A Stopped-Flow Clinical Analyzer in Which Immobilized-Enzyme Reaction Loops Are Used

Martin D. Joseph, Daniel J. Kasprzak, and S. R. Crouch

A stopped-flow clinical analyzer is described that makes use of a reaction loop containing immobilized enzyme(s) for the determination of the analyte/substrate. The analyzer has been evaluated by determining glucose with immobilized glucose oxidase. The stopped-flow mixing system was constructed at a current cost of less than $500. The analyzer separates the enzymatic reaction from a followup, spectrophotometric indicator reaction. This separation allows the enzymatic reaction to be used in either a fixed-time, kinetic mode or in an equilibrium mode. Likewise, the indicator reaction can be used in either mode. Results for glucose in blood serum indicate that good precision and accuracy can be obtained.

In recent years immobilized enzymes have become increasingly useful as specific catalysts for determining clinically important substrates (1-4). Immobilized enzymes are attractive as reagents in the clinical laboratory because they possess the usual specificity and sensitivity of soluble enzymes and can be re-used, often in hundreds or thousands of determinations, thus greatly reducing the cost of routine use of an enzymatic kinetic or equilibrium method. In addition, immobilized enzymes are often more stable than they are in solution; calibration is thus simpler and less frequently required.

In this report, a stopped-flow clinical analyzer is described that combines the advantages of immobilized enzymes with the speed, mixing efficiency, and ease of automation of the stopped-flow mixing technique. The system utilizes a simple, low-cost, stopped-flow sampling and mixing unit together with a reaction loop that contains the immobilized enzyme. The reaction loop is similar in principle to the sample loop described by Pardue et al. (5, 6) except that the enzyme-catalyzed reaction occurs in the loop. After a suitable incubation period in the loop, the sample solution is rapidly mixed by the stopped-flow unit with any desired reagent(s) and sent to an observation cell for spectrophotometric monitoring of the appropriate indicator reaction. The separation of the enzyme-catalyzed reaction from the indicator reaction provides extreme versatility. The enzyme-catalyzed reaction can be allowed to go to completion (equilibrium method) or it can be incubated for only a short, fixed time interval (kinetic method). Likewise, either the reaction rate or the equilibrium absorbance of the spectrophotometric indicator reaction can be used in the final measurement. Because of stopped-flow mixing, measurements can be made on time scales ranging from milliseconds to hours.

The semi-automated stopped-flow clinical analyzer described below illustrates the measurement principle and chemical flexibility of the system. We evaluated the analyzer by determining glucose in blood serum with a reaction loop containing immobilized glucose oxidase (EC 1.1.3.4). Possible extensions of the system to multiple determinations of the same substrate or to near-simultaneous determinations of multiple substrates are discussed.

Materials and Methods

Instrumentation

The instrumentation we used to determine glucose is schematically represented in Figure 1. This system, except for the enzyme reaction loop, was constructed completely from commercially available items. Two 1-ml gas syringes with threaded ends, A and B in Figure 1 (from Glenco Scientific, Houston, Tex. 77007), are used to deliver indicator reagent and push liquid, respectively. Valves 1 through 4 are 0.060-inch bore, three-way slider valves complete with accuator-return mechanisms C and D and solenoid controllers S2 and S3 (from Altex, Berkeley, Calif. 94710). The syringes are driven by a 2-inch stroke pneumatic cylinder, PC, with solenoid controller S1 (Scovil, Wake Forest, N. C. 27587). Solutions are mixed by forcing them through E, a KEL-F tee (Altex). The indicator reaction is monitored in a 250-μl micro flow cell (F) with 10-mm light path (Thomas, Philadelphia, Pa. 19105). The flow cell is fitted into a single-beam uv-visible spectrophotometer (GCA McPherson EU-700, Acton, Mass. 01720). All tubing except for the enzyme reaction loop is 1.5 mm i.d., 3 mm o.d. Teflon with appropriate connector fittings (Altex).
The heart of the system is the enzyme reaction loop, prepared as described below and fitted with plastic high-pressure liquid chromatography connectors. The enzymatic reaction that occurs in the loop for the determination of glucose is:

\[
\text{Glucose} + \text{O}_2 + \text{H}_2\text{O} \xrightarrow{\text{glucose oxidase}} \text{gluconic acid} + \text{H}_2\text{O}_2
\]

The extent of this reaction is determined outside the loop by allowing the \( \text{H}_2\text{O}_2 \) produced to mix with and rapidly oxidize iodide to triiodide in the presence of a molybdate catalyst (7). The indicator reaction is:

\[
\text{H}_2\text{O}_2 + 3\text{I}^- + 2\text{H}^+ \xrightarrow{\text{Mo(VI)}} 2\text{H}_2\text{O} + \text{I}_3^-
\]

Triiodide, measured spectrophotometrically at 365 nm, indicates the concentration of glucose in the sample. The indicator reaction is followed by an equilibrium method in this case, because the absorbance of triiodide is measured at equilibrium. The enzymatic reaction, however, is of the fixed-time reaction-rate type. The enzymatic reaction time is fixed by the incubation time of the sample in the loop. This time is kept short (2 min) to ensure that the enzymatic reaction is pseudo-zero order. This combination of reaction-monitoring methods exemplifies the flexibility of the system.

The actual function of the enzyme reaction loop in the system is best described in terms of a typical sequence of events for the determination of glucose in a sample.

First, syringes \( A \) and \( B \) are filled with \( \text{KI}/\text{molybdate} \) reagent and a push liquid (phosphate buffer), respectively, by simultaneous switching of valves 1 and 2 to the fill position and retraction of \( \text{PC} \). Valves 1 and 2 are then switched to the "push-ready" position, as are valves 3 and 4 (positions shown in Figure 1). Next, \( \text{PC} \) is caused to push the contents of \( A \) and \( B \) through the system lines and out to the waste. This is repeated several times to remove air bubbles and fill all lines with solution. With \( A \) and \( B \) filled and valves 1 and 2 ready, valves 3 and 4 are simultaneously switched to the fill position, and a buffered glucose sample is aspirated into the enzyme reaction loop by a gentle negative pressure. Then valves 3 and 4 are switched to the "push-ready" position, which terminates sample introduction. The sample is incubated for a controlled time until \( \text{PC} \) is caused to push the contents of the enzyme loop through the mixer. Here the \( \text{H}_2\text{O}_2 \) produced by the enzymatic reaction is rapidly mixed with the \( \text{KI}/\text{molybdate} \) reagent, and the mixture is transported to the flow cell for observation and detection of \( \text{I}_3^- \). The flow is stopped upstream when the drive syringes reach the ends of their travel.

The system described above was constructed at a cost of less than $500, not including the specific flow cell and spectrophotometer mentioned here, for which there are suitable substitutes. The instrument can be assembled within a day or two due to the ease with which the valves and tubing can be connected via plastic fittings rated at 3450 kPa (500 psi). The simplicity, low cost, and versatility of this system make it an attractive analyzer for clinical determinations.

Reagents

**Buffer.** A 0.2 mol/liter solution of dibasic potassium phosphate in de-ionized water was adjusted to pH 6.30 ± 0.02 by use of a Heath pH meter (Model EU-302A) by adding concentrated hydrochloric acid. This buffer was then used to prepare all other solutions except where noted.

**Indicator reagents.** A sodium molybdate stock solution, 90 g/liter, was prepared and allowed to equilibrate for 48 h. The iodide/molybdate indicator reagent was then prepared daily by adding a fresh solution of potassium iodide to molybdate stock, resulting in a solution containing, per liter, 18 g of sodium molybdate and 0.5 mol of iodide.

**Glucose standards.** A glucose stock solution was prepared by dissolving 1.00 g of anhydrous granular D-glucose and diluting to 1.0 liter with de-ionized water. This solution was allowed to stand at room temperature for 24 h to ensure complete mutarotation, and was then stored at 5 °C. Aqueous glucose standards were then prepared by appropriately diluting this stock with the buffer.

**Deproteinization reagents.** We deproteinized the serum samples by adding chemically equivalent amounts of barium hydroxide and zinc sulfate. A barium hydroxide solution (20 g/liter) was titrated with zinc sulfate (20 g/liter) to a pH of 7 to determine the required volume ratio of the two reagents.
Preparation of serum samples. A control serum ("Montrol"; Dade Division, American Hospital Supply Corp., Miami, Fla. 33152) was reconstituted according to the manufacturer's instructions, and 0.5 ml of the solution was pipetted into a centrifuge tube. The chemically equivalent amounts of barium hydroxide and zinc sulfate were added, and the contents of the tube were thoroughly mixed, centrifuged for 3 min, and 1.0 ml of the supernatant fluid was pipetted into 3 ml of the phosphate buffer. This solution was then drawn into the enzyme reaction loop for analysis.

Immobilization of glucose oxidase. A procedure reported by Inman and Hornby (8) was modified for immobilizing glucose oxidase on the inner surface of nylon tubing, 100 cm long and 0.86 mm i.d. Most of the immobilization steps were accomplished by slowly pumping the required reagent through the tube with the use of a Technicon AutoAnalyzer pump to provide a constant stream of fresh reagent. Amorphous nylon was first removed by filling the tube with a 200 g/kg solution of CaCl₂ in methanol and incubating at 50 °C. The nylon was then mildly hydrolyzed by pumping 1.0 mol/liter HCl through the tube at room temperature, followed by rinsing with water. Glutaraldehyde, 125 ml/liter in 0.1 mol/liter tris(hydroxymethyl)methylamine buffer, pH 9.2, was then attached to the hydrolyzed nylon at 0 °C, followed by rinsing with the buffer. An 8 g/liter solution of glucose oxidase (Sigma, Type II) in 0.1 mol/liter phosphate buffer, pH 6.3, was then pumped through the tube at 0 °C for 4 h in a closed loop, during which time covalent linkages were formed between the glutaraldehyde and free amino groups on the enzyme (8). Finally, the tube was rinsed with 0.1 mol/liter sodium chloride to remove any physically adsorbed enzyme. The enzyme reaction loop was filled with buffer and stored at 5 °C when not in use.

Results and Discussion
The analytical capabilities of the analyzer system were evaluated by determining glucose in aqueous samples and in blood serum, with use of a reaction loop containing immobilized glucose oxidase. The first generation analyzer we used in these studies was manually sequenced. That is, the solenoid controllers for each of the valves in Figure 1 were actuated in the appropriate sequence by a series of manual toggle switches, which applied or disconnected the ac line voltage from the controllers. The spectrophotometric readout was obtained from a strip-chart recorder, linear in transmittance. Thus, the final absorbance values recorded for I₃⁻ were obtained by converting chart-recorder readings from transmittance to absorbance. The temperature of the enzyme reaction loop was not controlled.

The optimum push volume of the stopped-flow system was determined to be 0.78 ml per syringe by filling a dummy reaction loop with dye (p-nitrophenol). This volume ensured thorough purging and refilling of the observation cell, but prevented significant dilution of the observed solution by the push liquid. The incubation time for the enzymatic reaction was fixed at 2 min. Shorter incubation times did not provide adequate precision because of decreased response and also because of imprecision in manual sequencing; longer incubation times only added to analysis time without greatly enhancing the signal-to-noise ratio. Hence, transmittance was directly read out slightly over 2 min after the sample was introduced into the reaction loop.

A calibration curve for glucose was prepared in the range 20–250 mg/liter by running aqueous standards in triplicate. Table 1 shows the results. The observed negative intercept is expected in such a fixed-time method, because the enzymatic reaction is known to exhibit an induction period that is inversely proportional to glucose concentration (9), and hence a two-point calibration should be done periodically. The results in Table 1 indicate the excellent linearity and precision which may be obtained with this simple analyzer. Each determination requires only 1 ml of sample (about 100 μl of blood serum). The major sources of imprecision in this simple system appear to be variations in incubation time due to manual control, fluctuations in reaction rate due to variations in room temperature, and imprecision in reading the transmittance from the chart recorder.

For determining glucose in blood-serum samples, we prepared a two-point calibration curve, using the 20 and 200 mg/liter aqueous glucose standards. Two reconstituted Monitrol samples were analyzed, and the results (Table 2) agree well with the reported values. Note that the first two values reported for sample 1 were obtained on a different day from the second two values, although the same two-point calibration was used. Clearly, because of the high stability of the immobilized enzyme loop, the same calibration curve may be useful for several days. A reaction loop prepared earlier than the one used in these determinations showed excellent activity for six months before the enzyme was de-activated inadvertently by incubation with a highly acidic solution. Research is underway to determine more precisely the activity loss of immobilized enzyme loops with time under various conditions of storage and use.

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<th>Table 1. Results for Aqueous Glucose Standards</th>
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<td>Glucose concn, mg/liter</td>
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Slope = $1.60 \times 10^{-3}$ A per mg/liter (CV, 0.24%)  
Intercept = $-6.39 \times 10^{-3}$ A  
Correl. coeff. (r) = 0.99990
In this study we have demonstrated the potential of the analyzer for the determination of glucose in serum samples. Although the system was evaluated by using one particular enzyme/substrate couple, many other clinically useful enzyme/substrate couples are obviously applicable. Versatility is inherent, not only in the enzyme chosen for the reaction loop, but also in the reagent chosen for the indicator reaction. Furthermore, any of several combinations of methods (kinetic or equilibrium) may be used to follow the separate enzymatic and indicator reactions. We are now undertaking investigations on the capabilities of this new system in the clinical laboratory. New immobilization techniques are being studied for bonding other, more expensive enzymes to various inert supports. The multiplexing potential inherent in the system will be used, to allow several reaction loops containing the same or different enzymes to be used nearly simultaneously, thereby minimizing the time lost in incubation and maximizing the use of one detection system. The entire system will then be automated by interfacing it to a minicomputer or microprocessor for sample handling, instrument control, and data acquisition and subsequent processing. A more extensive paper describing this work will be submitted at a later date.

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References