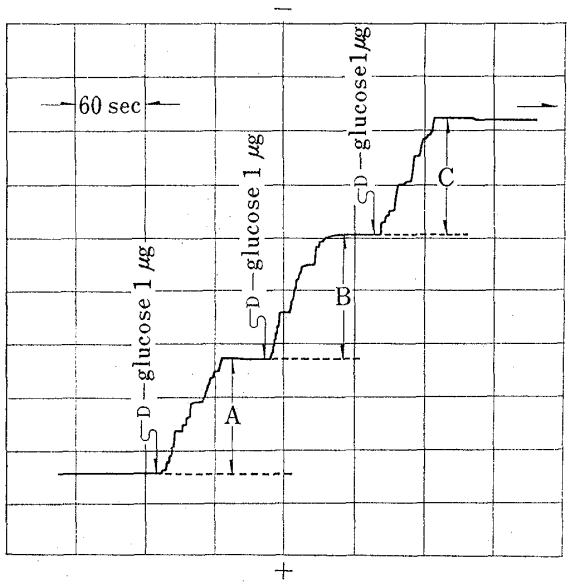

Fig. 2. Microdetermination of D-Glucose with β-D-Glucose Oxidase using Oxygen Electrode

A mixture of 1.0 ml of 0.2M sodium acetate–0.4% sodium chloride buffer (pH 5.6), 10 μl of 8 mg/ml β-D-glucose oxidase, and 10 μl of 0.1% sodium azide was put into the vial-1 under controlling temperature at 25° with a circulating system. After setting the oxygen meter reading at a proper position on the chart under a continuous stirring, 10 μl of 0.01% D-glucose (total 1 μg) was added with a microsyringe at the point as indicated. Oxygen consumption was recorded stepwise and ceased at the height of A. When the same amount of D-glucose was successively added, a similar oxygen consumption was observed (see B). Another addition of D-glucose repeated a similar oxygen consumption (see C). Recorder (EPR-2TC) was used at 2 mV (full scale) by cancelling 40 mV of the output voltage (42 mV) of oxygen analyzer with EPR-PREBOX (PB-30A).

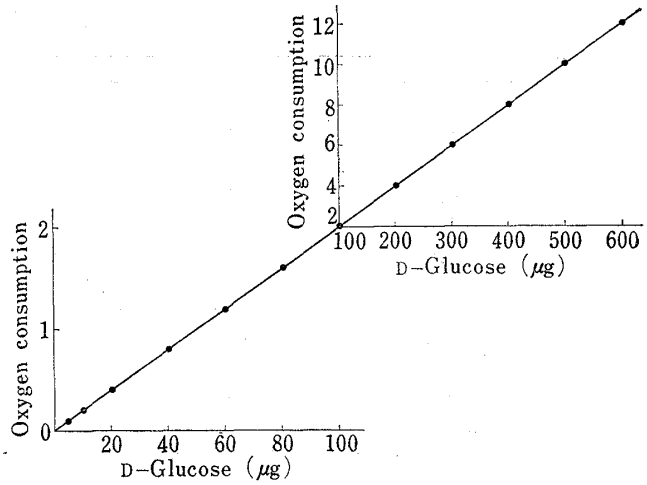

Fig. 3. Relation between Amount of D-Glucose and Relative Oxygen Consumption

2) Determination of D-Glucose in Serum: In a vial-1 with plastic holder, 40 μl of serum diluted in 1.0 ml of 0.2M sodium acetate–0.4% sodium chloride buffer (pH 5.6) and 20 μl of 0.1% sodium azide solution were put. After equilibrium of oxygen was reached, 20 μl of 8 mg/ml of β-D-glucose oxidase (in the case of crude enzyme, the enzyme concentration should be determined previously) was added. A rapid oxygen consumption was observed as shown in Fig. 4-A, and the consumption reached a maximum within *ca.* 1 min, and the curve slightly declined because of the diffusion of oxygen from the air. Then, 10 μl of 0.3% standard D-glucose (total 30 μg) was added. The second oxygen consumption was recorded as shown in Fig.4-B. The next experiment was done using the same amount of the sample, and 10 μl of 0.2% standard D-glucose (total 20 μg) was added as the internal standard to give the oxygen consumption C. From the values of the oxygen consumption of these samples and internal standards, the D-glucose amount in the sample could be exactly calculated as follows:

$$\text{in the case of } 30\,\mu\text{g standard, } \frac{A}{B} \times \frac{30}{40} = 0.795 \text{ mg/ml}$$

$$\text{in the case of } 20\,\mu\text{g standard, } \frac{A}{C} \times \frac{20}{40} = 0.803 \text{ mg/ml}$$

Two results agree with each other. The method with internal standards was found to be further accurate than the method with external standards. In this standard procedure using 40 μl of the sample, it is not necessary to use EPR-PREBOX (PB-30A).

Only 5 μl of serum is quite sufficient for the microdetermination of D-glucose in serum when EPR-PREBOX is connected with the recorder.

3) Determination of D-Glucose in Blood: D-Glucose in blood can be measured by the method as described in the case of serum. In this case, 40 μl of peripheral blood was first diluted in 0.5 ml of distilled water to hemorinize completely, and the diluted sample mixed with 0.8 ml of 0.2M sodium acetate–0.4% sodium chloride buffer (pH 5.6) was heated at 90° for 30 sec to decompose hemoglobin which interferes the oxygen consumption measurement, or the same volume (40 μl) of peripheral blood was first mixed with 0.2 ml of 0.7N NaOH to denature hemoglobin, and 0.2 ml of 0.7N HCl and 0.9 ml of 0.2M acetate buffer (pH 5.6) were added to neutralize the reaction mixture. Then, the heated sample after cooling or the neutralized sample was subjected for the determination of D-glucose as in the case of serum. The denatured hemoglobin did not interfere the estimation. When EPR-PREBOX is connected with the recorder, only 5 μl of blood is sufficient to determine the D-glucose amount in blood.

Comparison of Results from the Present Method with Those from *o*-Toluidine-borate Method for Representative Human Serum Samples——Table II shows a direct comparison of a representative portion of the data by the present method with the *o*-toluidine-borate method.[15] It should be noted that good agreement was obtained over the entire range of D-glucose values found. It was reported by some investigators[16] that the values by glucose oxidase method are rather a little lower than those by any other methods. As shown in Table II, the values by our method were mostly

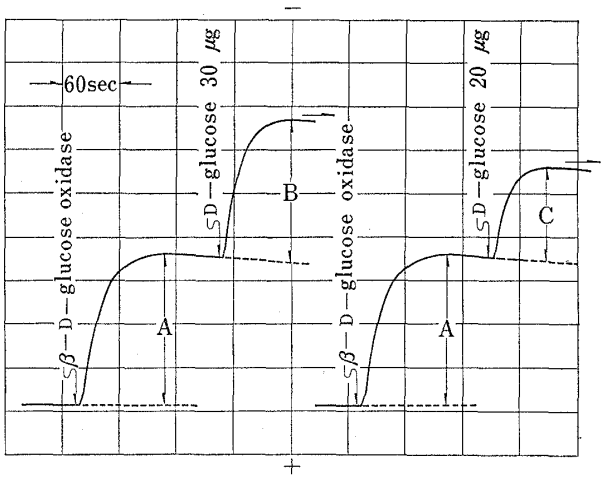

Fig. 4. Measurement of D-Glucose in Serum with β-D-Glucose Oxidase by using Internal Standard

A mixture of 40 μl of serum diluted in 1.0 ml of 0.2M sodium acetate–0.4% sodium chloride buffer (pH 5.6) and 20 μl of 0.1% sodium azide was put into the vial-1. After equilibrium of oxygen was reached at room temperature, 20 μl of 8 mg/ml β-D-glucose oxidase was added at the point as indicated. Oxygen consumption reached a maximum (see A), then the curve slightly declined without becoming flat because of the diffusion of oxygen from the air. Then, 10 μl of 0.3% standard D-glucose (total 30 μg) was added at about 1 minute after the maximum point as indicated, because it was needed to know the baseline for oxygen consumption by standard D-glucose. The oxygen consumption due to standard D-glucose should be regarded as B. The next measurement was carried out using the same amount of the sample and 10 μl of 0.2% standard D-glucose (total 20 μg). The oxygen consumption due to the sample was A and that to standard D-glucose was C. Recorder was used at the range of 50 mV (full scale). The output voltage of oxygen analyzer was 42 mV.

Table II. Comparison of the Results from the Present Method with Those from *o*-Toluidine–borate Method for Representative Human Serum Samples (D-Glucose mg/dl)

No.	Present method	*o*-Toluidine–borate method	No.	Present method	*o*-Toluidine–borate method
1	67.3	72.0	10	203.8	212.0
2	80.2	85.0	11	80.4	82.0
3	86.1	88.0	12	86.5	89.5
4	95.2	94.5	13	85.9	88.0
5	117.1	125.0	14	76.9	79.0
6	96.2	100.0	15	85.0	83.0
7	87.4	92.0	16	103.7	111.0
8	133.3	131.5	17	80.8	83.0
9	147.5	148.0	18	74.5	75.5

15) T. Sasaki, *Jap. J. Clin. Path.*, **11**, 40 (1967).
16) M. Kitamura, *Jap. J. Clin. Path.*, supplement **15**, 35 (1968); L.K. Jacobsen, *J. Clin. & Lab. Invest.*, **12**, 76 (1960).

a little lower than those by *o*-toluidine–borate method, too. From these results, it was confirmed that there were only very small amount of aldohexoses except D-glucose in blood. The data of the D-glucose microdetermination in blood by the present method will be compared with those by any other methods and the differences will be discussed from the point of existence of other sugars (*e.g.* D-galactose) in the near future.

Discussion

The present polarographic method using oxygen electrode in direct oxygen consumption mode is useful for determination of D-glucose in the small clinical and biochemical laboratories requiring urgent results. In the present method, the authors added only a trace amount of sodium azide, a strong inhibitor for catalase contained in the biological materials and glucose oxidase preparation, to the reaction mixture so as to measure the full oxygen consumed by the glucose oxidase reaction, while Kadish, *et al.*[7] added an iodine-iodide-molybdate mixture which decomposes hydrogen peroxide formed by the glucose oxidase reaction. By connecting a voltage prebox with the recorder (2 mV full scale) to cancel unnecessary voltage of the output voltage of oxygen analyzer, we could obtain an increase of sensitivity by about 25 times. By this microdetermination using voltage prebox, one can estimate to the extent of 0.2 μg of D-glucose. Only 5 μl of peripheral blood is needed at such a high sensitivity to determine the amount of blood glucose, and therefore this method is quite useful in the field of pediatrics and diabetes requiring small amount of blood in many times. The method has also advantages in accuracy and reproducibility, that is, the standard deviation is only 2% in the estimation of 50 μg of D-glucose and 6% in the estimation of 1 μg of D-glucose.

Although a calibration curve had been usually used in the D-glucose determination of many biological materials by oxygen electrode, the authors estimated D-glucose in the sample with internal standards, because the method with internal standards was found to be further accurate than the method with external standards from our experience. One of the benefits of this polarographic technique is to be able to omit deproteinization procedure. No. interference was observed in the case of determination of D-glucose in serum and heated or alkali-treated blood with turbidity.

This microdetermination technique using oxygen electrode and recorder connected with voltage prebox is also applicable to the microdetermination of dissolved oxygen,[17] some oxidoreductases and their substrates (above 5 mμmoles).

17) J. Okuda, T. Inoue and I. Miwa, in preparation