

CONF-870940--5

DETECTION OF POLYAROMATIC COMPOUNDS USING
ANTIBODY-BASED FIBEROPTICS FLUOROIMMUNOSENSORS

CONF-870940--5

by

DE88 009065

T. Vo-Dinh⁽¹⁾, B. J. Tromberg^(1,2), G. D. Griffin⁽¹⁾, K. R. Ambrose⁽¹⁾,
M. J. Sepaniak⁽²⁾ and J. P. Alarie⁽²⁾

1) Advanced Monitoring Development Group
Health and Safety Research Division
Oak Ridge National Laboratory
Oak Ridge, Tennessee 37831-6101

2) Department of Chemistry
University of Tennessee
Knoxville, Tennessee 37996-1600

This research is jointly sponsored by the National Institutes of Health (Grant No. 5R01 GM 34730-02) and the Office of Health and Environmental Research, U.S. Department of Energy under contract DE-AC05-84OR21400 with Martin Marietta Energy Systems, Inc.

The submitted manuscript has been authored by a contractor of the U.S. Government under contract No. DE-AC05-84OR21400. Accordingly, the U.S. Government retains a nonexclusive, royalty-free license to publish or reproduce the published form of this contribution, or allow others to do so, for U.S. Government purposes.

MASTER

DISTRIBUTION OF THIS DOCUMENT IS UNLIMITED

INTRODUCTION

Human health protection against environmental pollutants, such as potentially carcinogenic polynuclear aromatic hydrocarbons (PAH), requires sensitive and selective monitoring instrumentation to analyze trace amounts of toxic substances in complex biological samples. The PAH compounds, which are produced in occupational and residential activities as a result of incomplete combustion of organic matters, are particularly important pollutants since many of them are carcinogenic (1,2). With the advent of immunochemical techniques, many traditional aspects of chemical and biological monitoring technologies are experiencing dramatic changes. Radioimmunoassays (RIA) utilize radioactive labels and comprise the most widely used immunoassay methods. The usefulness of RIA, however, is limited by several shortcomings, including the cost of instrumentation, the limited shelf life of radioisotopes, and the potential deleterious biological effects inherent to radioactive materials. For these reasons, extensive research efforts are aimed at developing simpler, more practical, equally sensitive, and selective analytical instrumentation.

The use of fiberoptics-based sensors has recently been reviewed (3,4). For the past few years our laboratories have been involved in the development of various fiberoptics-based sensors (5-13). In this study the performance and selectivity of an antibody-based fiberoptics sensor using laser-induced immunofluorescence for benzo(a)pyrene (BP) and its tetrol derivatives are investigated. Benzo(a)pyrene was selected as the model PAH compound because this important compound is found in many industrial and residential environments (chemical, petroleum, coke oven, and synfuel industries: wood-burning fireplace and cigarette smoke) and is known to be carcinogenic (1,2). Studies have shown that BP is metabolically activated to electrophilic

intermediates, which can bind to DNA. Measurements of BP-DNA adducts or related products, such as BP-tetrols (BPT), provide an important indication of the carcinogenic activity of BP. Recent advances in luminescence instrumentation, laser miniaturization, biotechnology and fiberoptics research have permitted the development of sensors that are capable of measuring environmental and human exposure to toxic chemical and biological materials. In one type of sensor, antibodies to BP, produced by polyclonal techniques, were covalently bound to a fiberoptics sensing probe of a fluoroimmuno-sensor (FIS). In another type of sensor, monoclonal antibodies against BPT were bound to microbeads and encapsulated at a membrane-tip probe. A helium-cadmium laser was used as the excitation source. Results illustrate the real-time measurement capability and the selectivity of detection.

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

MATERIALS AND METHODSInstrumentation

Figure 1 shows a diagram of the FIS instrument. A detailed description of the FIS instrument development is given elsewhere (10-13). Only the main features of the device are provided here. The excitation source was a helium cadmium laser (Omnichrome, Model 3112) operated at 325 nm. The laser radiation was directed through an optical beam splitter and focused onto the incident end of an optical fiber (excitation path). A 50-mm focal-length $f/2$ lens was used to focus the laser radiation onto the end of a 600- μm core diameter, multi-mode, fused silica optical fiber (Math Associates Inc., Model QSF-600). The FIS device developed here utilized the back scattering of light emitted at the remote sensor probe. A single fiber was used to transmit the excitation radiation into the sample and collect the fluorescence emission. An optical fiber micro positioner was used to provide fine adjustments of the fiber's incident end and to optimize transmission of the focused laser beam onto the sample. The sensor tip can have different designs. In one design, antibodies are covalently bound at the end of the fiber's tip. In another design, the sensing probe consists of a membrane-enclosed microcavity containing antibody solutions or antibodies bound on beads (12,13).

The laser radiation reached the sensor probe and excited the BP bound to the antibodies immobilized at the fiberoptics probe. The BP fluorescence was isolated by a bandpass filter and monochromator (Instruments SA, Model H-10) with an 8-nm bandpass and transmitted back through the beam splitter to the detector. The fluorescence was detected with an RCA 1P28B photomultiplier tube operated at 800 volts. The photocurrent was processed using a Keithley

(Model 485) picoammeter. Data were stored in the memory of the picoammeter and recorded using a strip-chart recorder.

Antibody Production and Characterization

Polyclonal antibodies against BP were produced as described previously (14-16). Briefly, BP was conjugated to bovine serum albumin (BSA) by chemical modification of BP, and the resulting conjugate (BP-BSA) was used to immunize rabbits. Following initial immunization and reimmunization, serum samples were isolated and assayed for BP-antibodies by appropriate immunological assays [i.e., passive hemagglutination and/or enzyme-linked immunosorbent assays (ELISA)]. A large blood collection was made from donor animals with the highest BP-antibody titers, and following isolation of the serum, this crude antiserum was used for the experiments described below.

Radioimmunoassay of BP antisera preparations was carried out essentially following a procedure described previously (17). This assay depends upon the ability of dextran-coated activated charcoal to bind free BP antigen, while antibody-bound antigen is not attached to the charcoal. A simple centrifugation is thus sufficient to separate free from antibody-bound BP, following appropriate incubations to allow antigen-antibody interaction. In all radioimmunoassays, ^3H or ^{14}C -BP (from New England Nuclear or Amersham) was diluted successively in acetone and dimethyl sulfoxide to obtain a solution in a solvent compatible with aqueous solutions. Varying amounts of the radiolabeled BP were added to antisera aliquots in phosphate-buffered saline containing 10% fetal calf serum to make a final volume of 0.5 mL. Incubations were carried out at 37° C in a shaking water bath. Following incubation, charcoal suspensions were added as described (17). Aliquots of the final supernatant were analyzed in a liquid scintillation counter.

Sensor Probe Preparation

The experimental procedures used to prepare optical fibers and samples for fluorescence and radiolabeled BP measurements were as follows: quartz fibers were stripped of their cladding for a length of 7-8 mm; bare fibers were then derivatized with 3-glycidoxypropyltrimethoxysilane (GOPS) using described procedures (10,11). Following oxidation with periodic acid, the fibers were incubated for 36-48 h in solutions containing 2 mg/mL of rabbit IgG (for the control fibers) or 2 mg/mL crude IgG fraction from sera of rabbits immunized with BP-BSA (for the BP-antibody fibers). Phosphate buffered saline (PBS) was used as the diluent for the IgG preparations. After covalently linking the IgG protein to the fibers, the final step was reduction with sodium borohydride (NaBH_4). The fibers were then rinsed with PBS and ready for use.

The membrane-enclosed sensor was constructed with 200/300- μm diameter core/cladding plastic-clad fused silica fiber (numerical aperture = 0.26) supplied by General Fiber Optics, Inc. Very fast cellulose dialysis membrane (7- μm thick, molecular weight cutoff = 10,000; Diachema AG) was stretched across the face of a piece of plastic heat-shrink tubing and positioned with a band of heat shrink. The tip was assembled so that it could slide on and off the fiber and, when in place, a tight seal would form between the fiber jacket and the plastic tubing. Approximately 2-3 mm of bare fiber core was exposed. The plastic heat-shrink tip was tapered, resulting in an inner diameter of roughly 300 μm . The distance between the membrane and the fiber face was adjusted to about 0.5 mm, yielding an approximate sensor volume of 40 nL.

Chemicals and Reagents

Benzo(a)pyrene and 3-Glycidoxypropyltrimethoxysilane were purchased from Aldrich Chemical Co. Bovine serum albumin and phosphate buffered saline were

purchased from Sigma Chemical Co. Multi-mode, fused-silica optical fibers were obtained from Math Associates. Monoclonal antibodies to BPT were developed and provided by Dr. R. M. Santella and co-workers at Columbia University. The PAH mixture was obtained from the National Bureau of Standards (NBS):(sample No. 1647).

RESULTS AND DISCUSSION

Instrument Performance

Various types of fluoroimmunoassays can be performed using the FIS, depending on the type of antigens. For fluorescent antigens, direct in-situ measurements of the analytes bound to the antibodies on the sensor probe can be performed. Various types of detection schemes can be used for measurements. With the sensor probe having immobilized antibodies against BP, the fiber tip is placed in a solution of BP. Following incubation, the probe is removed from the solution, rinsed with a PBS solution and directly analyzed. Since BP is a fluorescent compound, there is no need to use fluorescent-tagged labels in the assay. The laser radiation transmitted along the optical fiber reaches the sensing probe and excites the BP molecules that have conjugated with the anti-BP antibodies covalently bound to the sensor. Continuation of the incubation procedure leads to a steady increase in fluorescence signal as more and more BP molecules reach the bound antibodies.

The temporal response of the FIS signal following incubation in a BP solution is shown in Figure 2. The plateauing of the sensitivity curve after 1 h may be due to the saturation of the antibodies by the BP molecules and indicated that steady-state conditions were reached. The results in this figure were obtained from a sample of BP ($2 \times 10^{-7}M$) in phosphate buffer/1% ethanol. After the first 10 min, the signal reached 50% of its maximum value.

During the next 50 min the fluorescence signals only increased to 75% of the maximum value.

With membrane-tip sensors, measurements can be obtained using a sequential and a stepwise procedure. Sequential measurements were performed by filling the membrane sensor tip with antibody solution (typically 0.3 mg/mL) for each sample. After each measurement the sensor head was refilled with fresh antibody. Each FIS was incubated in 1-mL stirred antigen solution for a given time interval and rinsed in PBS solution for about 5 min. During this period, fluorescent antigen diffused across the membrane and was conjugated to its specific antibody. When the sensor was rinsed in PBS, unbound antigens and/or interfering substances were dialyzed out of the sensing tip. Antibody-bound material remained, confined by the membrane to the fiber's viewing region. Signal was obtained either from the slope of the signal rise or from the difference between pre- and post-incubation signals in blank PBS solutions. Signal-to-noise (S/N) values were calculated using the peak-to-peak noise of blank PBS solutions.

Due to the large amount of antibody that remains unbound during these non-equilibrium dialysis measurements, stepwise calibration data were also obtained. These assays involved loading the sensor tip with antibody and performing fixed-time incubations. Rinsing the sensor in PBS served to mark the endpoint of the measurement and the new baseline for the next analysis. Both the slope and the signal difference were used to obtain data. Sensor response was independent of the measurement sequence of antigen solutions (i.e., signals obtained going from high to low concentrations were the same as those obtained going from low to high concentrations).

A temporal response curve of the membrane-tip sensor using sequential measurements for EPT (3×10^{-9} M in PBS) is illustrated in Figure 3.

Specificity Study

An important factor in the FIS's performance is the specificity of antibodies used at the sensor tip. A 15-component PAH mixture was obtained from the National Bureau of Standards (NBS sample No. 1647) and used as a complex mixture to investigate the specificity of anti-BP polyclonal antibodies. The composition of this mixture is shown in Table I. The mixture was evaporated to dryness and redissolved in dimethylsulfoxide (DMSO). Appropriate aliquots of this mixture were added to the reaction solution in competition experiments. Using this assay, and radiolabeled BP (usually ^{14}C), we have found that the rabbit antisera preparations can bind free BP (that is, not attached to bovine serum albumin). This result indicates that the BP antibody, which was produced using the protein-BP complex, can recognize the free hapten (BP) that was used as part of the immunogen complex, and does not only recognize the hapten as part of a larger molecular structure (the protein-hapten complex). That this finding is due to antigen-antibody interaction and not just adventitious or nonspecific attachment of BP to serum protein was demonstrated by experiments in which normal rabbit serum was used instead of serum from immunized animals. In these experiments, the amount of BP in solution following centrifugation (i.e., not charcoal-bound) was always equivalent to that observed in experiments where no rabbit serum was included in the incubation mixture. Further evidence that the BP-protein interaction detected arises from antigen-antibody binding was obtained in experiments in which the amount of BP antisera in the reaction mixture was increased. The amount of BP found in solution (antibody-associated) was increased in a

proportionate manner. Appropriate control experiments in which normal rabbit serum amounts were increased showed no such effect.

In order to carry out meaningful competition experiments with PAH other than BP, it was first necessary to establish optimal conditions for the amount of BP antigen bound to a given amount of antibody. Experiments indicated that incubation times of 30 min or more were sufficient to produce a steady-state level in amount of BP bound to the antisera; 250 pmoles of BP in the reaction mixture was found sufficient to saturate the antigen-binding capacity of 25 μ L of antisera from the most highly immunized animals.

To investigate the degree of specificity of the antisera preparations for BP, competition experiments were performed in which varying amounts of the PAH mixture were added to the reaction mixture. If BP antibodies in the antisera react equally well with other PAHs, addition of increasing amounts of these other PAHs should produce an incremental decrease in the amount of radiolabeled BP bound to the antisera.

The PAHs present in the mixture used for competition experiments ranged from 2-ring structures to multiple-ring structures comparable in complexity to BP. Benzo(a)pyrene was only ~3%, by molar ratio, of the total PAHs in the competition mixture. Because of the dilution effect of adding this nonradiolabeled BP to the standard amount of radiolabeled BP in each reaction mixture, the specific activity of the radiolabeled BP changed with the amount of competition mixture added, and appropriate corrections were made to calculate the amounts of BP found. The results of a competition experiment are shown in Figure 4. It is clear from this figure that the BP antibodies present in the antisera display considerable specificity for the molecular structure of BP. If the antibodies had no specificity for BP in relation to other PAHs,

then one would predict that at a molar ratio of 1:1 (total PAH:BP), the pmoles of antibody-bound BP would drop to one-half the amount observed when no competing PAHs were present (i.e. to ~22 pmoles). The fact that this does not occur suggests that most (or all) of the other PAHs in the competing mixture do not fit in the antigen binding site on the antibody as effectively as does BP. Even at molar ratios where there are 10 times as many molecules of competing PAHs as there are molecules of BP, the amount of BP bound is still 63% of the value for BP binding in the absence of competing PAHs. This may be contrasted with a predicted decrease to 9-10% of the initial value (dashed curve in Figure 4), if the antibody had no specificity for BP. It is, therefore, concluded that the antibody produced upon immunization with a BP-protein conjugate has strong specificity for the BP molecular structure. The use of this polyclonal antibody for measuring BP in the presence of a wide variety of other PAHs contributes strongly to the selectivity of the FIS technology.

CONCLUSION

In this work we have investigated the performance of an antibody-based fiberoptics sensor for the detection of the carcinogen benzo(a)pyrene and its DNA-adduct product BP-tetrol. The excellent sensitivity of this device-- femtomole limits of detection for BP (11) -- illustrates that it has considerable potential to perform analyses of chemical and biological samples at trace levels in complex matrices. The results indicate that fiberoptics-based FIS can be useful in a wide spectrum of biochemical and clinical analyses.

ACKNOWLEDGEMENTS

This research is jointly sponsored by the National Institutes of Health (Grant No. 5R01 GM 34730-02) and the Office of Health and Environmental Research, U.S. Department of Energy under contract DE-AC05-84OR21400 with Martin Marietta Energy Systems, Inc.

REFERENCES

1. Grimmer, G., Ed. (1983), Environmental Carcinogens: Polycyclic Aromatic Hydrocarbons, CRC Press, Boca Raton, FL.
2. Gelboin, H. V.; Ts'o, P. O. P., Eds., Polycyclic Hydrocarbons and Cancer, Academic Press, New York (1978).
3. Seitz, W. R., Anal. Chem. (1984), 56, 16A.
4. Peterson, J. I.; Vurek, G. G. Science (1984), 224, 123.
5. Vo-Dinh, T.; Gammage, R. B., Amer. Ind. Hyg. J. (1981), 42, 112.
6. Tromberg, B. J.; Eastham, J. F.; Sepaniak, M. Appl. Spectrosc. (1984), 38, 38.
7. Vo-Dinh, T.; Griffin, G. D.; Ambrose, K. R., Appl. Spectrosc. (1986), 40, 696.
8. Sepaniak, M. J.; Tromberg, B. J.; Estham, J. F. Clin Chem. (1983), 29, 1678.
9. Vo-Dinh, T.; Griffin, G. D.; Ambrose, K. R.; Sepaniak, M.; and Tromberg, B. J. (1985): Fiberoptics-based Immunofluorescence Spectroscopy for Monitoring Exposure to Polynuclear Aromatic Compounds, 10th International Symposium on Polycyclic Aromatic Hydrocarbons, Battelle, Columbus, Ohio, Oct. 21-24, 1985.
10. Tromberg, B. J.; Sepaniak, M. J.; Vo-Dinh, T.; Griffin, G. D., (1987), Anal. Chem., 59, 1226.
11. Vo-Dinh, T., Tromberg, B. J., Griffin, G. D., Ambrose, K. R., Sepaniak, M. J., and Gardenhire, E. M., (1987), Appl. Spectr., 41 735.
12. Vo-Dinh, T., Tromberg, B. J., Sepaniak, M. J., Griffin, G. D., Ambrose, K. R., and Santella, R. M. (1988): Immunofluorescence Detection For Fiber Optics Chemical and Biological Sensors, Proceedings of the Symposium on Laser Spectroscopy, SPIE's O-E LASE '88, Jan. 10-15, 1988, Los Angeles, CA.
13. Tromberg, B. J., Sepaniak, M. J., and Vo-Dinh, T. (1988): Development of Antibody-Based Fiber-Optic Sensors, Proceedings of Symposium on Medical Application of Lasers, SPIE's O-E LASE '88, Jan. 10-15, 1988, Los Angeles, CA.
14. Creech, H. J. J. Am. Chem. Soc. (1941), 63, 576.
15. Creech, H. J.; Jone, R. N. J. Am. Chem. Soc. (1941), 63, 1661.

16. Griffin, G. D., Ambrose, K. R., Thomason, R. N., Murchinson, C. M., McManis, M., St. Wrecker, P. G. R. and Vo-Dinh, T. (1985): Production and Characterization of Antibodies to Benzo(a)Pyrene, 10th International symposium on Polycyclic Aromatic Hydrocarbons, Battelle, Columbus, Ohio, Oct. 21-24, 1985.
17. Herbert, V., Lan, K-S., Gottlieb, C. W. and Bleicher, S. J. (1985) "Coated Charcoal Immunoassay of Insulin," J. Clin. Endocrinol., 25, 1375.

FIGURE CAPTIONS

- Figure 1 Schematic Diagram of the FIS Instrument Having Antibody-bound Fiber Sensor
- Figure 2 Temporal Response of the FIS with BP Antibody-bound Fiber Sensor (Concentration of BP = 2×10^{-7} M in PBS)
- Figure 3 Temporal Response of the FIS with Membrane Tip Sensor for BPT (Concentration of BPT = 3×10^{-9} M in PBS)
- Figure 4 Specificity Study of the Anti-BP Antibody Using a Complex PAH Mixture

TABLE I. Composition of the PAH Complex Mixture Used for Specificity Study NBS Sample No. 1647

PAH Compound	Concentration (nmoles/mL)
Anthracene	18
Benz(a)anthracene	22
Benzo(a)pyrene	21
Fluoranthene	50
Pyrene	49
Naphthalene	176
Acenaphthylene	126
Fluorene	30
Phenanthrene	28
Chrysene	21
Benzo(b)fluoranthene	20
Benzo(k)fluoranthene	20
Dibenz(a,h)anthracene	13
Indeno(1,2,3-cd)pyrene	15

THE LASER-BASED FIS INSTRUMENT

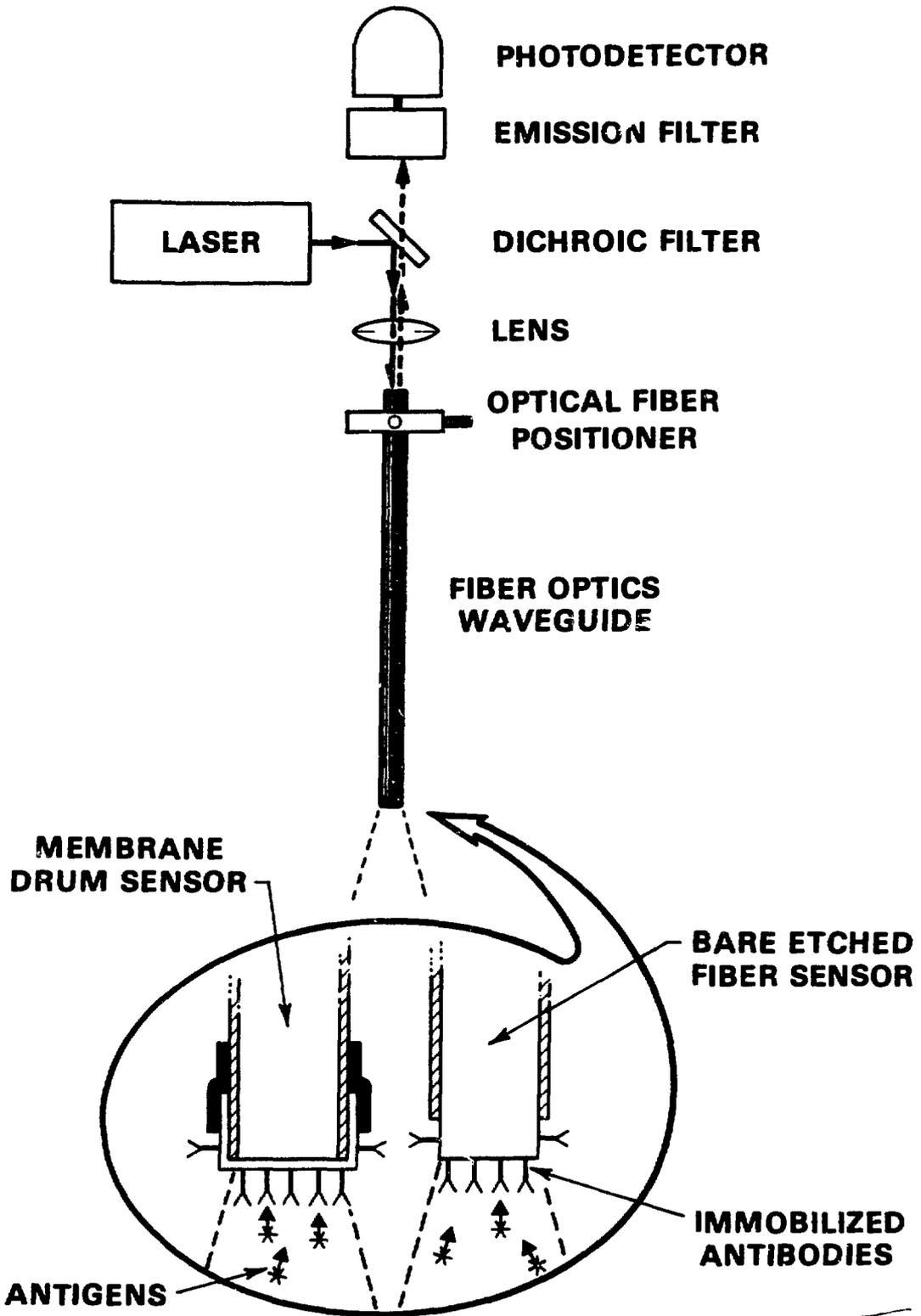


Fig. (1)

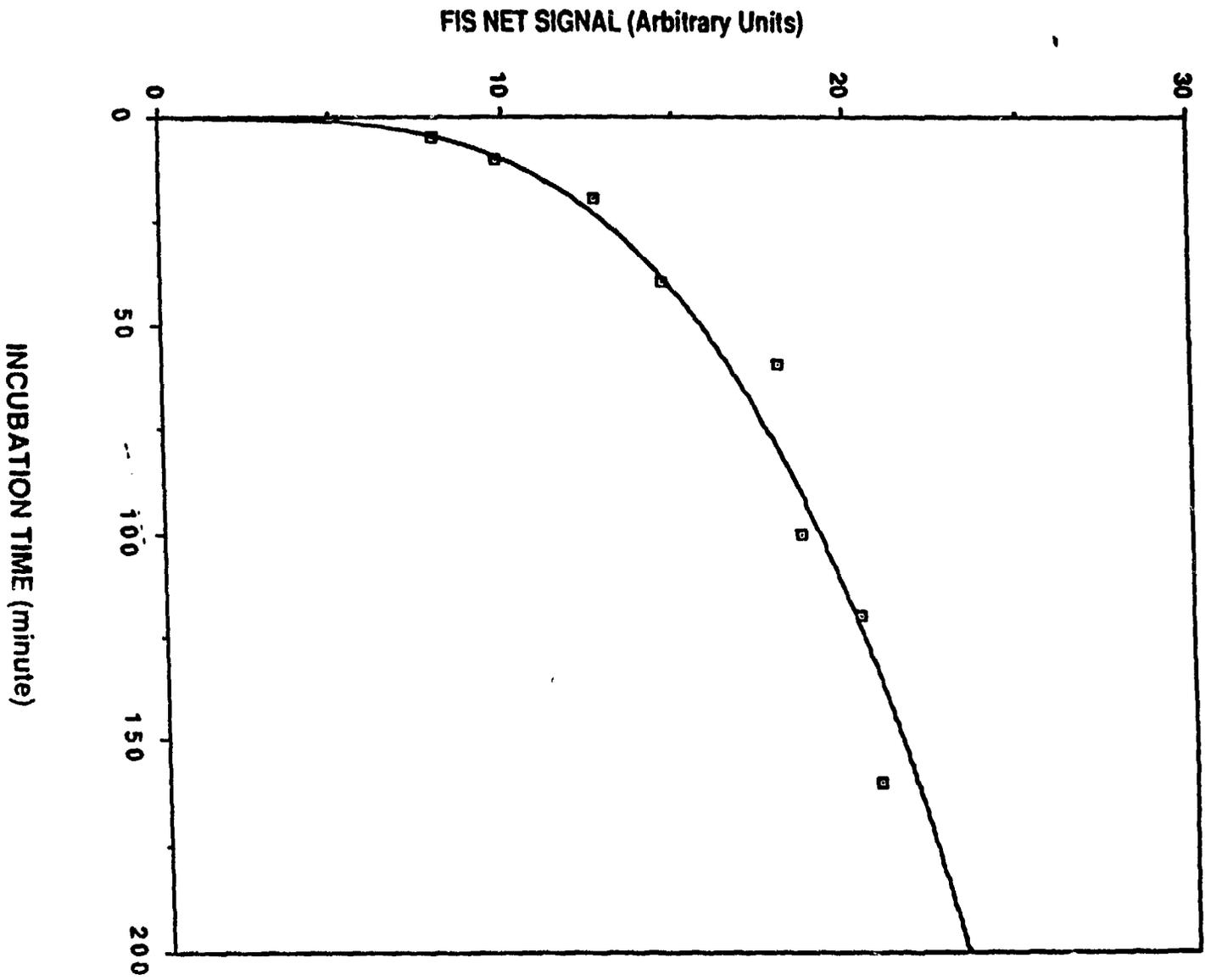
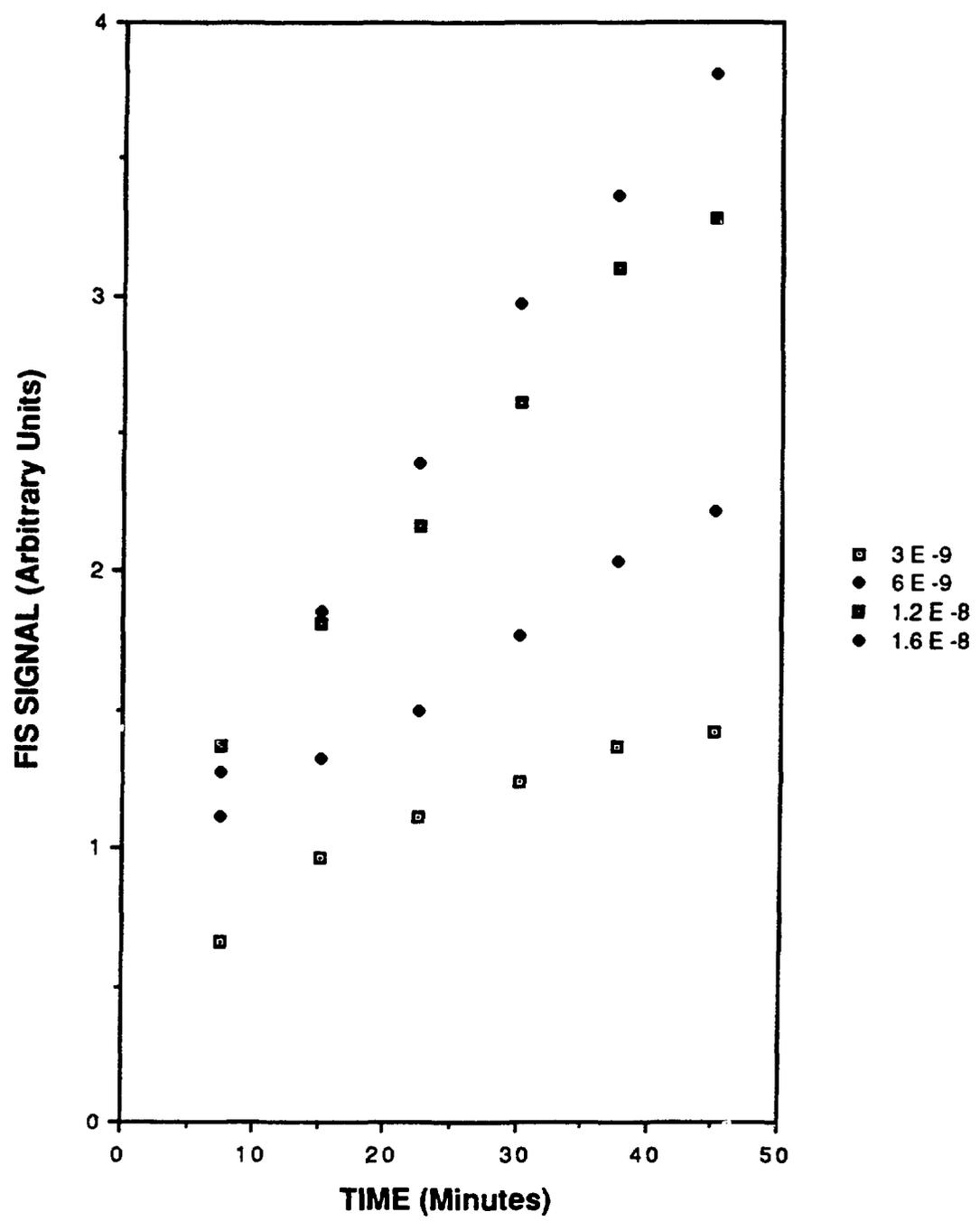
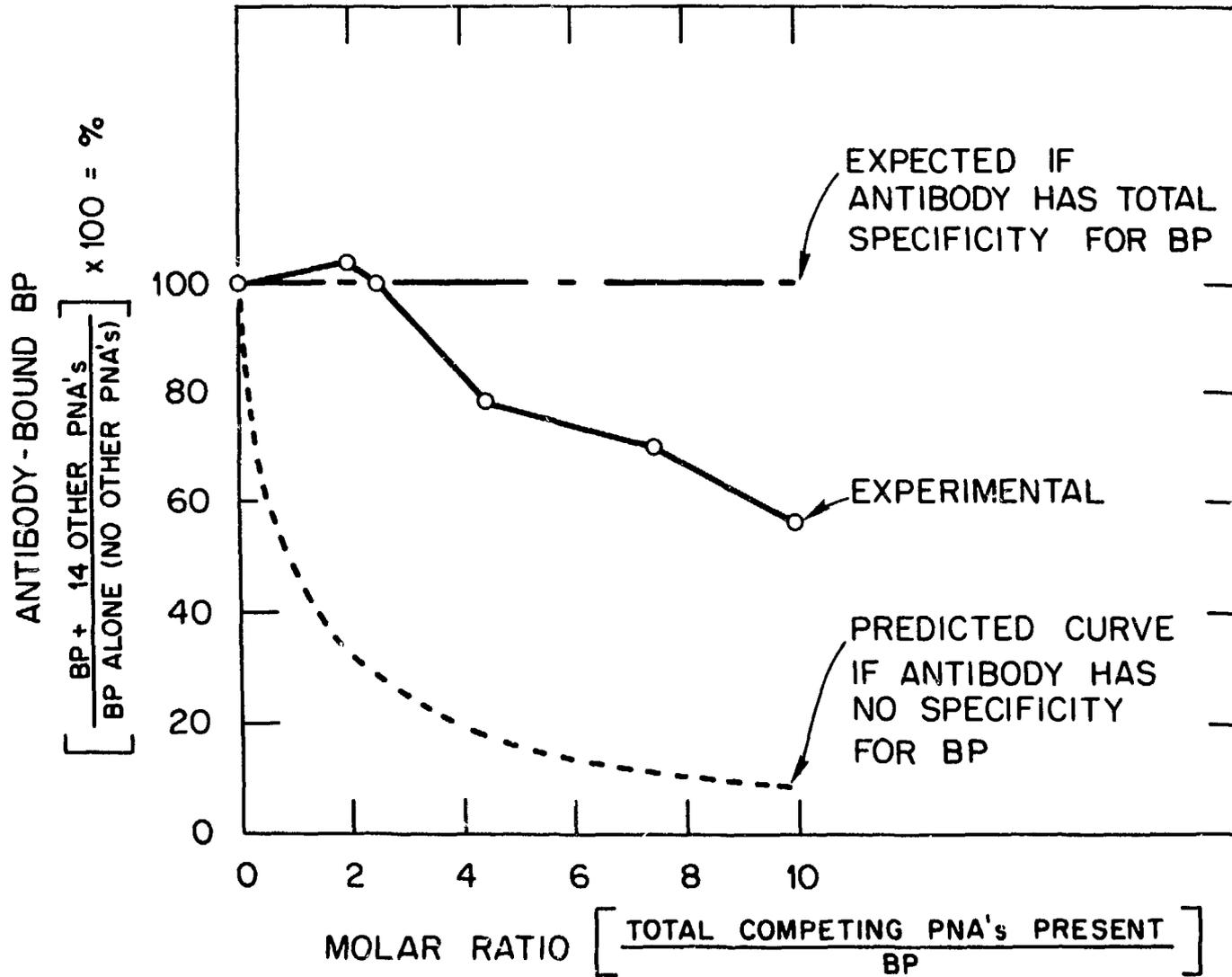


Fig 2

Fig (3)

Data from "FIS/membrane beads"





Decrease In Amount Of Antibody-bound BP With Increasing Amounts Of Competing PNA's

Fig (4)