# Fluorometer for Flow Injection Analysis with Application to Oxidase Enzyme Dependent Reactions

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**A simple effective flow fluorometer has been assembled and operated in conjunction with a flow injection analysis (FIA) system. A submicroliter capillary sheath flow cell is used for fluorescence detection with a laser source. The instrument has been tested for stability, precision, accuracy, sensitivity, dynamic range, and speed. Rhodamine 6G was used as a standard fluorophore. Application was also made to the clinical analysis of oxidase enzyme dependent reactions by reacting $H_2O_2$ with leucodiacetyldichlorofluorescein to produce fluorescent dichlorofluorescein. This produced dye is excited at 488 nm with the argon laser. Concentrations of $10^{-10}$ M hydrogen peroxide could be detected when injecting 25 μL aliquots.**

Automation within the clinical laboratory has been a major source of improvement in the quantity, precision, analysis time, cost, analytical control, and flexibility of tests performed. Skeggs (and the commercial appearance of the AutoAnalyzer) contributed significantly to that development with the application of segmented continuous flow analysis (CFA) (*1*). In comparison to discrete and centrifugal automatic analyzers, CFA is claimed to be generally simpler and more versatile in operation (*2*). In the mid-1970s, flow injection analysis (FIA) emerged as a faster, even simpler variation on single-channel CFA, with sample rates commonly over 120 per hour and bubble segmentation eliminated (*3–5*). FIA is the reproducible introduction of a sample plug into a laminar flowing stream, with mixing by diffusion; a response peak results (*6*).

Fluorescence spectroscopy has long been known as a very sensitive technique. Its application as a means of detection for FIA is relatively new, however (*7–9*). The potential of such a combination is great, although problems with stability and efficiency have generally precluded successful straightforward interfacing and fluorescent detection. A similar situation is found with the use of fluorescence detectors in HPLC and standard flow systems, where several technical variations exist. Several optical systems (*10–14*), excitation sources (*11, 15–19*), and flow cells (*12, 15, 20*) have been used with varying degrees of success, in improving the sensitivity, selectively, precision, and/or speed of analysis.

Reported here is a simple, effective fluorometric detector used in conjunction with FIA. A laser source is focused on a capillary sheath cell. Scattered light is eliminated with a single cutoff filter. The change in photomultiplier tube (PMT) current is seen as a peak on a strip-chart recorder. The system was characterized with the use of the fluorescent compound, Rhodamine 6G.

A determination of glucose, and potentially any oxidase enzyme dependent reaction, was made according to the reaction scheme

$$\text{glucose} + O_2 \xrightarrow{\text{GOD}} \text{gluconic acid} + H_2O_2 \quad (1)$$

$$\text{LDADCF*} + H_2O_2 \xrightarrow{\text{HRP}} \text{DCF} + 2H_2O \quad (2)$$

LDADCF* represents an activated form of leucodiacetyldichlorofluorescein (nonfluorescent, Figure 1), DCF is dichlorofluorescein (fluorescent), GOD is glucose oxidase, and HRP is horseradish peroxidase. Reaction 2, originally described for the sensitive determination of $H_2O_2$ (*21*), works well as an indicating reaction for oxidase systems (*22*).

## EXPERIMENTAL SECTION

**Flow System (Figure 2).** Compressed air is used to drive the system. Separately regulated streams are forced from respective reservoirs (500 mL) through Teflon tubing (Altex tubing and fittings; 0.8 mm i.d., 1.5 mm o.d.). Both lines pass through the flow metering system. A bubble of fixed volume is injected into each stream and timed over a known length of tubing. Flow rates were calculated according to the following derived equations:

$$F_r = \frac{k}{t_r} \quad (3)$$

$$F_s = \frac{k}{t_s} \quad (4)$$

$$F_T = \frac{k}{t_r} + \frac{k}{t_s} \quad (5)$$

where $F_r$, $F_s$, and $F_T$ are the flow rates (mL/min) of the reagent stream, sheath stream, and total flow, respectively, and $t_r$ and $t_s$ are the times (min) required for the bubbles to pass through the designated length of tubing (reagent and sheath, respectively); $k$ is a constant experimentally determined according to the volume of tubing through which the bubbles were measured ($k$ = 0.6960 mL). Pressures from 0.2 to 0.6 atm gave flow rates of 0.5–3.0 mL/min for the reagent stream and 1.0–5.0 mL/min on the sheath line.

The sheath line feeds directly into the flow cell while the reagent line proceeds via the injector and reaction tube. An Altex syringe-fed loop injector reproducibly injects 25 μL plugs into the reagent stream. The plug passes through 40 cm of coiled tubing (to ensure mixing) before going into the flow cell. The flow cell (Figure 3) establishes a sheath flow through the glass capillary and the laser beam is focused on this stream.

Depending on the relative stream flow rates, the diameter of the reagent or sample stream (surrounded by the sheath solvent stream under laminar flow conditions) varied from 0.04 to 0.22 mm. The laser was focused to illuminate a 0.12 mm length of the reagent stream. This corresponds to an effective illuminated volume range of 0.2–4.6 nL. Standard pressure settings of 0.50 and 0.25 atm gave respective reagent and sheath flow rates of 2.2 and 1.0 mL/min and an illuminated reagent volume of 3.8 nL. The sheath flow cell was from a Biophysics (now owned by Ortho Instruments) Corp. Cytofluorograf.

**Fluorescence Instrumentation.** The simple filter fluorometer is shown in Figure 4. Laser light is focused on the flow cell. The fluorescent light is collected at 45° by a microscope equipped with a cutoff filter and baffle and focused onto the face of a photomultiplier tube. The laser was an argon ion laser (Spectra Physics Model 162) generating 8 mW of power at 488 nm. It was focused on the reagent stream within the flow cell using a combination of lenses that resulted in a final focal length of 1 cm. The 10X, 0.25 numerical aperture objective of the Leitz microscope carried the emitted light through a 520-nm cutoff, antifluorescent filter (to remove scattered radiation) and the slit of a baffle onto an S-20 type PMT (EMI 9785A at 500 V). A Keithley electrometer was used to convert the PMT current into a voltage recordable on a Linear Instruments dual-pen strip chart recorder. Extraneous noise was minimized using an RC filter with a 1.7-s time constant.

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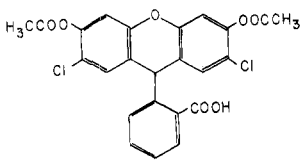


**Figure 1.** Structure of leucodiacetyldichlorofluorescein (LDADCF) (3′,6′-diacetyl-2′,7′-dichlorofluorescein).

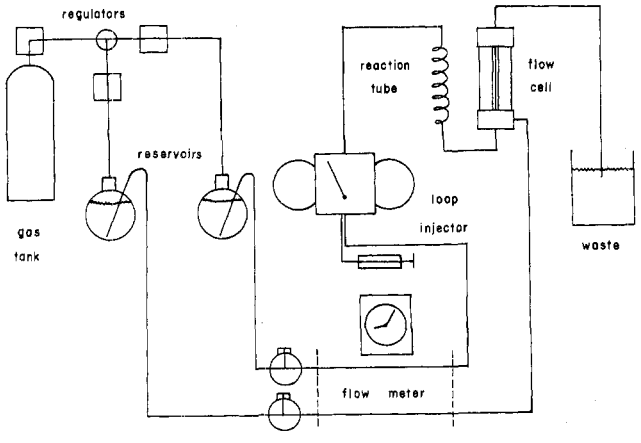


**Figure 2.** Schematic diagram of the flow system.

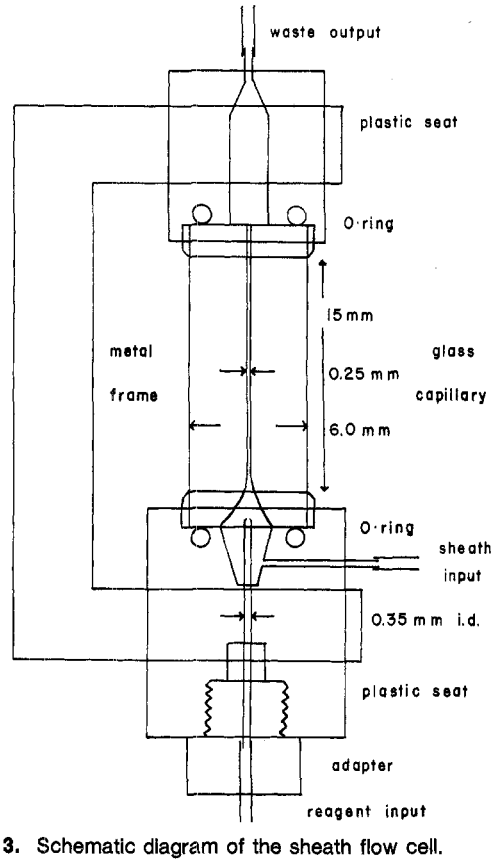


**Figure 3.** Schematic diagram of the sheath flow cell.

**Reagents.** Distilled, deionized water was used in making all the solutions. In the evaluation of the fluorometric flow system, a stock solution ($2.33 \times 10^{-4}$ M) of Rhodamine 6G tetrafluoroborate (Allied Chemical Co.) was used to prepare daily working solutions to be injected into the reagent stream of distilled, deionized water.

LDADCF was synthesized according to the procedure of Brandt and Keston (*23*). Dichlorofluorescein was obtained from Sigma (D-6132). A TLC plate of the synthesis product gave three spots (assumed to be product intermediates at each stage of the synthesis). LDADCF was therefore separated from 10% HCl by extraction (3×) into diethyl ether. The ether extract was dried over anhydrous $MgSO_4$, filtered, and evaporated to give crude crystals. This batch was then recrystallized twice from chloroform and hexane, along with being decolorized with charcoal and then filtered. The white crystalline product, as expected, had a melting point of 199.5–200.2 °C. A stock solution ($1 \times 10^{-4}$ M) of the compound was prepared in absolute ethanol.

For $H_2O_2$ and glucose determinations, phosphate buffer (0.025 M $NaH_2PO_4$ with 0.04 mg/mL $ZnSO_4 \cdot 7H_2O$), adjusted to the appropriate pH with 1 M NaOH and 1 M HCl, was used. LDADCF was activated with 0.01 M NaOH (1:4) and diluted appropriately with buffer. [Activation with dilute base is presumed to hydrolyze one or both of the acetyl groups on LDADCF; without this, no fluorescent product is formed on reacting with $H_2O_2$ (*21*).] The above buffer was used as well in the preparation of the enzyme solutions. HRP and GOD were both obtained from Sigma (P-8250, G-6125, respectively). Standards were prepared daily from aqueous stock solutions (3% $H_2O_2$ (MC&B), 100 mg/dL glucose (Baker Chemical Co.)). Serum studies were performed on commercial control sera (preanalyzed Dade Patho-trol and Lab-trol).

**Procedure.** For the characterization of the flow system, numerous pressure and flow settings were used. After a few minutes, the instrument was warmed up and the flow had stabilized. Flow rates were measured and Rhodamine samples were injected into a distilled, deionized water reagent stream. Several readings were taken of each signal before conditions of flow and/or concentration were varied.

Prior to their measurement with the fluorometer, the enzyme reactions (both separately and coupled together) were evaluated and optimized with respect to pH and the various concentrations and activities involved. Measurements were performed on a Perkin-Elmer 650-10S fluorescence spectrophotometer with a standard 3 mL (1.0 cm) rectangular cuvette. The direct adaptation of these optimum concentrations for the use on the flow system was somewhat arbitrary due to dilution of the reagent stream by the injected sample, as will be discussed later.

For clinical applications, pressures of 0.50 and 0.25 atm, corresponding to 2.2 and 1.0 mL/min flow rates for reagent and sheath streams, respectively, were used. An injection was made by switching the 25-μL loop in the reagent stream. It was switched back after reaching the signal peak and reloaded and reinjected just as a base line reading was obtained. For $H_2O_2$ analysis, the reagent stream was made up of activated LDADCF ($4 \times 10^{-6}$ M) in phosphate buffer (pH 8.5). Sample pretreatment involved adding HRP (8 IU/mL) before injections were made. In the glucose determination, the reagent reservoir contained activated LDADCF ($4 \times 10^{-6}$ M) and GOD (24 IU/mL) in phosphate buffer (pH 7.0). HRP (4 IU/mL) was added to the aqueous standards or diluted serum (1:1000 with distilled, deionized water), prior to injection. Similar treatment was used for spiked serum samples, for recovery studies, and in interference and inhibition studies. Serial dilutions were made in establishing calibration curves.

## RESULTS AND DISCUSSION

**Flow Fluorometer.** The establishment of a stable flow system was critical. Spherical reservoirs with a large solution surface to volume ratio minimized the effect of gravity on the flow rate. A few minutes (ten) allowed the flows to stabilize after each change in the regulated applied pressures. Due to the common source (one air tank) and common end (mixing in the sheath flow cell) of the two streams, the flow rates were very dependent on one another. Care was necessary in reproducibly injecting bubbles to measure the flow rates. With these precautions noted, the flow over a 1-h period (17 measurements) exhibited a relative standard deviation within 2% ($\bar{t}_r = 32.60 \pm 0.71$ s, $\bar{t}_s = 11.64 \pm 0.21$ s, and $\bar{F}_T$(measd) = 4.88 ± 0.078 mL/min). The reservoirs were large enough to provide over 3 h of stable operation before refilling. A value of $k$ was determined from the mean of the measured $F_T$ values of some fifty previous runs using eq 5. The determined $k$ value ($k$ = 0.6960 mL) was accurate to 0.2% in calculating the corresponding flow rates from $\bar{t}_r$ and $\bar{t}_s$ in the above 17 measurements, i.e., the means of sizable populations of values were reproducible to within 0.2% [$\bar{F}_r$(calcd) = 1.281 mL/min,

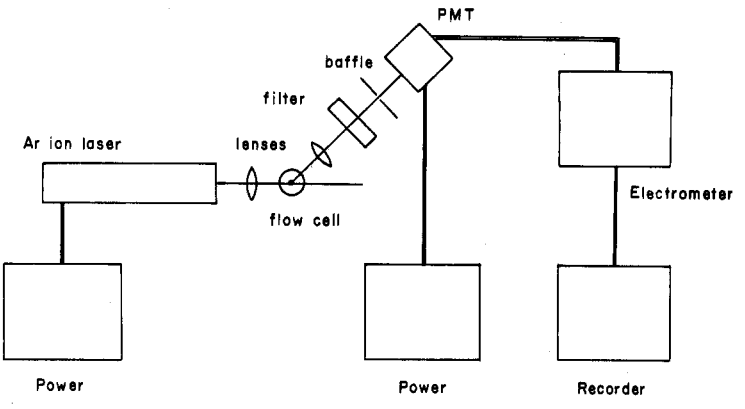


**Figure 4.** Schematic diagram of the fluorescence instrumentation arrangement (top view).

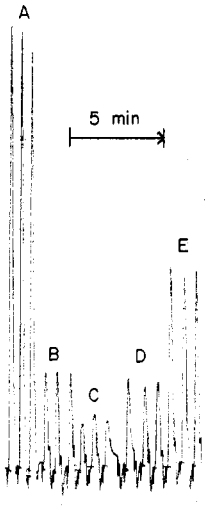


**Figure 5.** Sample peaks of triplicate runs of aqueous glucose standards (A = 0.50 mg %, B = 0.10 mg %, C = 0.05 mg %), serum (D = Dade Lab-trol), and spiked serum (E = D + 0.10 mg % glucose) samples.

$\bar{F}_s$(calcd) = 3.588 mL/min, and $\bar{F}_T$(calcd) = 4.869 mL/min)].

Operation of the injector gave smooth peaks (Figure 5), generally Gaussian in shape, indicating sample dispersion primarily by diffusion (*6*). Due to the switching back of the bypass loop (rinsed with solvent) into the stream, slight blemishes appeared on the trailing edge of each peak. The flat base line indicated minimal fluctuations in either the flow or the background signal.

The use of Rhodamine 6G as a fluorescent standard adversely affected the precision (8.5%) and sampling time (less than 100 runs per hour) of the instrument. Its high adsorbability on surfaces caused rapid contamination of the tubing, injector, and syringes, especially at higher concentrations (greater than $10^{-8}$ M). The sampling rate does decrease with decreasing reagent flow rate (the time for the response to return to base line or the peak base width increases) (Figure 6) as observed by Mottola (*24*). Lengthening the tubing allows for slower reactions (on the order of 2 min) to be analyzed before peak broadening becomes too great (Figure 7). With the rapid enzymatic reactions used here, short tubes (71 cm) were used to reduce dispersion.

Two particular relationships were experimentally inseparable. The fluorescence response increased with faster total flow rates. Increased flow rates over this short length of tubing reduces the residence time of the sample and thus constrains the plug to a smaller, more concentrated volume. The signal in the sheath flow cell was also proportional to the diameter of the reagent stream as indicated by the ratio $F_r/F_s$. Therefore, linearity was observed for the fluorescent response vs. the combined factor, $F_T(F_r/F_s)$ ($r$ = 1.000). These rela-

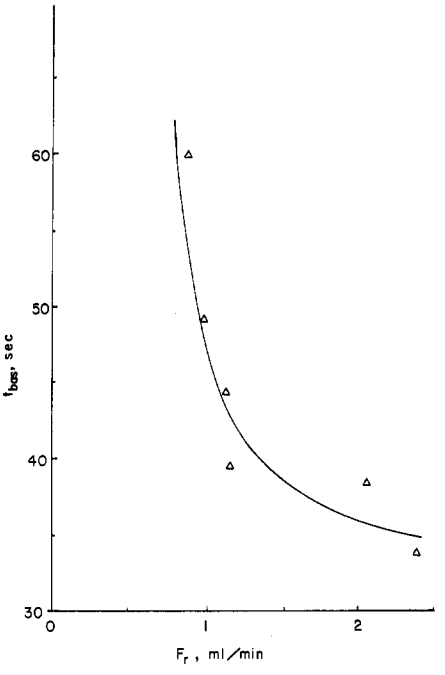


**Figure 6.** Relationship of $F_r$ (reagent flow rate) to $t_{bas}$ (time for response to return to base line). Rhodamine 6G (0.13 ng) injected each time.

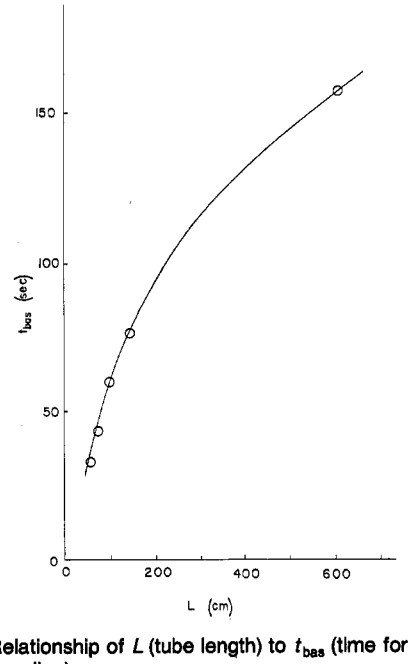


**Figure 7.** Relationship of $L$ (tube length) to $t_{bas}$ (time for response to return to base line).

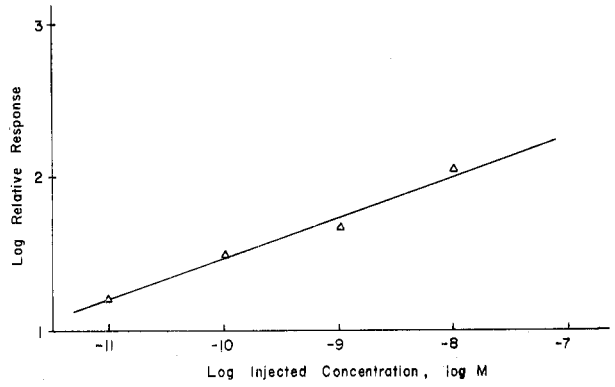


**Figure 8.** Range of the fluorescence response with concentration (of aqueous Rhodamine 6G standards).

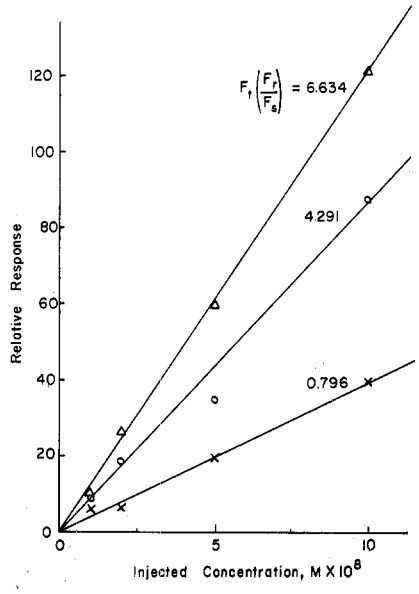


**Figure 9.** Short range linearity of fluorescence response with concentration (of aqueous Rhodamine 6G standards) at sets of flow conditions.

tionships were observed over the entire range of flow conditions.

The pressure range was limited to 0.2–0.6 atm due to failure of the tubing system at high pressures and inability to adjust the gas regulators at lower pressures. Particular standard flow conditions (2.2 mL/min reagent and 1.0 mL/min sheath) were chosen arbitrarily to enhance sensitivity without adversely compromising the consumption of reagents or the sampling rate of 100 runs per hour.

The range of the fluorometer's response is indicated in Figures 8 and 9. A relative response to the fluorphore (Rhodamine 6G) concentration is demonstrated over 4 orders of magnitude (Figure 8, $r = 0.982$). The nonlinear response for Rhodamine 6G over this broad range may be due to self-absorption and/or adsorption on the walls of the tubing at higher concentrations as indicated above. Sensitivity is based on the minimum detectable quantity of Rhodamine 6G apparent above the base line (noise was minimized with an RC filter). At this level, the concentration injected was $10^{-11}$ M, in which a 25-$\mu$L plug contains 130 fg of compound. The optimum wavelength of excitation for Rhodamine 6G is 530 nm with an emission maximum at 560 nm. The slopes of calibration curves and the minimum detectable quantity were observed to vary slightly from day to day depending on the exact flow parameters used and possible shifts in the optics. A few extra minutes to realign the microscope and to adjust the flow rates gave results that varied less than 5% from day to day. A linear response is obtained over a narrower range

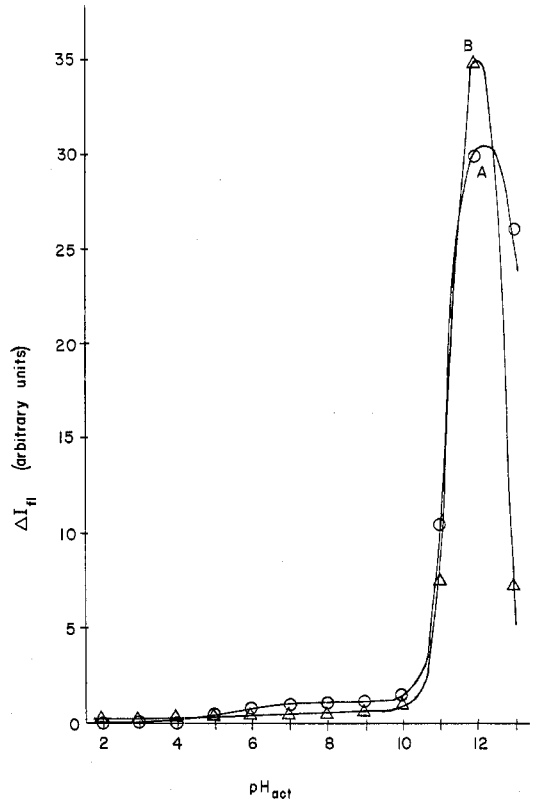


**Figure 10.** The changes in fluorescence intensity in 1 min for (A) activation of LDADCF ($2 \times 10^{-6}$ M) in unbuffered solutions of varying pH and (B) subsequent reaction of activated LDADCF ($4 \times 10^{-8}$ M) in 0.1 M phosphate buffer (pH 8.5) containing 4 IU/mL HRP, $10^{-6}$ M $H_2O_2$.

and is seen to hold over a range of flow conditions (Figure 9, $r = 0.999$, 0.994, and 0.996 for top, middle, and bottom curves, respectively). Preliminary studies have been performed by using a previously described laminar flow cell (*20*) with similar sensitivity and dynamic range results.

**$H_2O_2$ and Glucose Determinations.** Reaction 2 was initially studied separately as a means of quantitatively determining $H_2O_2$. The LDADCF reagent was chosen because the dichlorofluorescein product can be excited at the 488 nm wavelength of the argon ion laser. The optimum wavelength of excitation was determined to be 505 nm, with an emission maximum at 525 nm. The stability of LDADCF to activation by base hydrolysis and subsequent activity of the activated form (Figure 10) verified the use of $10^{-2}$ M NaOH for that hydrolysis. The blank signal (A) is from a 50-fold larger concentration of the dye than used to obtain the analytical signal (B). Hence, the blank signal represents 1.8% of the analytical signal at equimolar dye concentrations when activated at pH 12. The fact the blank signal occurs when the dye is activated at pH 12 indicates that the activated form of the dye undergoes a small amount of autooxidation.

Keston and Brandt employed a pH of 7.2 with $2 \times 10^{-6}$ M LDADCF and 0.001 mg/L HRP for $H_2O_2$ measurement. We have found a sharp pH dependence, with the maximum response at pH 8.5, being 200% of the response of pH 7.2. The other variables were optimized at pH 8.5 using a simplex technique (*25*) (0.2 IU HRP, $8 \times 10^{-7}$ M LDADCF). An increase of about 10-fold in the signal was obtained by adjusting conditions from those of Keston and Brandt to our optimized conditions. A calibration curve established linearity of at least 3 orders of magnitude ($r = 0.998$) and detection levels at $10^{-10}$ M $H_2O_2$, corrsponding to 85 fg of $H_2O_2$ or 1 pg of DCF produced. The precision of such measurements was anywhere from 0.5 to 5.5%, generally better than 2.6%.

In an identical manner, the optimum conditions for both reactions together were obtained. For the glucose analysis,

Table I. Effects of Various Interfering and Inhibiting Compounds on the Glucose Response

| compound | concn injected, M | rel effect, % |
|---|---|---|
| amylose | $1 \times 10^{-5}$ | +14 |
| maltose | $3 \times 10^{-5}$ | +39 |
| lactose | $8 \times 10^{-5}$ | 0 |
| starch | 10[a] | +9 |
| uric acid | $1 \times 10^{-4}$ | −5 |
| ascorbic acid | $2 \times 10^{-4}$ | −1 |
| bilirubin | $1 \times 10^{-7}$ | 0 |

[a] μg/mL.

optimum conditions were found to be pH 7.0, 0.36 IU HRP, 0.55 IU GOD, and $1 \times 10^{-6}$ M LDADCF. The rather sharp pH dependence was a compromise between the optimum pH value of 5.5 for the glucose oxidase reaction and 8.5 for the dye reaction. It was possible to measure as little as 2 ng of glucose (25 μL of $5 \times 10^{-7}$ M or 0.01 mg/dL aqueous glucose standard). Overall precision was the same as that of $H_2O_2$ analysis. Serum samples should be diluted a thousandfold. This will tend to minimize the effect of interferences. Table I shows the effects of several possible interfering or inhibiting compounds at greatly elevated concentrations from those found in serum. The elevated signals were from amylase, maltase, and glycogenase as impurities in the GOD preparation. Therefore with better reagent purities, negligible effects would be seen at possible serum concentratons of potential interference. With respect to the serum itself, an average recovery of glucose of 102% was seen from spiked serum samples, and the mean determined glucose level was 99% of the established levels present.

In the application to oxidase systems, the LDADCF method performed exceptionally well. It has also been applied successfully in the determination of uric acid using uricase (22). Concerning the adaptation of concentrations from the optimized static system to the flow system, another tradeoff became evident, that being between the detection power and the convenience and expense of analysis. Absorbance measurements of injected colored solutions indicated an approximate average dilution of the reagent stream by sample of 10:1 (i.e., the sample is diluted about 10% in the stream) from diffusional mixing, corresponding to a situation of limited dispersion. Therefore the amounts of LDADCF and GOD were significantly lower than at optimum static conditions. In fact, because of this, the actual FIA response increase in optimizing to conditions established using static solutions was 400% rather than 10-fold. The actual dynamics of relative concentrations involved with FIA are complex enough so as to require optimization on the particular system in use. The sample pretreatment with HRP was also a necessity. The presence of HRP in the dye reagent gave an undesirable background fluorescence possibly due either to some inherent fluorescence in the preparation or to an alternative side-reaction mechanism involving $O_2$ rather than $H_2O_2$. This was equivalent to $<10^{-10}$ M $H_2O_2$ and limited the detection power to $10^{-10}$ M. A simple solution would be to merge third stream or plug containing HRP with the reagent stream after the point of sample injection or possibly to place immobilized HRP downstream.

The effectiveness of a new fluorometer FIA system has been demonstrated. A simple combination of techniques has resulted in an accurate, precise, sensitive, stable, and versatile instrument that is easy, fast, and inexpensive in its construction and operation. Also presented was an enzymatic fluorescence reaction scheme of broad application to oxidase systems that can be used in this flow system using argon ion laser excitation at 488 nm.

## ACKNOWLEDGMENT

The authors express their gratitude to Leon Hershberger and James Callis for their assistance and suggestions in the construction and operation of the instrument and to Paul DePree for his work in separating and analyzing the dyes in this study.

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RECEIVED for review May 18, 1981. Accepted July 3, 1981.