Routine Glucose Determination in Serum by Use of an Immobilized Glucose Dehydrogenase Nylon-Tube Reactor

P. V. Sundaram, B. Blumenberg, and W. Hinsch

We report a method for immobilizing glucose dehydrogenase on the inside surface of nylon tubes to produce an immobilized-enzyme nylon-tube reactor. The glucose dehydrogenase reactor is integrated into the flow system of a continuous-flow analyzer to facilitate routine analysis of serum glucose at 50 samples/h. We compared results with those by the reference hexokinase/glucose-6-phosphate dehydrogenase solution method. The coefficient of correlation was $r = 0.996$. A glucose dehydrogenase reactor made starting with 1 mg (250 U) of enzyme was stable during eight weeks of continuous use, that is, for nearly 3500 tests. This reduced the cost of the assay by at least 50-fold, compared with that for a commercial glucose dehydrogenase test pack method.

Additional Keyphrase: enzymatic method

Chemical methods for glucose determination (1) are used less and less in the clinical laboratory because of their lack of specificity. The enzymic methods of the recent years have been based on (a) oxidation of glucose (catalyzed by glucose oxidase), followed by an optical measurement of the product $\text{H}_2\text{O}_2$ (2) or by electrometry (3), or (b) the hexokinase/glucose-6-phosphate dehydrogenase method (4). Colorimetric procedures associated with glucose oxidase methods often rely upon the use of peroxidase to transfer oxygen from $\text{H}_2\text{O}_2$ to an acceptor molecule, which is mostly either 3-methyl-benzothiazolin-2-on-hydrazine in combination with dimethyl-aniline (5) or 2,2'azino-di-3-ethyl-benzthiazoline-2-sulfonic acid (6).

The hexokinase/glucose-6-phosphate dehydrogenase method of glucose determination depends upon following the formation of NADPH for detection of glucose. Mclothlin and Jordan (7) used direct injection enthalpimetry in determining glucose. A recent development (8) is the use of a bacterial glucose dehydrogenase ($\beta$-D-glucose:NAD$^+$ oxidoreductase, EC 1.1.1.47) in conjunction with mutarotase (aldose 1-epimerase, EC 5.1.3.3), which is nearly specific for the metabolite and is available in high purity and specific activity. These enzymic methods of glucose determination are specific in most cases and involve the use of two enzymes in solution.

The increasing use of immobilized enzymes has amply demonstrated that the cost of routine analysis can be dramatically reduced. Guilbault and Lubrano (9) devised an enzyme electrode for glucose determination, and Inman and Hornby (10) immobilized glucose oxidase onto nylon tubes, as did also León et al. (11). León et al. (12) also immobilized hexokinase and glucose-6-phosphate dehydrogenase onto nylon tubes. Hicks and Updike (13) described an automated system for glucose determination with glucose oxidase entrapped in polyacrylamide gel. In a novel approach, Williams et al. (14) devised an enzyme electrode. Recently, Bisse and Vonderschmitt (15, 16) coupled glucose dehydrogenase to nylon tubes. Kuan et al. (17) immobilized the same enzyme on a stirrer.

As part of a series of papers (18–20) dealing with routine determination of urea, citrulline, uric acid, pyruvate, and lactate by use of immobilized-enzyme nylon-tube reactors, we now report a method for immobilizing glucose dehydrogenase on the inside surface of nylon tubes and describe a flow-system consisting of such a reactor (nylon tubing bearing immobilized glucose dehydrogenase) and a continuous-flow analyzer for routine analysis of serum glucose. When we compared the results of analysis by the continuous-flow method with those by hexokinase and glucose-6-phosphate dehydrogenase in solution, we found that the new method gives reliable and reproducible results. The operational and storage stabilities of the reactor were very good.

Materials and Methods

Apparatus

We used a Varioperpex (LKB Produkter, Bromma, Sweden) peristaltic pump to control the flow of the substrate through the reactor in the research laboratory in characterizing the system. For the clinical trials we used an AutoAnalyzer II (Technicon Instruments Corp., Tarrytown, N.Y. 10591).

Materials

"Nylon" tubing was obtained from Portex Ltd., Hythe, Kent, U.K. Glucose dehydrogenase isolated from Bacillus megaterium M 1286 (250 kU/g) was kindly supplied as a gift by Dr. W. Brümmer, E. Merck, Darmstadt, F.R.G. Triethylxylonium tetrafluoroborate was purchased from Aldrich–Europe Division (B-2340 Beerse, Belgium). Reagents used in preparing and characterizing the reactor included phosphate buffer (0.1 mmol/L, pH 7.0) containing 1 mmol of ethylenediaminetetraacetate (EDTA) and 1 mmol of dithioerythritol per liter, and substrate solution consisting of glucose (0.2 mmol/L) and NAD$^+$ (0.6 mmol/L) dissolved in the buffer.

The reagent used for routine assay with the reactor connected to the AutoAnalyzer consisted of phosphate buffer (0.12 mmol/L, pH 7.6) containing, per liter, 1.1 mmol of NAD$^+$, 1 mmol of EDTA, 1 mmol of dithioerythritol, 110 $\mu$mol of mutarotase, and 0.5 $\mu$L of Brij 35, the surfactant. Reference serum used for routine calibration of the AutoAnalyzer method was Q-Pak (Hyland-Travenol, 8000 Munich, F.R.G.).

Max-Planck-Institut für experimentelle Medizin, Abteilung Chemie, Hermann-Rein-Strasse 3, D-3400 Göttingen, F.R.G.

1 Medizinische Universitätshklinik, Abteilung für Klinische Chemie, D-3400 Göttingen, F.R.G.

The routine application of this immobilized enzyme is part of a doctoral thesis of B. Blumenberg at the Medical Faculty of the University of Göttingen, 1979.

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Control sera used for precision studies were Precinorm U (Boehringer, 6800 Mannheim, F.R.G.), Kontrolofgen L and LP (Behring-Werke, 3550 Marburg, F.R.G.), Monitrol I and II (Merz + Dade, 8000 Munich, F.R.G.), and Eurocoenz N and P (Hyland—Travenol).

Preparation of the Reactors

The reactors we used in the clinical trials reported in this study were made by cross-linking the enzyme to a nylon-polyethyleneimine copolymer by use of glutardialdehyde (27). Before preparing the copolymer, we first activated nylon tubing (1 mm i.d.) by O-alkylation. We coiled 1 m of tubing around a plastic rod and filled it with a 0.1 mol/L solution of triethyloxonium tetrafluoroborate in dichloromethane, allowing it to react for 4 min at room temperature before we emptied the tube. The tube was then quickly washed with ice-cold methanol, followed by ice-cold de-ionized water.

We also coupled the enzyme to hydrolyzed nylon by an earlier method (22), in which nylon-polyethyleneimine copolymer was made by the method of Sundaram and Apps (23): 10 g/L dilution of a stock solution of polyethyleneimine (supplied by Serva GmbH, 6900 Heidelberg, F.R.G.) in a bicarbonate buffer (pH 9.4, 0.1 mol/L) was pumped for 4 h at room temperature through an alkylated nylon tube made as described above. The tube was then washed well with de-ionized water, filled with a freshly made solution of glutaraldehyde (25 g/L) in the bicarbonate buffer, and allowed to stand for 40 min at room temperature. The tube was again washed well with water and immediately filled with about 250 U, or 1 mg of a 1 g/L solution, of glucose dehydrogenase dissolved in phosphate buffer (0.1 mol/L, pH 7) containing EDTA (1 mmol/L). We tried the coupling in the absence or presence of NAD⁺ (1.5 mmol/L) dissolved along with the enzyme in the above solution. The enzyme was allowed to react overnight for about 18 h at 4 °C, then washed successively with a 0.1 mol/L NaCl and de-ionized water, to remove any adsorbed protein.

An enzyme solution of the same composition, but without NAD⁺, was used in cross-linking the enzyme to hydrolyzed nylon.

Analytical Methods

Assay with enzyme in solution: To characterize the enzyme used in this study, we did some simple kinetic studies, not only to see the results of varying the concentrations of glucose and NAD⁺ but also to see whether NADP⁺ and 6-thio-NAD⁺ (Boehringer Mannheim) act as coenzymes for this enzyme.

We performed these assays by putting the substrates into a 1-mL cuvette and starting the reaction by adding 25 μL of a 2 g/L solution of the enzyme made up in pH 7 phosphate buffer (0.1 mol/L) containing 1 mmol/L of EDTA. The reaction was followed at 340 nm with a recording spectrophotometer.

Measurement of activity of the reactors: Glucose was determined after perfusing substrate solution containing NAD⁺, 0.6 mmol/L, and glucose of various known concentrations made up in phosphate buffer (pH 7, 0.1 mol/L) through the reactor tubing at a flow rate of 0.32 mL/min. (A coefficient of molar absorptivity of 6290 L·mol⁻¹·cm⁻¹ for NADH was used in the calculation of activity.)

Routine analysis with a continuous-flow analyzer: The flow diagram for glucose determination with a reactor linked to a Technicon AutoAnalyzer is depicted in Figure 1. The method that uses hexokinase/glucose-6-phosphate dehydrogenase in solution in the assay of glucose was performed on one channel of a Technicon SMA 12 multichannel analyzer with Boehringer reagents, according to the flow diagram given by Boehringer Mannheim (24).

Results and Discussion

The reactions conducted with glucose dehydrogenase in solution and the different cofactors, such as NAD⁺, NADP⁺, and 6-thio-NAD⁺, revealed that NAD⁺ elicited the maximum activity when used with glucose as the substrate, even though both 6-thio-NAD⁺ and NADP⁺ have a lower Kₘ than NAD⁺. However, it is interesting to note that 6-thio-NAD⁺ also acts as a cofactor, albeit a poor one. Table 1 contains the kinetic constants obtained with these substrates.

We tried coupling the enzyme by cross-linking to partly

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<th>Table 1. Kinetic Constants of Reactions Catalyzed by Free and Immobilized Glucose Dehydrogenase with Various Substrates</th>
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* All at pH 7.8 in 0.1 mol/L phosphate buffer.

References

1. For example, see the discussion in: The Enzymes, Vol. 1, Academic, New York (1941).
3. The profile for the enzyme in solution possesses one of the recognized shapes in enzyme reactions (25), indicating an ionizing group at pH 6.8, whereas the enzyme coupled to nylon-polyethyleneimine copolymer or to hydrolyzed nylon shows two optima. This anomalous shape is probably caused by immobilization and micro-environmental effects at the nylon-water interface, where the enzymatic reaction occurs.

Routine Analysis with the AutoAnalyzer-Linked Reactor

We used a reactor prepared by cross-linking glucose dehydrogenase on a 1-m nylon-polyethyleneimine copolymer tube and performed the analysis at pH 7.6. The average specific activity of reactor was about 8.5 mU/ml per minute.

Under the conditions specified in the flow diagram (Figure 1), the standard curve for the test was linear up to 4000 mg of glucose per liter, with standards in the range of 500-4000 mg/L. The rate of analysis in these experiments was 50 samples/h with a sample-to-wash ratio of 1:1. This ensured a low enough carryover between samples (i.e., a 40 mg/L carryover from a 4000 mg/L sample followed by a 500 mg/L sample), thus meeting the standards of routine clinical analysis. This 8% carryover is considered to be reasonable for immobilized enzyme nylon-tube reactors. For daily routine measurement, the enzyme was calibrated with reference sera of known concentration, interspersed between every 25 samples, and was readjusted when necessary. Under these conditions, the precision of this immobilized glucose dehydrogenase method is good for different control sera with high and low glucose concentrations, as seen in the day-to-day precision values (Table 2).

The new method was compared to the reference method for glucose determination in clinical chemistry (24), the hexokinase/glucose-6-phosphate dehydrogenase method in solution, performed with a Technicon Multichannel AutoAnalyzer SMA 12. The line of regression, given in Figure 4, is based on

| Table 2. Precision of the Reactor Method for Determination of Glucose |
|------------------------|-----------------|-----------------|
| Avg. concn. (mg/L)     | Within-day CV, % | Day-to-day CV, %|
| 750                   | 3.2             | 3.9             |
| 880                   | 2.9             | 3.3             |
| 1020                  | 1.9             | 2.9             |
| 2020                  | 1.9             | 2.1             |
| 2100                  | 2.1             | 2.8             |

The pH–activity profiles of glucose dehydrogenase in solution and immobilized on nylon tubing are shown in Figure 3.

![Figure 3](image-url)  
**Fig. 3.** pH–activity profiles of glucose dehydrogenase in solution and of glucose dehydrogenase reactors

Absorbance values \( A_{540} \) for reactions catalyzed by the enzyme in solution and by immobilized enzyme are not comparable because enzyme concentrations are different in each case. O——O, free enzyme; X——X, nylon polyethyleneimine-immobilized enzyme; □——□, nylon-immobilized enzyme.

![Figure 4](image-url)  
**Fig. 4.** Comparison of results of glucose determination by the reactor method with those by the hexokinase/glucose-6-phosphate dehydrogenase (HK-G6PDH) (solution) method
Fig. 5. Decay curve of activity of a reactor made with nylon-polyethyleneimine copolymer tubes during continuous operation in the clinic for over 3500 tests

250 patients' serum samples. The correlation is excellent ($r = 0.996$).

We made no attempt to co-immobilize mutarotase, the second enzyme involved in the reaction that accelerates the conversion of $\alpha$-D-glucose to $\beta$-D-glucose. Because this enzyme is inexpensive and easily available, it was added in solution. The activity of the reactors is high enough to enable us to measure lower concentrations of glucose than are present in whole blood. Thus, determination of glucose in deproteinized capillary blood or hemolysates is the next obvious move.

Operational and Storage Stability

The reactors are quite stable. When not in use they can be stored for several months at 4 °C, if filled with phosphate buffer (pH 7.0, 0.1 mol/L) containing 1 mmol each of EDTA and diithioerythritol per liter without a significant loss in activity. After intermittent use for 32 days with an average of 160 samples for each day of use (nearly 3500 tests), the reactor retained 20% of the original activity (Figure 5).

The stability of the reactor is dependent upon the number of tests carried out with it rather than the span of time over which these tests are carried out. This was confirmed when the same number of tests (3500) was carried out with another reactor during seven days with an average of 500 tests per day. The reactor suffered a similar loss in activity when the same number of glucose determinations were made over a longer period of time. The amide bond formed by coupling an NH$_2$-group of an enzyme to O-alkylated nylon (Fig. 1 in ref. 19) has now been found to be reversible (26), which leads to the slow loss of enzymic activity. Considerably more tests should become possible when micromethods for a Technicon SMAC are applied to the reactor. This method would facilitate up to 150 tests/h and use low flow rates.

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References


