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Enzymatic Determination of Serum Glucose[1)]

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The optimal reaction conditions and kinetic properties of glucose dehydrogenase were studied in order to develop a method for serum glucose determination. The K_m value for glucose of this enzyme was influenced by the medium pH and ionic strength. Suitable conditions for the use of glucose dehydrogenase in rate assay and end point assay were identified. These methods showed very good reproducibility and were essentially unaffected by other reducing agents in the serum. The values obtained by these methods showed excellent correlations with those obtained with the hexokinase method. The present methods are rapid, simple and accurate.

Keywords——serum glucose; enzymatic determination; glucose dehydrogenase from *Bacillus megaterium*; rate assay; end point assay

Several enzymatic methods for serum glucose determination have been reported so far, and they may be classified into two categories; (a) glucose oxidase/peroxidase methods,[3)] (b) hexokinase /glucose-6-phosphate dehydrogenase method.[4)] In the case of the glucose oxidase method, various interfering substances have been reported, including uric acid, bilirubin, glutathione and ascorbic acid.[3,5)] On the other hand, the hexokinase method has the drawback that two enzymatic steps are required. Recently, glucose dehydrogenase has become readily available, and methods using this enzyme to determine serum glucose have been reported.[6)] However, the properties of this enzyme have not yet been examined in sufficient detail, and the above methods are not satisfactory from the view point of enzyme kinetics.

Glucose dehydrogenase from *Bacillus megaterium* has been isolated in a homogeneous from and its physicochemical properties have been studied.[7)] However, the K_m value and heat stability of this enzyme were not examined in detail. We examined the enzymatic properties and found that the K_m value for glucose and the heat stability were influenced by the medium pH and ionic strength. Thus, the K_m value of glucose dehydrogenase can be adjusted to be suitable for two different glucose determination methods: rate assay and end point assay.

Materials and Methods

Reagents and Enzymes——Glucose dehydrogenase from *Bacillus megaterium* was kindly supplied by Amano Pharm. Co. Ltd. (Japan). Nicotinamide adenine dinucleotide (NAD^+) was purchased from Oriental Yeast Co. Ltd. (Japan) and Glucoquant® (hexokinase method) was obtained from Boehringer Mannheim Co. Ltd. Other chemicals were of reagent grade.

Assay of Enzyme Activity——Glucose dehydrogenase activity was assayed by monitoring the rate of NADH formation spectrophotometrically at 340 nm and 25°. The reaction mixture contained 0.1 M Tris-HCl buffer (pH 8.0), 0.2 M glucose and 1.4 mM NAD^+ in a total volume of 3.0 ml. The reaction was started by the addition of glucose dehydrogenase. In each assay, 1 unit of glucose dehydrogenase was defined as the amount which catalyzed the formation of 1 μmol of NADH/min under the specified conditions.

Standard Procedure for the Determination of Serum Glucose——Rate Assay: 100 mg of NAD^+ and 1.3 ml of glucose dehydrogenase (11×10^3 U/l) were dissolved in 100 ml of 0.1 M Tris-HCl buffer (pH 8.0) containing 3 M NaCl. A 3 ml aliquot of the reaction mixture was preincubated at 25° for 5 min, and then 100 μl of the serum was added. After mixing, the increase in absorbance at 340 nm was recorded with a spectrophotometer (Hitachi 200-10) for 2 min.

End Point Assay: 150 mg of NAD^+ and 11 ml of glucose dehydrogenase (11×10^3 U/l) were dissolved in 150 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 0.1 M NaCl. A 1.5 ml aliquot of the above incubation mixture was preincubated at 37° for 5 min, and then 5 μl of the serum was added. After incuba-

tion of the mixture at 37° for 30 min, the absorbance was measured at 340 nm against a reagent blank.

Conventional Method——The Manual Hexokinase Method: The reagent solutions and the method were those recommended by the manufacturer of this kit [Boehringer; No. 158062 (Gluco-quant®)].

Results and Discussion

Effect of the Medium pH and Ionic strength on the Stability and K_m Value

The initial velocities were measured as a function of glucose concentration in various buffers, and the K_m values were calculated by means of a double reciprocal plot.[7] Figure 1 shows the K_m values for glucose as a function of pH at two different NaCl concentrations. As the NaCl concentration increased, the K_m value of glucose dehydrogenase for glucose increased. This observation suggested that the K_m value for glucose of glucose dehydrogenase could be controlled by varying the medium pH and ionic strength. For the rate assay, glucose dehydrogenase is used at pH 8—9, at which it has a high K_m value, and for the end point assay, it is used at pH 6—7.5, at which it has a low K_m value.

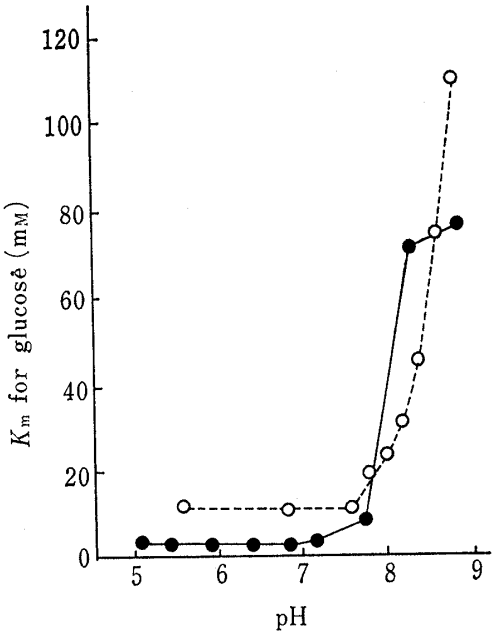

Fig. 1. pH Dependence of the Michaelis Constant of Glucose Dehydrogenase for Glucose in the Presence and Absence of 2.5 M NaCl

pH profiles of K_m for glucose with (--○--) and without (—●—) 2.5 M NaCl.
pH 5—7; 50 mM phosphate buffer,
pH 7.2—9; 50 mM Tris-HCl buffer.

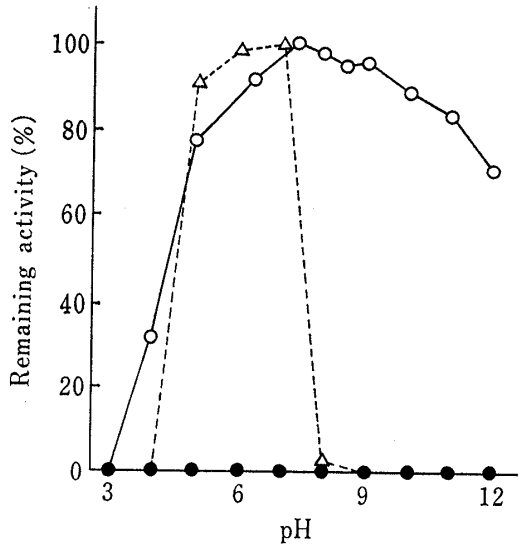

Fig. 2. Effect of pH on the Stability of Glucose Dehydrogenase

pH profiles of the enzyme stability at 56° for 1 hr with (—○—), without (—●—) 3 M NaCl and at 37° without NaCl (--△--).
pH 3—7; 0.2 M McIlvaine buffer,
pH 7.1—9; 0.1 M Tris-HCl buffer,
pH 9.1—12; 0.1 M glycine-NaOH buffer.

When glucose dehydrogenase was incubated without NaCl at 56° for 1 hr at various pHs, the enzyme irreversibly lost activity (see Fig. 2), but it was stable at pH values between 6—7 at 37°. In the presence of a high concentration (2.5 M) of NaCl, glucose dehydrogenase showed a high stability at 56° at pH above 5.0. Similar results were obtained with various electrolytes, such as NaCl, KCl and $(NH_4)_2SO_4$, so the enhancement of stability by NaCl may be due to ionic strength.

Glucose Determination by Rate Assay

Glucose dehydrogenenase was used in the pH range at which the K_m value for glucose was high.

A calibration curve for glucose determination by the rate assay was plotted by using standard glucose solutions; it was found that the calibration curve passed through the origin and that the initial reaction velocity (OD at 340 nm/min) increased linearly with glucose concentration up to 600 mg/dl glucose (the initial reaction velocity at a concentration of 600 mg/dl glucose was 0.069 OD/min).

Table I. Recovery of Glucose in added to Serum

No.	Rate assay		End point assay	
	Glucose added (mg/dl)	Glucose recovered (mg/dl)	Glucose added (mg/dl)	Glucose recovered (mg/dl)
1	100	100	100	99
2	100	99	100	100
3	100	101	100	103
4	100	100	100	100
5	100	99	100	101
6	100	101	100	101
Average		100		101

The recovery of glucose in the serum was investigated by the standard method and the average recovery of glucose was 100% (Table I). Reproducibility was examined with human serum. The within-day precision (C.V.) was 1.2% ($n=20$) and the day-to-day precision (C.V.) was 1.3% ($n=15$). The proposed method was thus found to be reproducible. We evaluated the specificity of the proposed method by adding potentially interfering substances to 100 mg/dl standard glucose solution. The following compounds at the concentrations (mg/dl) indicated did not show any interference: ascorbic acid (15), glutathione (20), bilirubin (20), and uric acid (20).

Glucose Determination by End Point Assay

Glucose dehydrogenase was used in a pH range at which the K_m value for glucose was low.

The necessary concentrations of glucose dehydrogenase and NAD^+ were examined by the standard method; in order to obtain a constant glucose value, 1.25 U and 1.5 mg, respectively, were required in the reaction system. The plots of absorbance at 340 nm *vs.* the amount of glucose (0—800 mg/dl) were linear, passing through the origin, and the absorbance of NADH at 340 nm in the presence of 800 mg/dl glucose was 0.915.

The amounts of added glucose were equal to those of glucose calculated from the observed increases in absorbance and the theoretical extinction coefficient for NADH. On the other hand, the reaction time required was investigated with or without mutarotase (30 U/tube). It was found that the time used in the standard method without mutarotase was only a little longer than that required with mutarotase, so no mutarotase was added in the standard assay mixture. The average recovery of glucose in the serum was 101% (Table I), and under these conditions, the precision of this method was good; the within-day and day-to-day, coefficients of variation were 1.2% ($n=10$) and 1.3% ($n=10$), respectively. The possibility of interference with the proposed method by reducing agents in the serum was examined. There was little if any interference by uric acid, bilirubin, glutathione or ascorbic acid.

Comparison with the Hexokinase Method

We compared the results of serum glucose determination by the rate and end point assays with those obtained by the hexokinase method using linear regression analysis. The coefficients of determination of both comparisons are given in Table II.

Lutz and Flückiger[6b] reported that a special formula was essential to determine serum glucose because the calibration curve for the rate assay was not linear. In other words, if

Table II. Comparison of the Rate Assay (y_1) and the End Point Assay (y_2) with the Hexokinase Method (x) by Linear Regression Analysis[a)]

Method	a	b	r	n
Rate assay (y_1)	0.99	−2.67	0.998	40
End point assay (y_2)	1.05	−6.23	0.997	40

a) According to the equation $y=ax+b$, with the regression coefficient (r).

the K_m value was low, the calibration curve for the rate assay would be nonliner in the high concentration range of substrate. Thus, we used glucose dehydrogenase under conditions such that its K_m value for glucose was high and the calibration curve was linear over a sufficient range to determine the serum glucose. On the other hand, in the end point assay, glucose dehydrogenase could be used at pH below 7.5 and low ionic strength.

Thus, the characteristic variation of K_m of glucose dehydrogenase for glucose was utilized to determine the serum glucose. The rate assay seemed to be particularly suitable for the determination of glucose, firstly because glucose dehydrogenase was very stable in the rate assay mixture containing 3 M NaCl, and secondly because only 2 min was required for a sample determination. The mechanisms of the variation of the stability and K_m value (for glucose) of glucose dehydrogenase, with changes of pH and ionic strength remain to be determined.

References and Notes

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