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TITLE: PULSED LASER FLUOROMETRY FOR ENVIRONMENTAL MONITORING

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Pulsed Laser Fluorometry for Environmental Monitoring

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Air sampling

Abstract

A compact pulsed laser fluorometer has been incorporated into a continuous flow system developed to detect acetylcholinesterase (AChE) inhibitors and/or primary amine compounds in air and water. A pulsed nitrogen laser pumped dye laser excites fluorescent reactants which flow continuously through a quartz flow cell. Data are collected, analyzed, and displayed using a Macintosh II personal computer. For detection of cholinesterase inhibitors the fluorogenic substrate N methylindoxyl acetate is used to monitor the activity of immobilized enzyme. Presence of inhibitors results in a decrease of steady state fluorescence. In water 100 ppt of isopropyl nitrophenyl methylphosphonate (INP) can be detected within two minutes. In conjunction with an in-house designed ultrasonic micromist air sampler and concentrator, we estimate that air borne levels of less than 0.1 ppt of INP will be detectable. Detection of compounds containing primary amines is based on their reaction with fluorescamine to rapidly produce intensely fluorescent products. Compounds of interest to our research were amino acids, peptides, and proteins. An increase in steady state fluorescence could be cause to evaluate the reasons for the change. The detection limit of the protein, bovine serum albumin (BSA) in water is 10 ppt. Nebulized BSA concentrated by the LANL air sampler can be detected at sub ppt original air concentration.

<H1> Introduction <H1>

Fluorescence chemistry offers the potential for extremely sensitive detection analyses. At the Los Alamos National Laboratory, we have been developing for several years both instrumentation and analytical procedures to detect and measure fluorescence at the molecular level. (Reviewed in Jett et al., 1990). Recently, single chromophores of Rhodamine 6 G have been reliably detected in a flowing sample stream using laser-induced fluorescence (Seitzinger et al., 1990). While this level of sensitivity requires very sophisticated instrumentation, moderately priced ($5-10 K) commercially available instrumentation can detect fluorescent compounds in the nanomolar to picomolar range. One such instrument is a pulsed laser fluorometer, a nitrogen pumped dye laser with the capability to excite and measure fluorescence from the near-ultraviolet to the near-infrared spectral regions. The authors were tasked to develop a point detector capable of detecting both acetylcholinesterase inhibitors and biological molecules (amino acids, peptides, and proteins) in water or air. As part of this system, we incorporated a pulsed laser fluorometer into a continuous flow monitor for each class of compounds above. The instrument should prove useful for the detecting and monitoring any compound or class of compounds for which fluorescent chemistry exists.

Fluorescence detection of the organophosphorous nerve agent, sarin, was reported in 1957 (Gehauf and Goldenson, 1957). Their method was based on the formation of highly fluorescent indoxyl by the direct reaction of sarin with indole and sodium perborate. Sensitivity of the method approached 1 ng of sarin ml^{-1}. A problem with this procedure is that the indoxyl is a short-lived (less

than 1 min) chemical intermediate in the reaction sequence between indole (the starting compound) and indigo (the end compound).

Guilbault and Kramer (1965A) combined immobilized acetylcholinesterase (AChE) with a fluorogenic substrate to monitor the reaction of the enzyme with anticholinesterase compounds. AChE was insolubilized within a starch gel placed on a polyurethane foam pad. A solution of 2-naphthyl acetate (non-fluorescent) was slowly passed through the enzyme pad, where it was enzymatically converted to the highly fluorescent 2-naphthol (ex 320 nm, em 410 nm). A drop in the baseline fluorescence indicated the presence of an inhibitor, and the rate of fall provided some idea of the activity of the inhibitor. The reported system easily detected 1 μg sarin ml^{-1} water.

Other fluorogenic substrates for AChE include resorufin butyrate and indoxyl acetate (Guilbault and Kramer, 1965 B), N-methylindoxyl acetate (Guilbault et al, 1968), and 1-methyl-7-acetoxyquinolinium iodide (Prince, 1966). The most sensitive of these substrates for AChE detection is reported to be N-methylindoxyl acetate (Guilbault, 1973). More recently, Parvari (Parvari et al, 1983) described a very sensitive two-step fluorometric reaction for detecting AChE activity. Acetylthiocholine is first converted by the enzyme to thiocholine, which is then reacted with coumarinylphenylmaleimide (CPM) to form a fluorescent product.

We explored both N-methylindoxyl acetate and CPM chemistries for use in our monitoring system.

Many methods are currently available for sensitive detection of amino acids, peptides, and proteins. For a fluorescent chemical process to be practical in a continuous flow water monitor, it must be sensitive and quick,

and the starting reactants should not be fluorescent. The reactants and products should also be compatible with an aqueous environment. For our experiments, we chose to examine both CPM, which reacts with thiols, and fluorescamine, which reacts with primary amines. CPM initially was of great interest to us because a single laser dye and set of detection optics would allow us to monitor both AChE activity and proteinaceous material. CPM was initially synthesized and used for the histochemical staining of protein thiol groups on tissue sections (Sippel, 1981 A,B). Fluorescamine (4-phenylspiro [furan-2(3H) 1'-phthalan]-3, 3'dione) was introduced in 1972 (Weigele et al, 1972 A, Udenfriend et al, 1972). This reagent reacts with primary amines to form the same fluorophors as are produced in the ninhydrin-phenylacetaldehyde reaction (Weigele et al, 1972 B). At room temperature the fluorescamine reaction with primary amines is extremely rapid, proceeding with a half time of a fraction of a second. CPM reacts more slowly but significant reaction does occur within one minute.

As stated earlier we were tasked to develop a monitoring system suitable for continuous sampling of both water and air. Because the monitoring system was designed to perform continuous aqueous phase wet chemistry, we required an air sampler that would scrub contaminants from the air into an aqueous phase suitable for injection into the detector. We were unable to obtain a compact, reasonably priced device from commercial sources to perform this task. Therefore we proceeded to build and test a compact air sampler/scrubber based on our own unique design (described later). A prototype system sampling at the rate of 50 L of air per minute has concentrated protein aerosols from 3-10, 000 times.

<H1> Materials and Methods <H1>

<H2> Chemicals <H2>

N-methyl indoxyl acetate (NMIA), purified electric eel acetylcholinesterase (AChE), fluorescamine, acetylthiocholine, all buffer chemicals, amino acids, (BSA) bovine serum albumin (fraction V), and solvents were obtained from Sigma Chemical Company, St. Louis, MO, 63178, USA. 7-diethylamino-3-(4'-maleimdylphenyl)-4-methylcoumarine (CPM) was obtained from Molecular Probes, Inc., Eugene OR 97402, USA. CNBr-activated Sepharose 4B was obtained from Pharmacia AB, Uppsala, Sweden. Isopropyl p-nitrophenyl methylphosphonate (INP) was a gift from Dr. Thomas Whaley, Biochemistry/Biophysics Group, Life Sciences Division, Los Alamos National Laboratory, Los Alamos, NM 87545, USA.

<H2> Immobilization of Acetylcholinesterase <H2>

One unit (about 1 μg) of AChE in 5 ml of 0.1 M carbonate buffer, pH 9.7 was added to one gm of CNBr-activated Sepharose 4B and gently rocked for 4 h at room temperature. The mixture was refrigerated overnight to hydrolyze remaining active sites. The Sepharose-AChE was washed with 300 ml of PBS, resuspended in 10 ml of PBS containing 5 mg of BSA. This reagent was stable for at least 6 months at 4^{0} C.

<H2> Stock and Working Reagent Solutions <H2>

NMIA: stock, 0.62 M in ethylene glycol monomethyl ether (stable at least 3 months); working, 75 μl of stock in 50 ml 0.1 M PO_4 buffer, pH 7.5 (stable for at least 24 h). CPM: stock 2 mg in 10.0 ml of acetonitrile; working, small amounts of stock were added directly to the sample to be analyzed. Aqueous CPM solutions were not stable because of spontaneous hydrolysis. INP: stock,

100 μg ml^{-1} in acetonitrile (stable at least 3 months); working dilutions of INP were made fresh daily in acetonitrile from stock. Sepharose-AChE: 50 μl (0.005 unit AChE per test). Acetylthiocholine: Stock (prepared weekly), 2 mg ml^{-1} 0.1 M PO_4 buffer, pH, 7.5; working dilutions varied. CPM: stock, 2 mg in 10 ml of acetonitrile; working dilutions varied but were prepared fresh daily from stock. Fluorescamine: stock/working, 200-400 μg ml^{-1} in acetonitrile (stable at least 3 mo). Fluorescamine rapidly hydrolyzes in the presence of water. The buffer used for amino acid/protein reactions with fluorescamine was 0.05 M sodium borate, pH. 10.0. Small amounts of stock fluorescamine were added directly to the buffered sample to be analyzed.

<H2> Continuous Flow Monitor for AChE Inhibitors, Figure 1 <H2>

Silicon tubing is used throughout. The sample and NMIA substrate are individually pumped (Peristaltic Pump P-3, Pharmacia, Uppsala, Sweden) into a mixing chamber and then flow through a reaction chamber (figure 2) which contains sepharose-AChE. The reaction chamber consists of a small disposable pipette tip in which a large glass bead is fitted The large bead is sequentially layered with small glass beads and sepharose-AChE. A stopper fitted with a 20 gauge needle seals the chamber. The fluorescent product flows into the measurement chamber, which is a small volume flow cuvette (Hellma Cells Inc., Box 544, Jamaica,NY 11424; flow cell Model No. 176.751-QS Z-8.5). Typically about two minutes is required for the sample to arrive at the measurement chamber. At this point the beam of a nitrogen pumped pulsed dye laser (Laser Sciences Inc., 80 Prospect St., Cambridge, MA 02139; Model 337500) impinges on the flow channel of the cuvette. The fluorescence signals are electronically processed and acquired and stored in a MAC-II computer. Presence of AChE inhibitors is indicated by a reduction of fluorescence

intensity.

<H2> Continuous Flow Monitor for Proteins, Peptides, Amino Acids, Figure 3 <H2>

The sample is pumped by a peristaltic pump and is mixed with both fluorescamine and borate buffer (pumped with a Harvard 22 Syringe Pump, Harvard Apparatus, South Natick, MA 01760, USA). The fluorescent reaction products pass through the flow cuvette where the fluorescence intensity is determined by the pulsed laser fluorometer. The fluorescence intensity increases with increasing levels of compounds containing primary amines. Signals are processed and stored as above.

<H2> Pulsed Laser Fluorometer <H2>

A nitrogen pumped pulsed dye laser (Laser Sciences Inc., 80 Prospect St., Cambridge, MA 02139; Model 337500) impinges on the flow channel of the cuvette. For the detection of primary amines we have used the laser dye known as BBQ (Laser Sciences Inc. Cat. No. 337876) to obtain output at a wavelength at ~ 395 nm for the excitation of the fluorescent product of fluorescamine. For detecting AChE inhibitors we have used the laser dye Coumarine 450 (Photochemical Research Associates, 45 MEG. Drive, London, Ontario, Canada NGE2U2; Cat. No. 2A446) to obtain output at a wavelength of 436 nm for the excitation of the fluorescent product n-methylindoxyl. The pulsed laser is externally triggered usually at a rate of about one pulse per second. We chose this slow rate for two reasons: first, it maximizes the laser life time (the nitrogen laser tube will last for about twenty million shots or about eight months at this rate) and second, it minimizes the amount of data stored by the computer based data acquisition system.

The fluorescence detection system is contained in a light tight housing.

The dye laser beam enters the housing after passing through a band pass filter. This filter blocks spontaneous fluorescence emission from the laser dye which is an undesired background for the fluorescence measurement. Though the flow cuvette is centered in the laser beam most of the laser energy is not absorbed by the sample but passes on through the cuvette and is absorbed by a beam dump. Sample fluorescence detected at right angles to the laser beam, passes through a long pass (and/or band pass) filter to block any laser light that might have been scattered by the sample or the cuvette. The sample fluorescence is detected by a small diameter (1.125 inch) photomultiplier tube (PMT, Thorn EMI Gencom Inc., 23 Madison Rd., Fairfield, NJ 07006; Model 9924B and Model QL/30 PMT housing) attached to the detector housing. The PMT high voltage is operated in the range of 500 to700 volts DC. Fluorescence emission from the sample during each laser pulse gives rise to a current pulse from of the PMT. The PMT signals are converted to voltage pulses by a transimpedance amplifier (Hiebert, 1990) Without further amplification these signals are routed to a Gated Peak-Sense and Hold circuit (GPS&H), (Steinkamp and Hiebert, 1982). The Peak-Sense and Hold circuit is gated on only at the time of each laser pulse, thus the detection electronics is processing signals only for each laser pulse. The signal amplitude is held for about 20 μs then cleared after the signal is digitized.

The signal amplitude is digitized by a 12 bit Analog-to-Digital Converter (ADC) contained on a data acquisition board from G.W. Instruments (35 Medford St., Somerville, MA 02143; Model No. GWI-625) which is interfaced to a Macintosh II (Apple Computers Inc., 20525 Mariana Ave., Cupertino, CA 95014). This board has several useful features. Its ADC can standardly digitize four signals and up to 12 signals using an expansion daughter board. The board can generate up to four logic output signals with programable control of timing and pulse width. These logic signals are used for external triggering of the pulse

laser and for triggering the GPS&H circuit. The board also contains two Digital-to Analog Converters (DAC) under programmable control. The DACs can be used to control the PMT high voltage power supply output or to generate special purpose pulses with programable waveforms.

The fluorescence data is recorded and stored by a Mac II program based on GW Instruments' control function library for the ModelGWI-625 data acquisition board. The data record is displayed in chart recorder format giving a history of the fluorescence intensity. The program also has a threshold alarm which analyzes the data for significant excursions from the background fluorescence as it is recorded. The usual alarm analysis tests the running average of the ten most recent measurements and inspects for a greater than ten percent change from the long term average. An alarm is read out several ways: 1) as a message on the computer terminal, 2) as a DAC output used to generate an audio alarm and to turn on a flashing light, and 3) as a trigger for a remote alarm either by a direct wire hookup or by a microwave link. The response time of the computer alarm discriminator is close to real time, ten seconds of data inspection. The total system response time is
the sum of the sample delay time and the software discriminator time or about two minutes and ten seconds.

<H2> Data Acquisition And Analysis <H2>

Data acquisition and analysis are accomplished using a Macintosh II (Apple Computers, Sunnyvale, CA, USA) computer with an internal analog-to-digital converter (ADC) board (GW Instruments, Inc., Somerville, MA, USA). The computer, ADC board, and laser are controlled by a program written in binary basic (Microsoft, Redmond, WA, USA) and a library of ADC control functions supplied by GW Instruments.

The program triggers the laser at a user controlled frequency. At a specific time after each trigger, the ADC converts the voltage available at the analog input to the board into integers with 12 bit precision (10 volts/4096). The data are plotted on the screen of the computer and (optionally) written into a file. Two averages are calculated after each conversion. The short average is the average of the current and previous relative few (on the order of 10) conversions. The long average is the average of the current and previous relative many (on the order of 200) conversions. The numbers of conversions in each average are user selectable. If the short average is greater than the long average by a user selectable factor, a 5 volt level is set at the analog output of the board (suitable for triggering an external alarm), and a bell is sounded by the computer. The factor could be related to the statistics of the measurements, for example, two standard deviations of the long average. The program can be set to run for any practical amount of time.

<H2> Ultrasonic Micromist Air Scrubber <H2>

Figure 4 schematically illustrates the principles used in the Los Alamos designed air sampler/scrubber. A small vacuum cleaner continuously draws air through the unit. A piezoelectric crystal oscillating at 2 million cycles per second generates a micromist (most of the mist will pass through a 0.5 um pore size filter) whose droplets scrub chemical vapors from the sampled air. A series of baffles (two are illustrated in the figure) condense and precipitate the contaminant containing droplets. The resulting fluid is pumped through the detector where it is analyzed for components of interest.

<H1> Results <H1>

<H2> Monitoring for Cholinesterase Inhibitors With NMIA <H2>

In the presence of AChE, the non-fluorescent NMIA is rapidly hydrolyzed to intensely fluorescent (ex 430 nm, em 501 nm) N-methylindoxyl (NMI) and acetic acid. NMIA stock solutions in ethylene glycol monomethyl ether were stable for at least one month. Working dilutions in 0.1 M phosphate buffer underwent slow spontaneous hydrolysis. Therefore the working solution is prepared just prior to analysis. INP was used as the AChE inhibitor in all of our experiments. The AChE inhibitory activity of this compound is estimated to be about 100 X less (per molecule) than the nerve agent sarin (T Whaley, LANL, personal communication). Stock and working dilutions were prepared in a biosafety cabinet. Rubber gloves, labcoat, and safety glasses should be used when one is working with this compound. Spills can be rapidly neutralized with molar sodium hydroxide solutions.

<H3> Preliminary Experiments in a Static System <H3>

Varying amounts of INP were placed into a cuvette containing 10^{-4} M NMIA. At time t=0 either 10^{-3} or 2.5 x 10^{-3} units of AChE were added with rapid mixing. Hydrolysis kinetics of the conversion of NMIA to NMI were monitored for 10 min. Examples of results obtained in this type of experiment are shown in figure 5. Under limiting AChE levels (10^{-3} units) significant inhibition of hydrolysis was seen at about 100 pg ml^{-1} of INP. In data not shown, we established that the INP solvent, acetonitrile, did not effect AChE induced NMIA hydrolysis. Reproducibility of results obtained in the static system were excellent (Figure 6). The data illustrates the overlay results of three separate experiments comparing spontaneous, slightly inhibited (130 pg INP ml^{-1}), and non-inhibited hydrolysis of 10^{-4} M NMIA. The amount of AChE used in fig 6 b

and c was 0.002 units ml^{-1}.

<H3> Measurements in Flow <H3>

The instrumental set up was as shown in figure 1. Flow rate through the reaction chamber was approximately 3 ml hr^{-1}. Figure 7a represents a six hour run with NMIA (10^{-4} M) in the abscence of AChE to illustrate the very low spontaneous hydrolysis rate observed. For the 6 hr experiment illustrated in figure 7b the reaction chamber contained 0.005 unit of Sepharose-AChE. The fluorescence intensity of the AChE catalyzed NMI remained remarkably constant throughout the run. Figure 8 illustrates the effect on NMI generation when either 10 ng (8a) or 1 ng (8b) of INP contained in 1 ml of NMIA solution passed through the reaction chamber. The gradual decrease in fluorescence intensity is caused by the poisoning of the immobilized AChE as the INP passes through the system. Once the bolus of INP has passed through the system no furthur decrease in fluorescence occurs, thus indicating that a new steady state exists which is directly proportional to the amount of active enzyme remaining. A reaction chamber could be used for experiments for a least 5 days without apparent loss of enzyme activity. However once the chamber was challenged with INP we did not attempt to reverse the poisoning; therefore a reaction chamber was used only once in a challenge experiment.

The generation of NMI reaction product was demonstrably effected by changes in the ambient temperature. Figure 9 illustrates this effect during a 48 hr run. Especially significant changes in reaction rate occur during afternoons when the black shade in our laboratory absorbed and released significant amounts of solar energy into the room. The data acquisition program (see Materials and Methods section for a description) we use compensates for these gradual changes in reaction rate. Alternatively the

reaction chamber could be placed in a temperature controlled compartment.

<H2> Monitoring AChE Activity With Acetylthiocholine and CPM <H2>

AChE catalyzes acetylthiocholine to thiocholine and acetic acid. The thiocholine can then react with CPM to yield an intensely fluorescent product (Parvari et al, 1983). We explored the use of this reaction sequence as a scheme to monitor AChE activity. Stock solutions of CPM were stable in acetonitrile for at least two weeks. However, working solutions of CPM in 0.1 M PO_4 buffer, pH 7.5 were not suitably stable for use in a continuous flow long term monitoring system, because of rapid (within 2 hr) spontaneous conversion of CPM (in the abscence of acetylthiocholine) to a fluorescent product, figure 10. No improvement was seen with CPM in tris buffer. Additionally, preliminary kinetic experiments in a non-flowing system indicated that the CPM scheme was significantly less sensitive than the NMIA scheme, figure 11. Using NMIA we could detect less than 1 ng of INP, figs. 5,8. We therefore decided not to pursue CPM as a fluorogenic reagent for a continuous monitoring system for AChE inhibitors.

<H2> Use of CPM To Monitor Proteins, Peptides, and Amino Acids. <H2>

Because of both the limitations discussed above and initial experimental observations that, while CPM rapidly reacts with proteins, it only slowly reacts with amino acids (data not shown) we chose not to pursue the use of CPM in our work.

<H2> Fluorescamine as a Fluorogenic Reagent For Proteins, Peptides, and Amino Acids <H2>

Fluorescamine rapidly reacts with proteins, peptides, and amino acids to form stable fluorescent products. Because fluorescamine rapidly hydrolyzes in

aqueous media, it must be dissolved in water miscible solvents and added rapidly to test solutions. All tests were performed in 0.05 M borate buffer, pH 10.0. Fluorescamine was in acetontrile at 0.2 mg ml^{-1}.

<H3> Static Experiments <H3>

The ability of fluorescamine to react with a number of amino acids was established by rapidly adding 100 ul of fluorescamine to 2 ml of buffer containing 0.5 ng of the amino acid. The pulsed laser fluorometer was used to measure the relative fluorescence intensity of each sample. The results of this experiment are shown in Table 1. All of the amino acids tested except proline, which contains a secondary rather than a primary amine, yielded detectable fluorescent products.

In similar experiments we examined the reaction between fluorescamine and the purified protein, BSA. An example of the results of this type of experiment is presented in Table 2. The reproducible detection limit was found to be 5×10^{-10} M.

<H3> Experiments in Flow <H3>

See figure 3 for the instrumental setup. In contrast to the AChE-NMIA reaction, the reaction between fluorescamine and amino acids, peptides, and proteins is not visibly temperature dependent. Therefore baseline fluorescence, representing ambient levels of these compounds, is relatively stable over long time periods.

Examples of detecting short pulses of BSA are shown in figure 12. The borate buffer flow rate was 0.4 ml min^{-1}; the fluorescamine (400 ug ml^{-1}) flow rate was 0.5 ml hr^{-1}. A pulse containing BSA at a concentration of 5 x

10^{-9} M can routinely be detected, fig. 12 b.

For long runs the borate buffer is pumped from a reservoir using a peristaltic pump, instead of the syringe drive. The fluorescamine is pumped via the syringe drive. Figure 13 illustrates continuous flow performance over 2 four day periods. Once each day the system was challenged with one ml of a 5 x 10^{-8} M BSA solution.

<H2> Ultrasonic Micromist Air Sampler <H2>

<H3> Efficiency of Aerosol Collection <H3>

3 ml of a 2.5 x 10^{-6} M BSA solution was placed into a small Hudson disposable nebulizer (obtained from our local hospital) which was attached to the intake stack of the air sampler. The nebulizer was driven at about 12 PSI with the lab air line. At this pressure approximately 0.45 ml of BSA solution was nebulized min^{-1}. The nebulized sample was introduced into the sampled air stream (flowing at 50 L min^{-1}) and mixed with the micromist which was ultrasonically generated from the borate buffer containing reservoir. The micromist was condensed by the baffles and precipitated. Precipitated samples were collected at various time intervals. Collected samples were analyzed for protein content by a Bio-Rad Protein Assay (Bio-Rad Laboratories, Richmond CA, USA) . The amount of protein collected was compared to the amount introduced, thus allowing us to determine the scrubbing efficiency of the device for BSA aerosols. In one experiment (whose results are shown in Table 3) 2.3 ml of the BSA solution was delivered into the sampler over a 5 minute period. As determined in the protein assay 9,844 optical density units of protein (402.4 ug) were delivered. Precipitated fluid samples were collected continuosly for 4 five minute intervals. About 2 ml of fluid min^{-1} was collected from the device. The optical density units contained in each 5 minute aliquot

were determined and totaled. A total of 2,803 OD units were obtained over the 20 minute period, indicating a collecting efficiency of about 28 %. Approximately 45 % of the collected OD were obtained in the first 5 min sample, and more than 86 % of the scrubbed sample was cleared within 10 minutes (5 minutes after collection ceased). Therefore about 24 % of the entering sample was scrubbed from 250 l air and delivered into 20 ml liquid within 10 minutes. This translates into a concentration factor of about 3,000. (402.5 ug BSA delivered in 250 l air = 1.61 ug l^{-1} or 1.61 ng ml^{-1}. Of this, 97 ug BSA was trapped and collected in 20 ml liquid. Each ml liquid contained 4.8 ug BSA, or about 3000 x as much BSA (4800/1.61) than was contained in 1 ml original air.)

<H3> Aerosolized BSA Detection In Conjunction With Fluorescamine Chemistry <H3>

Three ml of each BSA concentration was placed in the nebulizer. The solution was nebulized for 2 min at 10 psi into the flowing air sample (50 l min^{-1}). The reservoir contained borate buffer, pH 10. The condensed liquid precipitate was collected for 2 minutes for each BSA concentration nebulized. Fluorescamine (20 ul at 400 ug ml^{-1}) was added to the collected precipitate. The samples were read in the pulsed laser fluorimeter. The results were plotted as a function of the BSA concentration within the flowing air stream, fig 14. As can be seen one ppt of BSA in air, when concentrated by the air sampler and coupled to the fluorescamine detection chemistry, was readily detected.

<H1> Conclusions <H1>

This chapter describes the development and application of a pulsed laser fluorometer which has been incorporated into a continuous flow system for use

in environmental monitoring. The system provides a high degree of sensitivity in a compact, relatively inexpensive device, and should be capable of monitoring for any class or individual compound for which a fluorescent chemistry exists. By replacing the flow cuvette with a stationary cuvette, the pulsed laser fluorometer component can also be used as an instrument to measure fluorescence of individual reacted samples collected elsewhere.

The compact ultrasonic micromist air sampler described herein offers the environmental monitoring community a powerful and economical new tool for continuous air sampling and scrubbing of ambient air for an almost limitless number of contaminants. The device can serve as a "front end" to a variety of analytical systems.

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<H1> References <H1)

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Table 1. Detection of Amino Acid-Fluorescamine Reaction Products

Amino Acid[1]	**Relative Fluorescence Intensity**[2]
Buffer only	5
Buffer + Fluorescamine	42
Methionine	75
Cysteine	135
Lysine	108
Cystine	100
Phenylalanine	111
Tyrosine	60
L-glutamine	110
Arginine	147
Isoleucine	96
Proline	40
Asparagine	120
L-valine	102
L-serine	113
Threonine	167

1. 0.5 ng/tube
2. Arbitrary units

Table 2. Detection of BSA-Fluorescamine Reaction Products

BSA Concentration[1]	**Relative Fluorescence Intensity[2]**
Buffer	50
Buffer + fluorescamine	80
10^{-10}	95
5×10^{-10}	110
10^{-9}	135
5×10^{-9}	235
10^{-8}	>500 (off scale)

1. Mol. l^{-1}
2. Arbitrary units

Table 3. Collection Efficiency of the Ultrasonic Micromist Air Sampler[1]

Collection Period	Optical Density Units Collected
0-5 min	1260
5-10 min	1160
10-15 min	287
15-20 min	96

1. 402.5 µg BSA containing 9844 Bio Rad OD units were delivered into 250 l of air. Scrubbed samples were collected for four 5 min intervals. About 10 ml of sample was collected during each interval. The protein content, expressed as OD units, was determined for each sample. Approximately 24% of the delivered BSA was scrubbed from 250 l air and collected into 20 ml liquid within 10 minutes.

Figure legends.

Figure 1. Los Alamos chemical detector. Schematic diagram of system components. See text for narrative.

Figure 2. Drawing illustrating the components of the reaction chamber used for the generation of fluorescent reaction product which results from the interaction of immobilized AChE and a fluorogenic substrate.

Figure 3. Apparatus for detecting protein using fluorescamine. Schematic diagram of system components. See text for narrative.

Figure 4. Ultrasonic micromist air sampler. Schematic diagram which illustrates the principles of the device. See text for narrative.

Figure 5. Kinetics of the inhibition of AChE by INP. (a) Using 2.5×10^{-3} units of enzyme; (b) using 1×10^{-3} units of enzyme.

Figure 6. Results of three replicate kinetic experiments which illustrate the reproducibility of data obtained with the pulsed laser fluorometer. (a) spontaneous hydrolysis of 10^{-4} M NMIA; (b) hydrolysis of NMIA in the presence of 2×10^{-3} units AChE; (c) hydrolysis of NMIA in the presence of AChE and 130 pg INP.

Figure 7. Continuous flow analysis of the hydrolysis of 10^{-4} M NMIA in the abscence (a) and presence (b) of 5×10^{-3} units of immobilized AChE.

Figure 8. Sensitivity of detection of AChE inhibition by INP in flow. (a) injection of 1 ml of a sample containing 10 ng INP; (b) injection of 1 ml of a sample containing 1 ng INP.

Figure 9. Effect of changes in ambient temperature (see text) on the rate of enzymatic hydrolysis of NMIA during a 48 hr continuous run in flow.

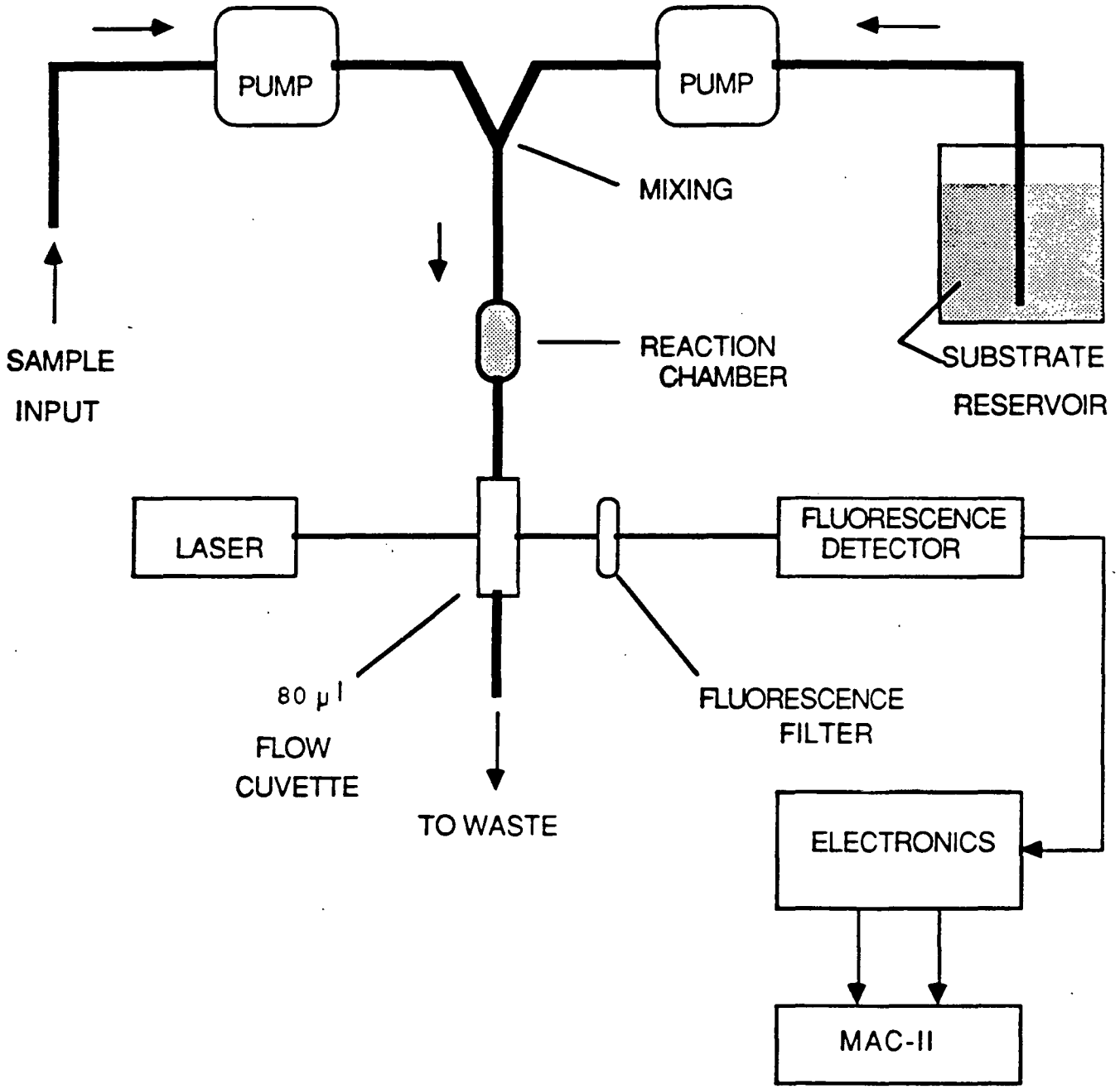
Figure 10. Kinetics of the spontaneous hydrolysis of 50 nM CPM in phosphate buffer.

Figure 11. Kinetics of acetylthiocholine (8×10^{-5}M) hydrolysis (as measured by CPM chemistry) by AChE (2.5×10^{-3} units) in the presence of various amounts of INP. CPM was at 50 nM. A comparison of these results with those obtained with NMIA (figure 5) indicates signifcantly less INP detection sensitivity when using the CPM scheme.

Figure 12. Examples of detecting short injection pulses of BSA by fluorescamine chemistry in flow. (a) injection of 2 ml 10^{-8} M BSA; (b) injection of 2 ml of 5×10^{-9} M BSA.

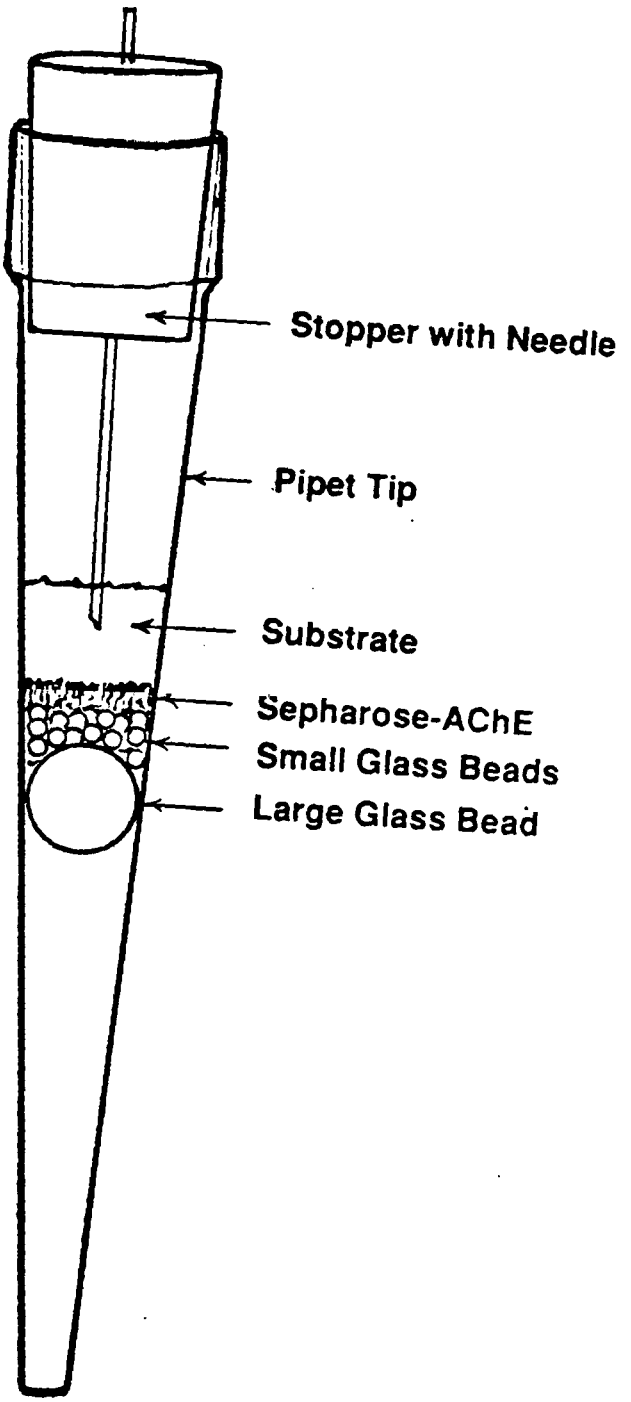
Figure 13. Results obtained in two 4 day water monitoring experiments using fluorescamine chemistry. Each day the system was challenged with an injection of 1 ml of a 5×10^{-8} M BSA (spikes).

Figure 14. BSA aerosol detection response curve. Aerosols were collected by the air sampler. The precipitated samples were reacted with fluorescamine and subsequently analyzed for fluorescence in the pulsed laser fluorometer. See text for details.

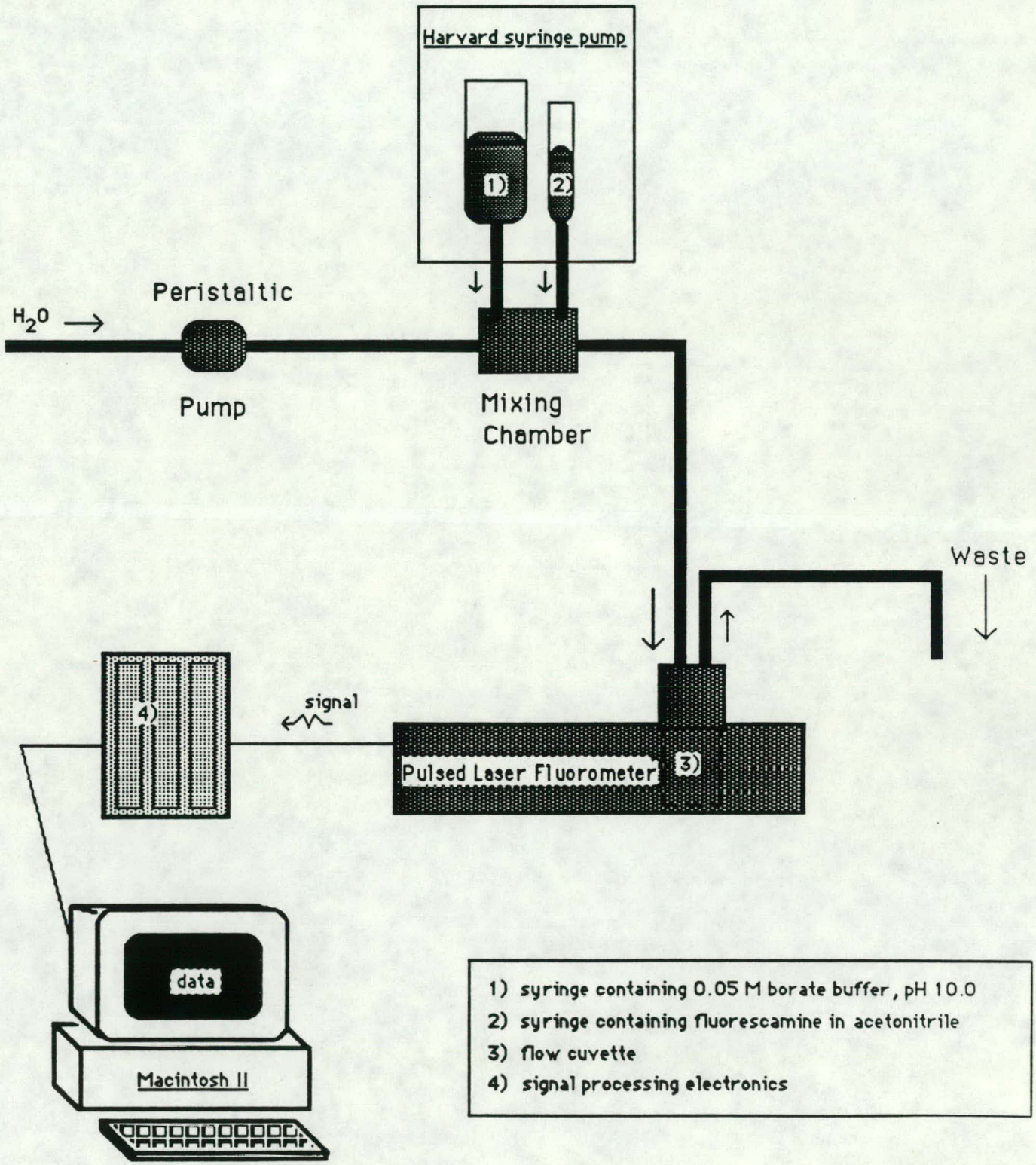


LOS ALAMOS CHEMICAL DETECTOR

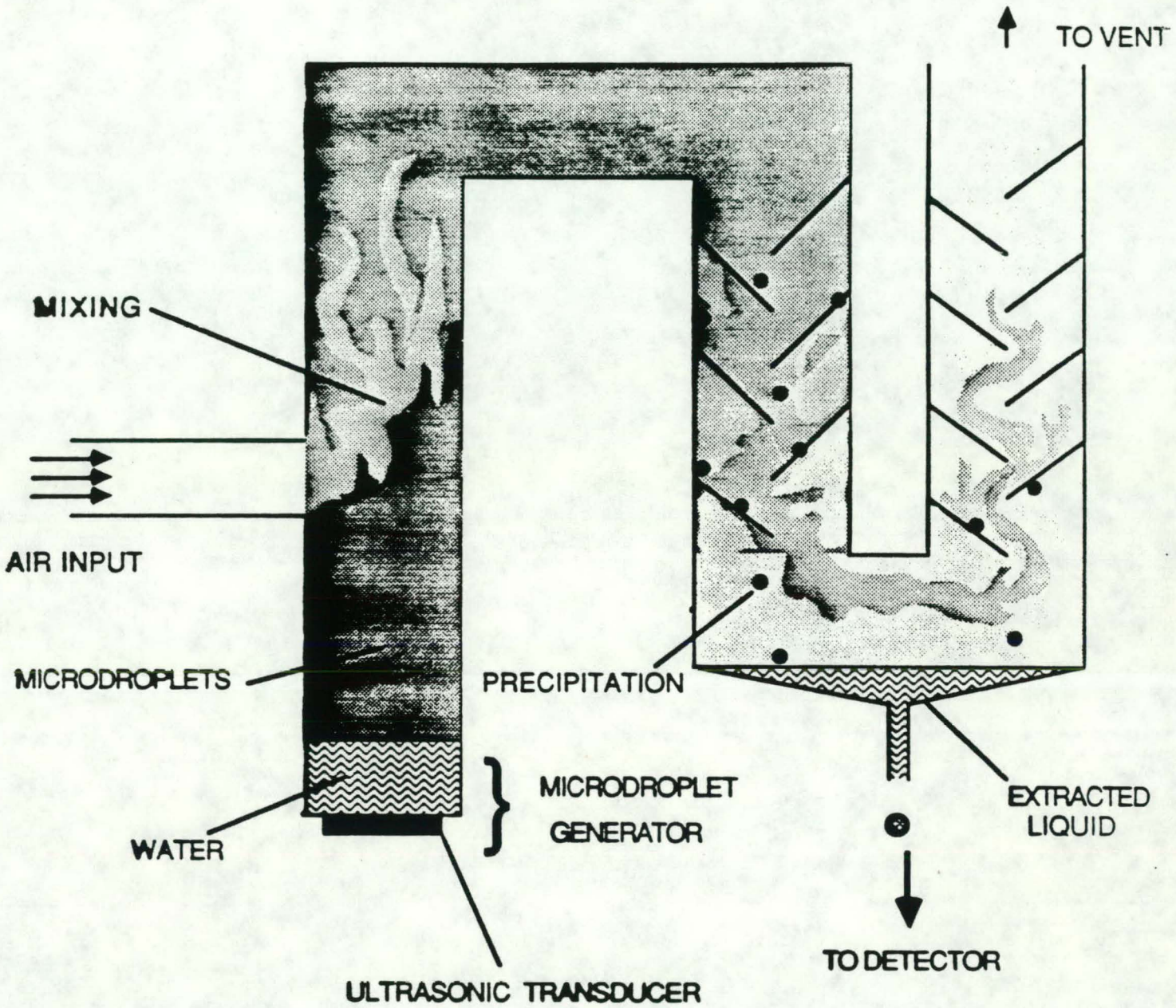
Reaction Chamber



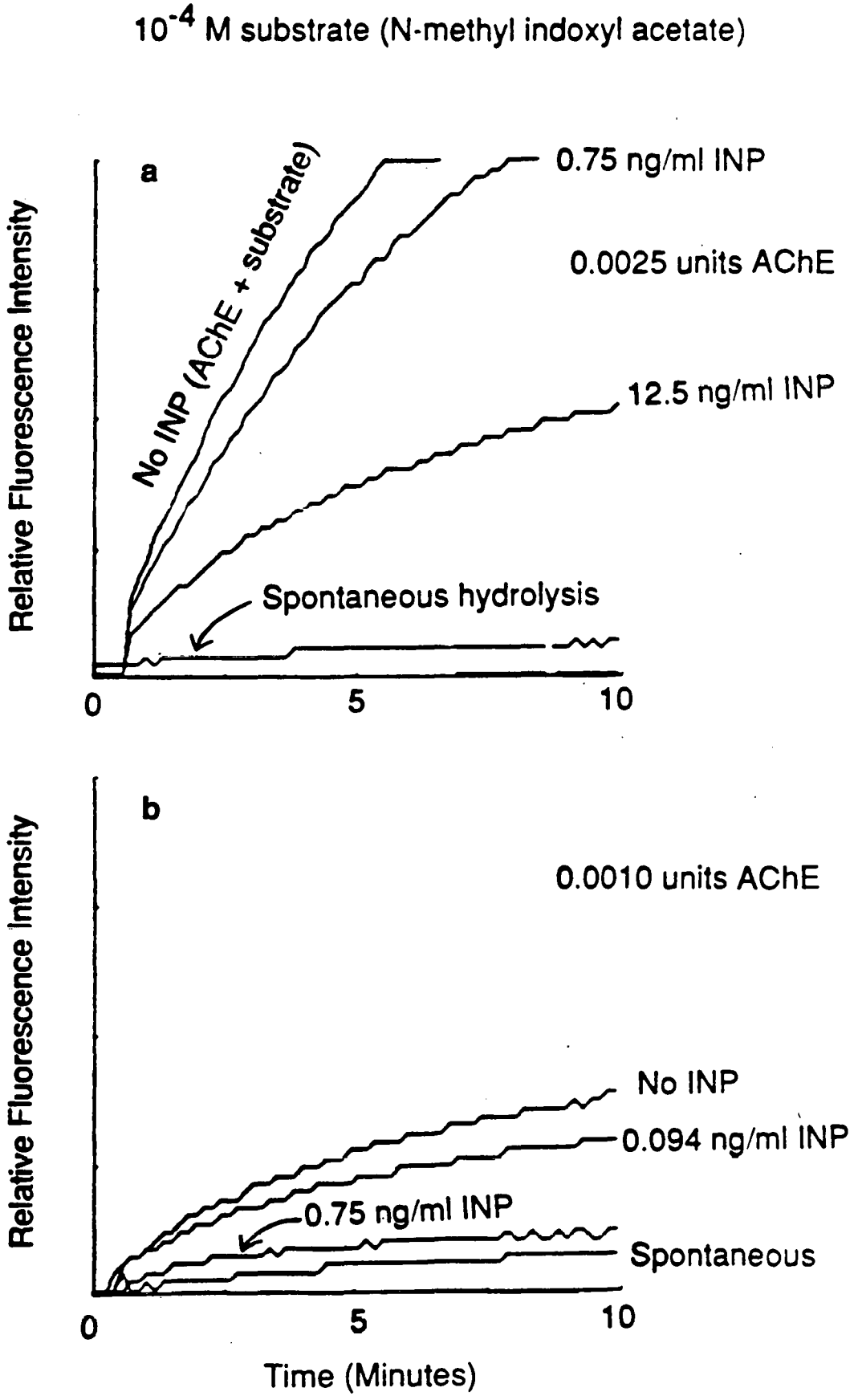
Apparatus for Detecting Protein Using Fluorescamine

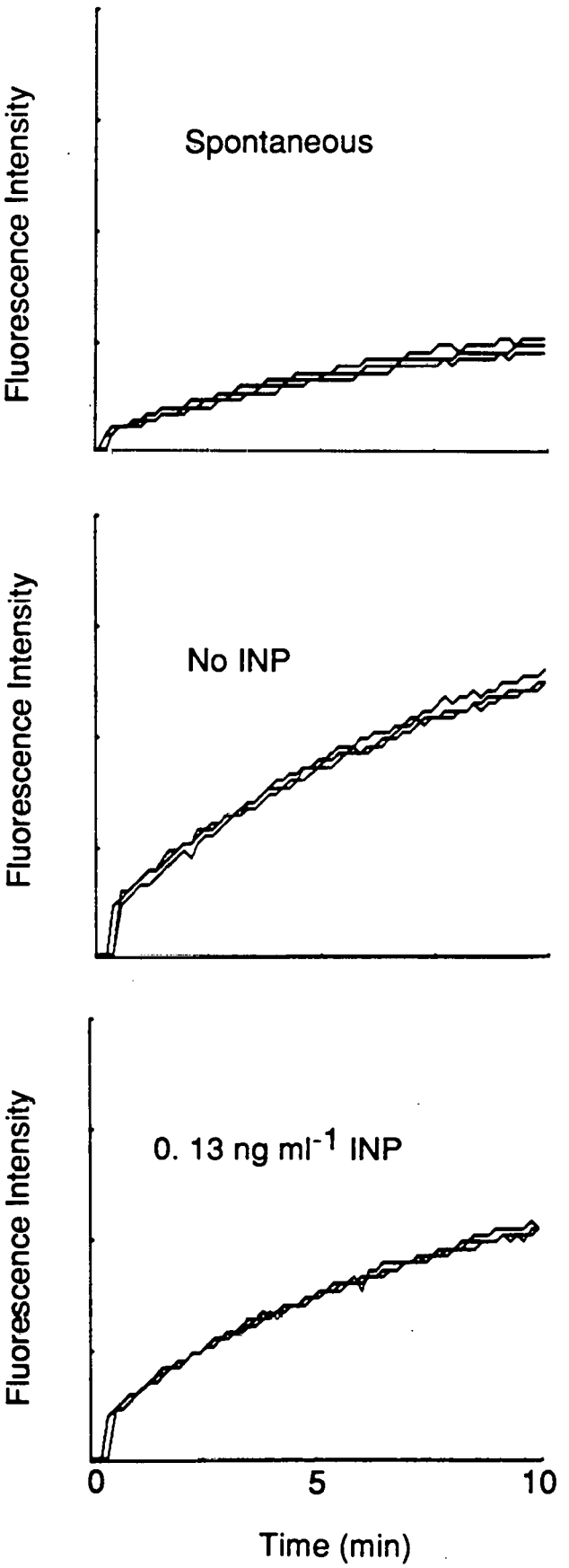


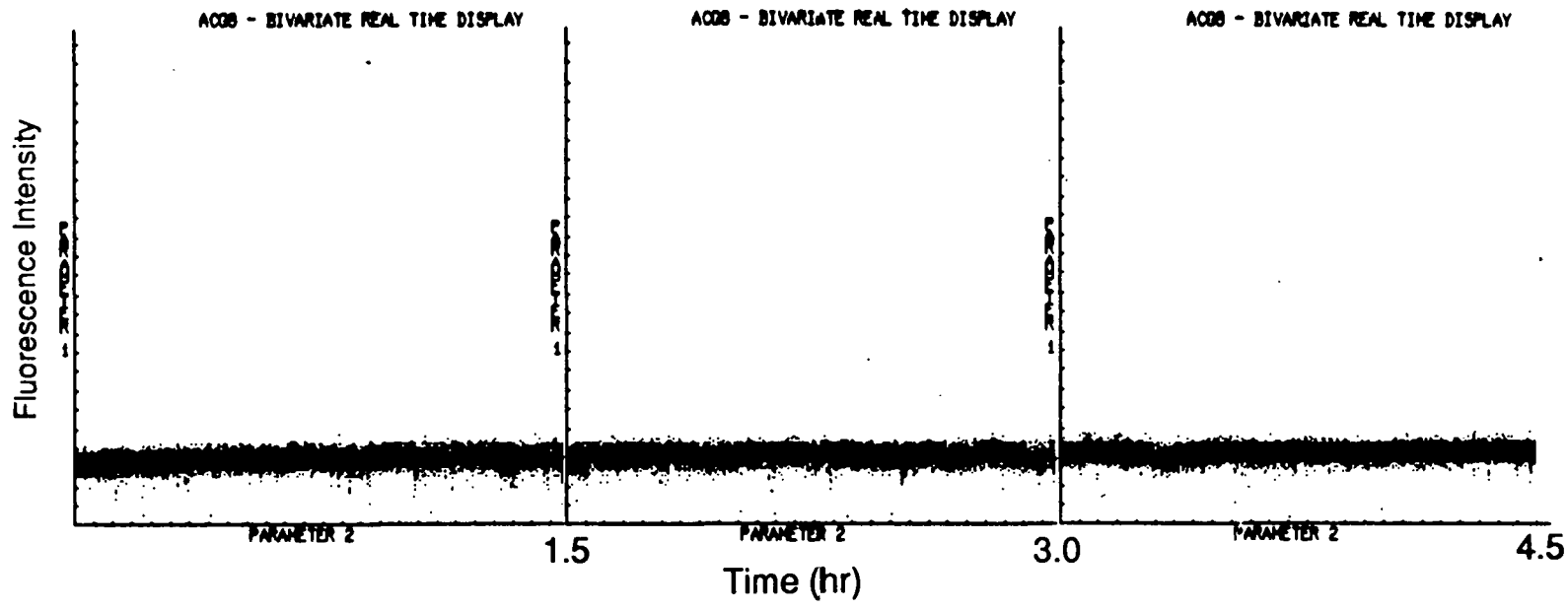
1) syringe containing 0.05 M borate buffer, pH 10.0
2) syringe containing fluorescamine in acetonitrile
3) flow cuvette
4) signal processing electronics

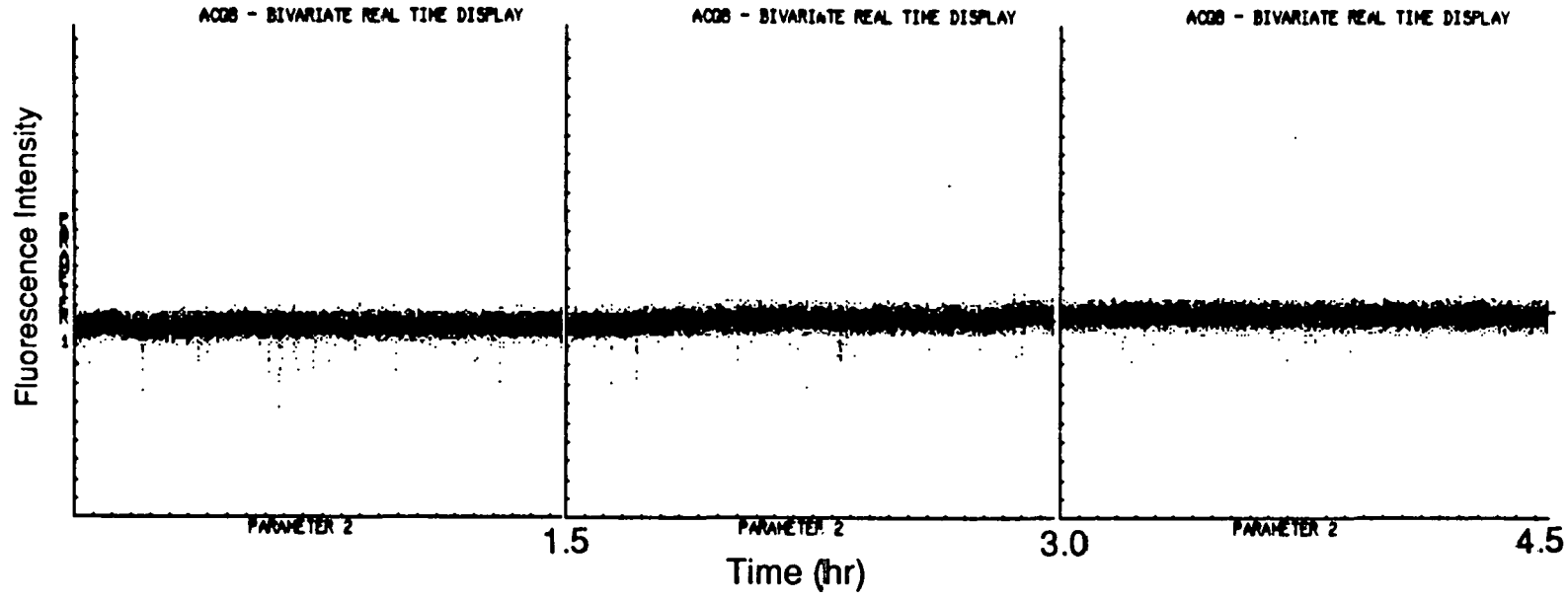


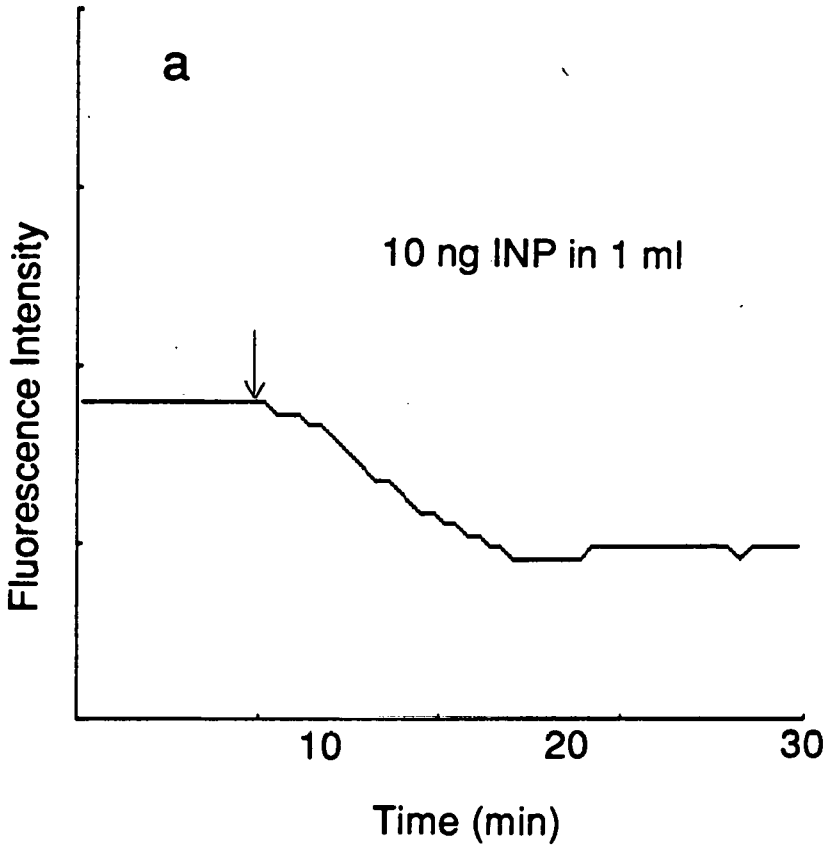
ULTRASONIC MICROMIST AIR SAMPLER

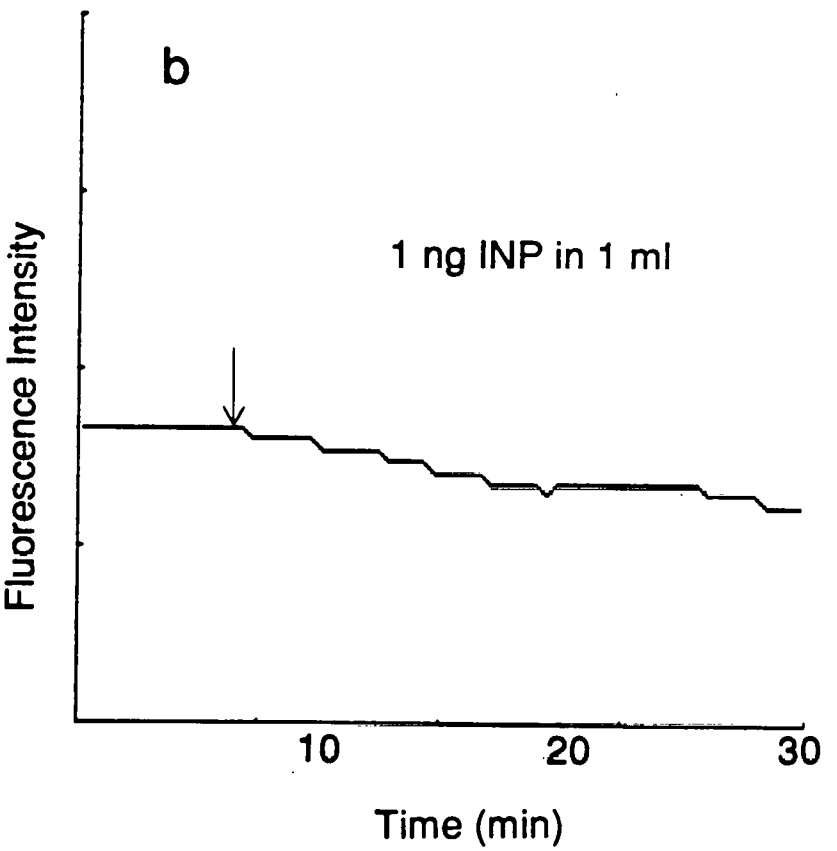

10^{-4} M substrate (N-methyl indoxyl acetate)
a
Relative Fluorescence Intensity
No INP (AChE + substrate)
0.75 ng/ml INP
0.0025 units AChE
12.5 ng/ml INP
Spontaneous hydrolysis
0
5
10
b
Relative Fluorescence Intensity
0.0010 units AChE
No INP
0.094 ng/ml INP
0.75 ng/ml INP
Spontaneous
0
5
10
Time (Minutes)

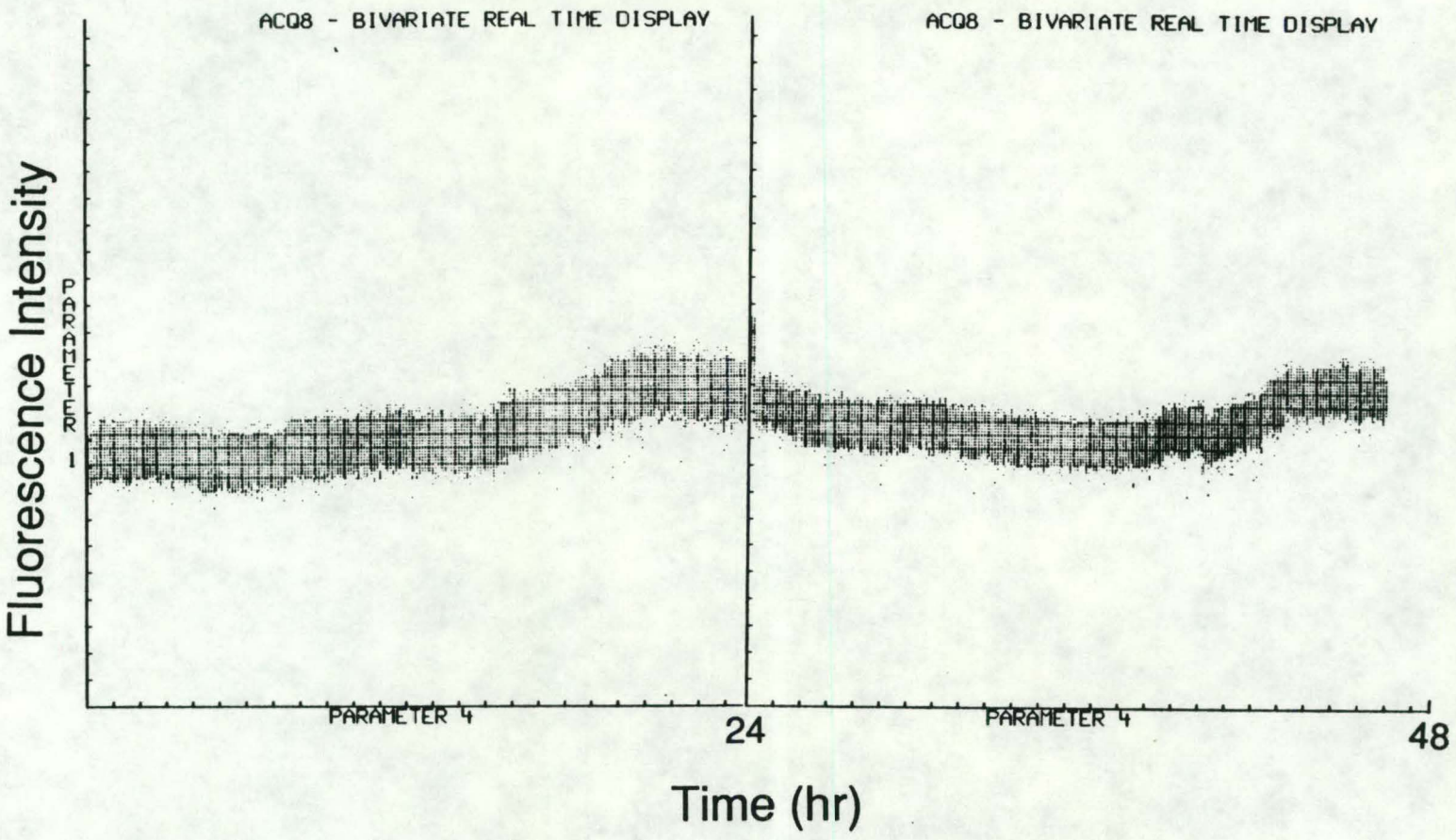

Fluorescence Intensity
Spontaneous
Fluorescence Intensity
No INP
Fluorescence Intensity
0. 13 ng ml-1 INP
0
5
10
Time (min)

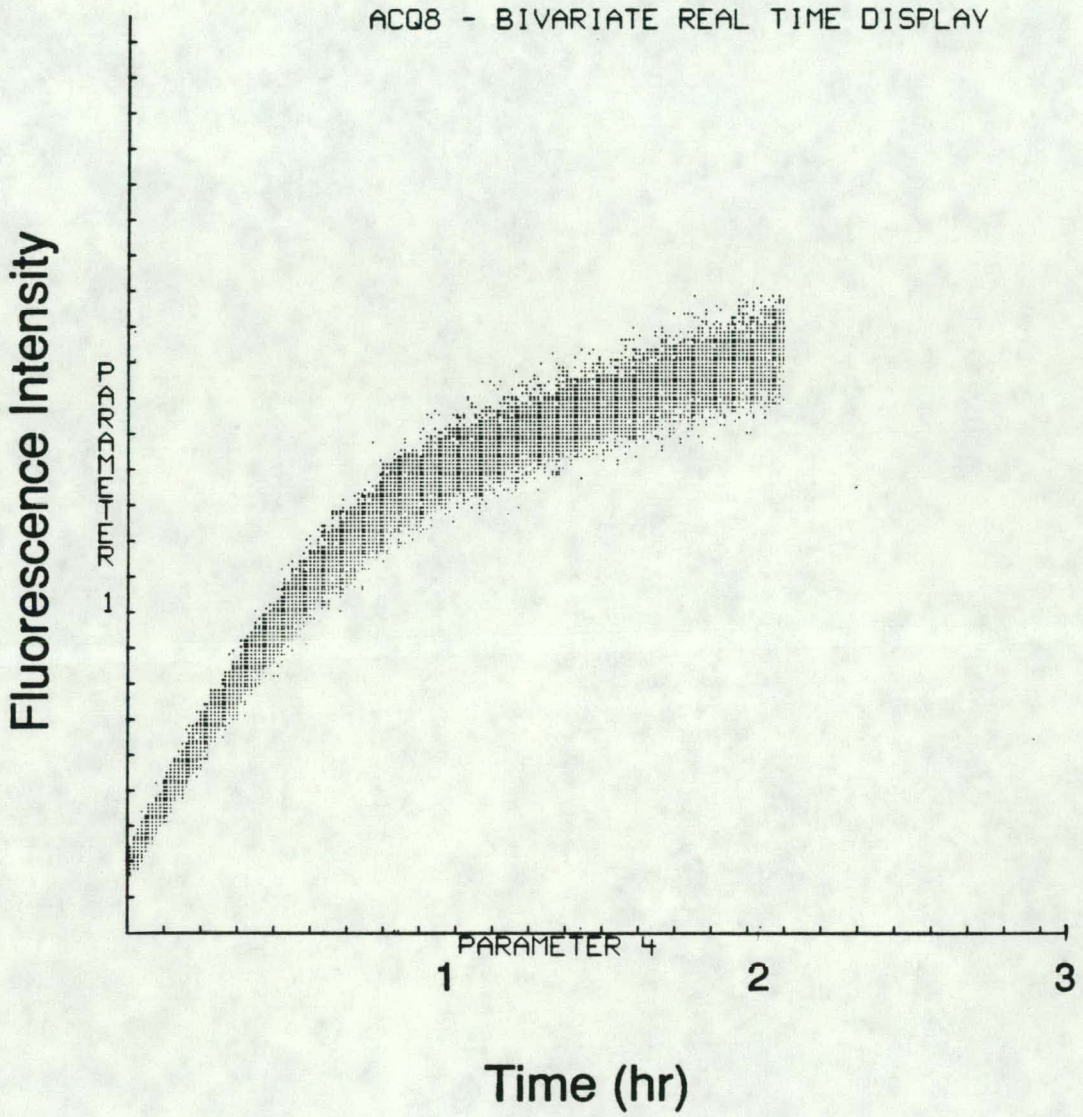

ACQ8 - BIVARIATE REAL TIME DISPLAY
ACQ8 - BIVARIATE REAL TIME DISPLAY
ACQ8 - BIVARIATE REAL TIME DISPLAY
Fluorescence Intensity
PARAMETER 2
1.5
PARAMETER 2
3.0
PARAMETER 2
4.5
Time (hr)

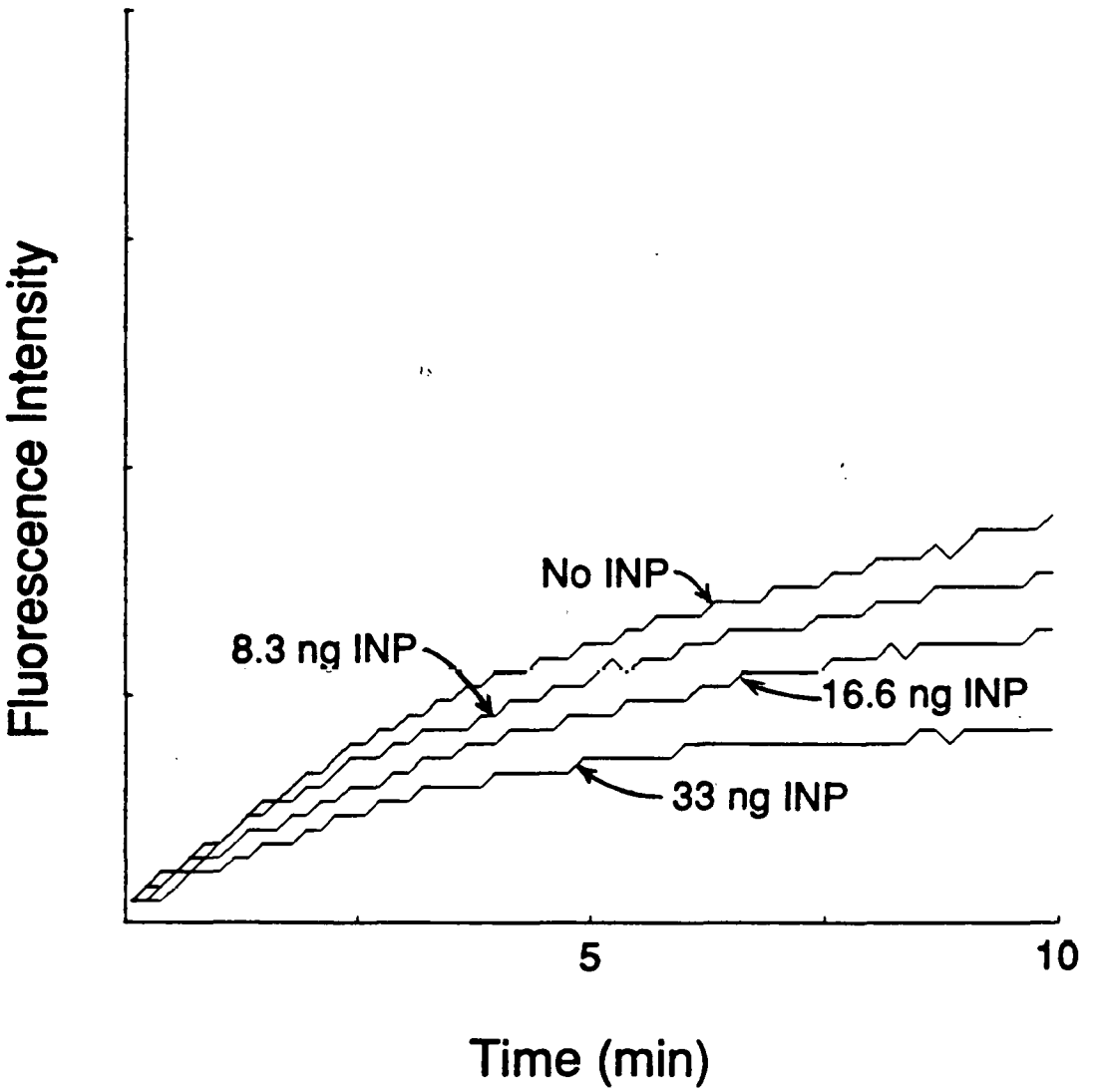

ACQ8 - BIVARIATE REAL TIME DISPLAY
ACQ8 - BIVARIATE REAL TIME DISPLAY
ACQ8 - BIVARIATE REAL TIME DISPLAY
Fluorescence Intensity
PARAMETER 2
1.5
PARAMETER 2
3.0
PARAMETER 2
4.5
Time (hr)


a
10 ng INP in 1 ml
Fluorescence Intensity
10
20
30
Time (min)

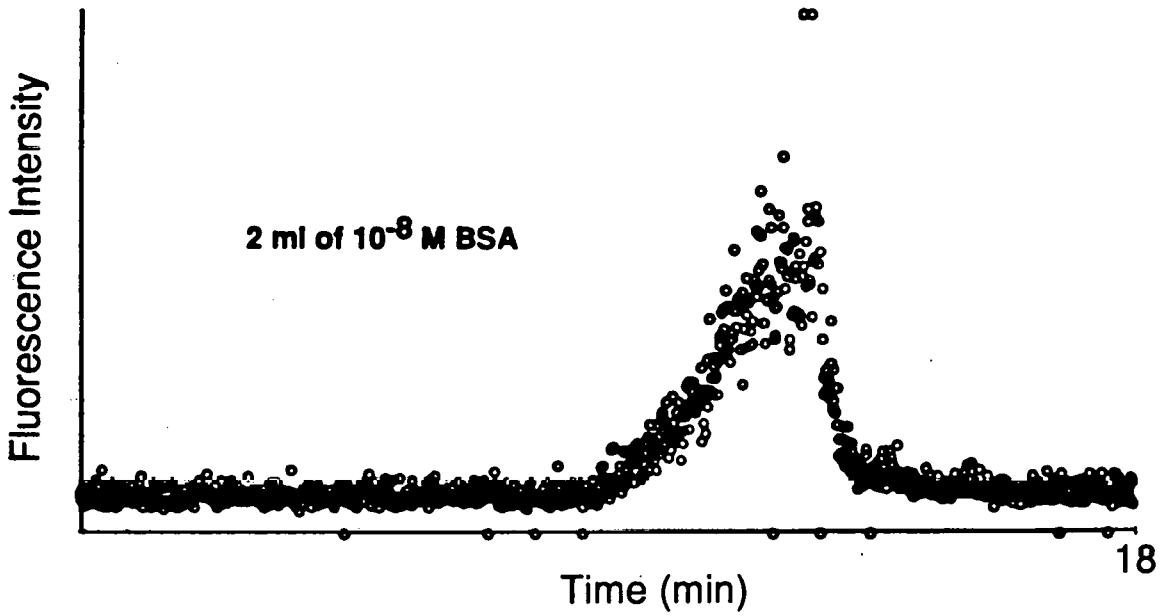

b
1 ng INP in 1 ml
Fluorescence Intensity
10
20
30
Time (min)

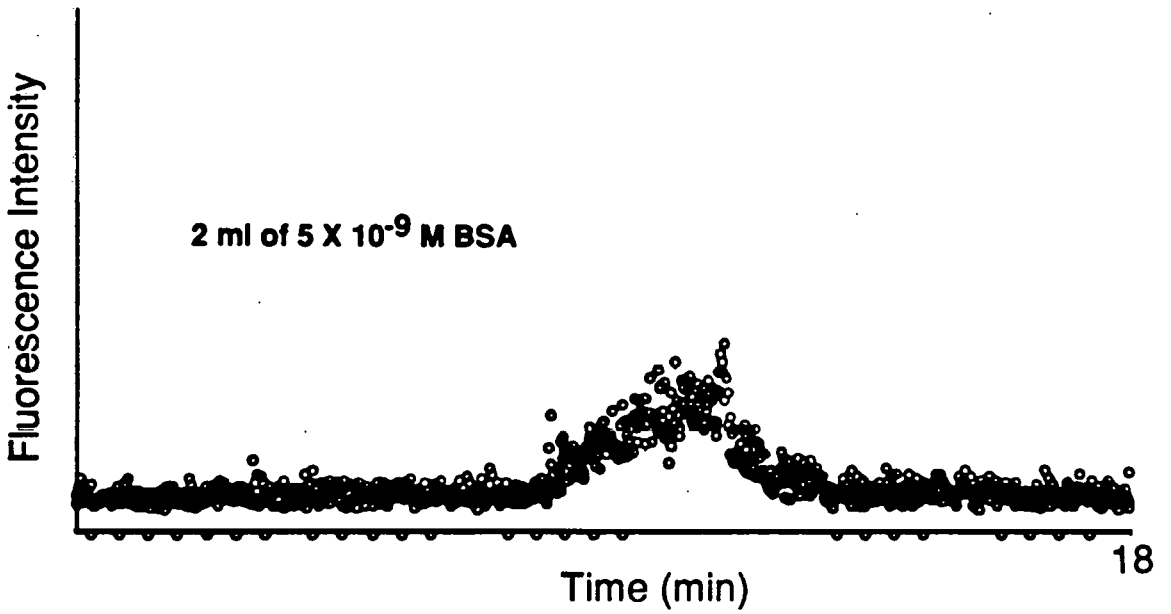

ACQ8 - BIVARIATE REAL TIME DISPLAY
ACQ8 - BIVARIATE REAL TIME DISPLAY
Fluorescence Intensity
PARAMETER 1
PARAMETER 4
24
PARAMETER 4
48
Time (hr)

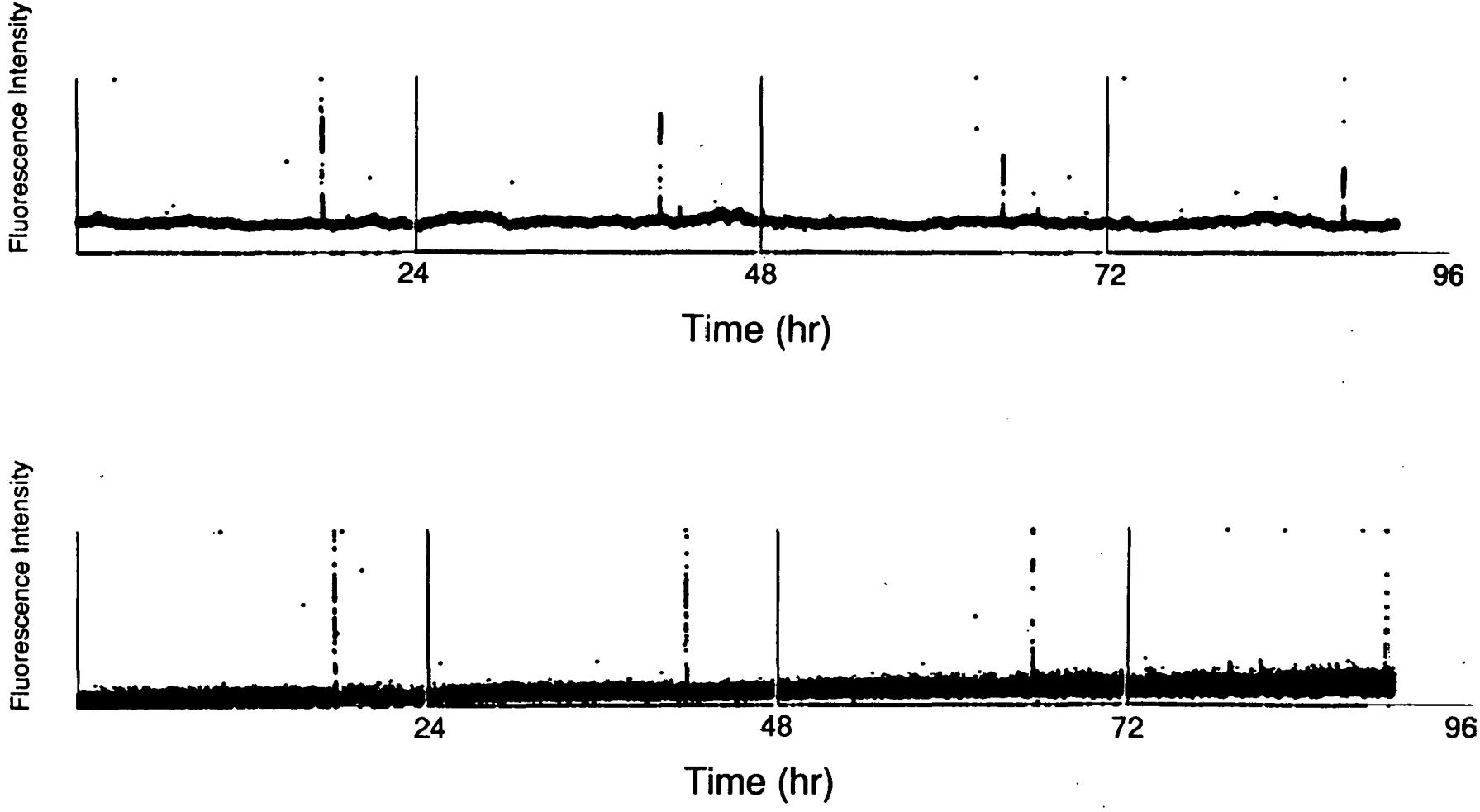

ACQ8 - BIVARIATE REAL TIME DISPLAY
Fluorescence Intensity
PARAMETER 1
PARAMETER 4
1
2
3
Time (hr)

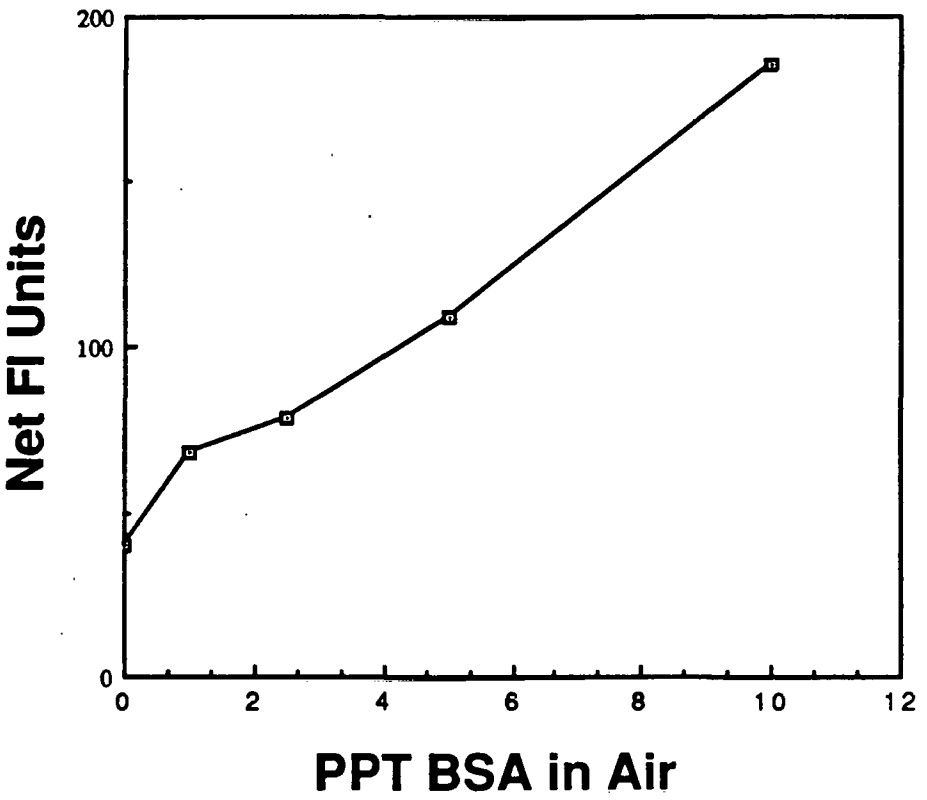

Fluorescence Intensity
No INP
8.3 ng INP
16.6 ng INP
33 ng INP
5
10
Time (min)

Examples of Detecting Short Pulses of Protein (BSA)






Fluorescence Intensity
24
48
72
96
Time (hr)
Fluorescence Intensity
24
48
72
96
Time (hr)


200
100
0
Net FI Units
0
2
4
6
8
10
12
PPT BSA in Air