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TITLE: ELECTRODE PROBES FOR THE RAPID ASSAY OF SEAFOOD TOXICANTS (SBIR 89-I)

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APPENDIX B
DOD No. 89.1

U.S. DEPARTMENT OF DEFENSE

# SMALL BUSINESS INNOVATION RESEARCH PROGRAM
# PHASE 1 — FY 1989
# PROJECT SUMMARY

Topic No. A89-082 Military Department/Agency Army

Name and Address of Proposing Small Business Firm

Universal Sensors, Inc.
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Name and Title of Principal Investigator

Dr. Graham Ramsay
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Proposal Title

Electrode Probes for the Rapid Assay of Seafood Toxicants

Technical Abstract (Limit your abstract to 200 words with no classified or proprietary information/data.)

An enzyme-limited immunosorbent-assays (ELISA) with electrochemical detection was developed for the seafood toxin okadaic acid (diarrhetic shellfish poison). At low ppb concentrations, competitive heterogeneous immunoassays were performed in which soluble sample antigen (50 µl) competed with immobilized antigen for soluble antibody (50 µl). Excess sample and antibody were removed by washing and a secondary antibody labeled with alkaline phosphatase was added. The enzyme label hydrolyzed phenyl phosphate to yield phenol which was oxidized at +870 mV vs. Ag/AgCl. The dynamic response was inversely proportional to soluble antigen concentration.

Anticipated Benefits/Potential Commercial Applications of the Research or Development

The electrochemical ELISA technology developed in Phase I will permit development of a family of electrochemical immunobiosensors for toxins, steroids, hormones and pesticides. These will be of great interest to DOD, USDA, DOC, FDA, NIH, and the seafood and healthcare industries.

List a maximum of 8 Key Words that describe the Project.

Electrochemical immunoassay, seafood toxins, okadaic acid, saxitoxin

Nothing on this page is classified or proprietary information/data

## A. INTRODUCTION

Biological toxins such as ricin are many times more lethal than hydrogen cyanide and could be used to poison military food and water supplies. The minimum lethal dose (MLD) of ricin is 0.02 μg/kg and the MLD of hydrogen cyanide is 700 μg/kg (Table 1). Ricin occurs in the castor bean from the plant Ricinus communis and is readily available. The ready availability and toxicity of ricin would make it an obvious choice as a bioagent.

Seafood toxins such as saxitoxin (MLD: 9 μg/kg) ciguatoxin (MLD: 0.45 μg/kg) and maitotoxin (MLD: 0.13 μg/kg) are also highly toxic, and could result in the poisoning of army personnel by ingestion of contaminated seafood and water. These toxins can produce intoxication or death at very low concentrations and there is a need for specific, highly sensitive toxin detectors for field use.

**TABLE 1. RELATIVE TOXICITIES OF SELECTED TOXINS**

| TOXIN | MLD (μg/kg) | SOURCE | REFERENCE |
|---|---|---|---|
| Hydrogen cyanide | 700.00 | Synthetic | 1 |
| Ricin | 0.02 | Ricinus communis | 2 |
| Maitotoxin | 0.13 | Gambierdiscus toxicus | 3 |
| Ciguatoxin | 0.45 | Gambierdiscus toxicus | 3 |
| Saxitoxin | 9.00 | Gonyaulax catenella | 2 |
| Tetrodotoxin | 10.00 | Spheroides rubripes | 1 |
| Microcystin | 100.00 | Microcystis aeruginosa | 2 |
| Okadaic acid | 200.00 | Dinophysis acuminata | 3 |
| Brevetoxin PbTx-3 | 500.00 | Ptychodiscus brevis | 1 |
| Brevetoxin PbTx-2 | 6600.00 | Ptychodiscus brevis | 1 |

These toxins can be detected by high pressure liquid chromatography (HPLC) or by mouse bioassays but such methods are

expensive, require laboratory conditions and can not be performed under field conditions.

Biosensors offer the possibility of rapid, inexpensive, in-field toxin assay using blood or urine samples. They are chemical detectors that use a biological sensing component, such as an enzyme or an antibody, and a transducer such as an electrode, to generate an analytical signal. These probes can provide rapid analyte determination in complex samples such as blood. For instance, diabetics can now use an electrochemical enzyme biosensor at home for a 30 second assay of blood glucose (4). Rugged toxin biosensors could be produced by the marriage of the well developed technique of enzyme linked immunosorbent assay (ELISA) to electrochemical detection. An ELISA test kit for the diarrhetic shellfish poison, okadaic acid, is available from UBE Industries Ltd., Japan, for sensitive (10 - 200 ppb, 50 $\mu$l samples) and rapid toxin assay. This kit is not suitable for field use but it demonstrates the applicability of the ELISA format. A robust toxin biosensor suitable for field use could be formed by immobilization of enzyme-labeled antibody from an ELISA kit to an electrode to form an electrochemical immunosensor.

## B. PROJECT OBJECTIVES

The objective of the Phase I work was to demonstrate that seafood toxins, such as okadaic acid, could be quantified by a simple, rapid and sensitive (ppb) electrochemical immunoassay technique. The technique had to be amenable to development into an

electrochemical immunobiosensor.

## C. MATERIALS AND METHODS

### 1. Materials

Soluble anti-okadaic acid/peroxidase (aOA/POD) and okadaic acid-ovalbumin conjugate immobilized to microtiter plates were supplied by UBE Industries, Ltd, Japan as a kit (DSP-Check) for the detection of okadaic acid (diarrhetic shellfish poison). Anti-mouse IgG(H+L)/alkaline phosphatase (aMIgG[H+L]/AP), anti-mouse IgG(γ)/alkaline phosphatase (aMIgG[γ]/AP), anti-mouse IgM(μ)/alkaline phosphatase (aMIgM(μ)/AP) were supplied by Kirkegaard and Perry Laboratories, Inc. (Gaithersburg, MD). Anti-saxitoxin (aSTX) from clone S3E.2 was generously donated by Dr. Tran Chanh (Southwest Foundation for Biomedical Research, San Antonio, Texas). Saxitoxin was a generous gift of Dr. Marc Poli (Pathophysiology Division, U.S. Army Medical Research Institute of Infectious Diseases, Fort Detrick, Frederick, USA). Phenyl phosphate and o-phenylenediamine were purchased from Sigma Chemical Co., St. Louis, USA. All other reagents were analytical grade and all aqueous solutions were prepared in deionized water.

### 2. Methods

**a. Fabrication of epoxy-coated Pt working electrode:** Pt wire was cleaned with fine sandpaper, washed firstly with deionized water, then washed with acetone and air-dried. The circumference of Pt wire (approximately one inch) was coated with water resistant epoxy resin ("2-Ton Epoxy", Trubond, Devcon Corp, USA) and cured

overnight, tip down, at 50 °C. The tip was cleaned with fine sandpaper and then polished with 0.05 μm alumina on a velvet pad (Polishing Kit; Bioanalytical Systems, West Lafayette, Indiana).

**b. Spectrophotometric determination of peroxidase activity in aOA/POD:** Fifty microliters of aOA/POD in 0.05 M sodium phosphate buffered saline/Tween-20 (0.05%) (PBS-Tween) and 50 μl of 45% methanol/55% $H_2O$ were added to each well of microliter plates coated with okadaic acid-ovalbumin conjugate (OA-OV) as supplied in the DSP-Check Kit. The antibody/MeOH solution was incubated for 30 minutes and then each well was washed 4 x 0.3 ml PBS-Tween. Fifty microliters o-phenylenediamine (4.0 mg/ml)/$H_2O_2$ (2 mM) in 0.05 M citrate buffer (pH 5.0) were added and the color was developed for 6 minutes in the dark. Fifty microliters of $H_2SO_4$ (1M) was added to stop the enzyme reaction. One hundred microliters of this solution were diluted with 400 μl of deionized water and the $A_{492}$ was measured (Sequoia-Turner Model 690 Spectrophotometer, Microcuvette, 3mm pathlength). In this experiment a control was incorporated in which there was no aOA/POD; 50 μl PBS-Tween and 50 μl of MeOH (45% in aquo) were incubated for 30 minutes as above. The control gave a color change due to non-enzymatic oxidation of o-phenylene diamine (OPD) and the control $A_{492}$ was subtracted from the sample to give a "true" absorbance change.

**c. Calibration of the spectrophotometric ELISA for okadaic acid:** Calibration standards were prepared that contained 0, 1, 4, 10,

40, 100, 400 ppb OA in 45% MeOH/55% $H_2O$ and analyzed as described above in section b. The corrected $A_{492}$ values were plotted versus log OA concentration.

**d. Electrochemical assay of peroxidase activity.** A two electrode system was used for all assays. The working electrode was Pt (0.2 mm DIA in glass) and the combined reference/counter electrode was an Ag/AgCl strip. The working electrode was poised at +650 mV vs Ag/AgCl (0.14 M KCl) with a Tacussel ED 110 potentiostat or a BAS LC-4B potentiostat and the current trace was displayed on a Houston Instrument strip chart recorder. Assays were carried out either in a 2 ml beaker or in the well of a microtiter plate. In the beaker assay the working electrode was equilibrated in 300 µl of stirred sodium phosphate buffer (pH 6.0, 0.1 M)/KCl (0.1 M). $H_2O_2$ (100 µl, 60 µM) and NADH (100 µl, 60 µM) and after attaining a stable background 100 µl of either dilute POD or aOA/POD were added. In the well assays 150 µl sodium phosphate buffer (pH 6.0, 0.1 M)/KCl (0.1 M) were added and the electrode equilibrated. $H_2O_2$ (25 µM, 1.6 µM) was added and after attaining a stable background NADH (25 µM, 1.6 µM) was added. The rate of decrease of $H_2O_2$/NADH concentration was measured.

**e. Electrochemical assay of alkaline phosphatase activity:** A two electrode system was used in all assays with a working potential of +870 mV vs Ag/AgCl (3.0 M KCl gel). The reference/counter electrode was separated from potential reactants in the bulk

solution by a ceramic porous frit and a gel containing 3.0 M KCl; bare Ag/AgCl electrodes could not be used due to the production of an unacceptably high interferant signal on addition of phenyl phosphate, which was the substrate for alkaline phosphatase. Experiments were performed in a 10 ml beaker in order to determine the effect of pH, phenyl phosphate concentration or $MgCl_2 \cdot 6H_2O$ concentration on the enzyme activity of alkaline phosphatase-antibody conjugate. The working electrode was a Pt wire (0.2 mm) encased in glass and the reference/counter electrode was a BAS Ag/AgCl (3.0 M KCl gel) electrode. The working electrode was equilibrated in 2.92 ml of the buffer (0.1 M)/KCl (0.1 M)/$MgCl_2 \cdot 6H_2O$ (1.5 mM)/ Tween-20 (0.05%). Fifty microliters of the AP labeled antibody were added and this was followed by 30 µl of phenyl phosphate (100 mM in aquo). The maximum slope of the resulting peak was measured.

**f. Calibration of electrochemical ELISA assay for okadaic acid:** Fifty microliters of OA calibration standard in 45% MeOH/55% $H_2O$ + 50 µl of aOA/POD were incubated in a microtiter plate containing immobilized OA for 30 minutes. After washing 4 x 0.3 ml with PBS-Tween, 50 µl of aMIgG(γ)/AP (10 mg/L in PBS) was incubated for 60 minutes and then excess secondary antibody was removed by washing 4 x 0.3 ml with PBS-Tween. The last wash was left in the well until immediately before electrochemical assay to prevent loss of enzyme activity by desiccation. The well was then washed with 4 x 0.3 ml PBS and 190 µl of sodium carbonate buffer (pH 10.1, 0.1 M)/KCl (0.1 M)/$MgCl_2 \cdot 6H_2O$ (5 mM)/Tween 20

(0.05%) were added. A Pt/epoxy working electrode (1.0 mm DIA) attached to a BAS Ag/AgCl (0.3 M KCl gel) reference/counter electrode was poised at +870 mV vs Ag/AgCl and, on attaining a stable background, 10 µl of phenyl phosphate (100 mM in aquo) were added. The maximum slope of the current vs. time trace was recorded for OA acid standards (1, 4, 10, 40, 100, 400 ppb) in 45% MeOH/55% $H_2O$. A control experiment with no primary antibody and 0 ppb OA was performed immediately before calibration in order to measure the nonspecific binding of the secondary antibody (aMIgG(H+L)/AP or aMIgG(γ)/AP).

## D. RESULTS AND DISCUSSION

### 1. Use of a Peroxidase Label for the Electrochemical ELISA of Okadaic Acid

It was rapidly found that the peroxidase (POD) label, although effective for spectrophotometric procedures, resulted in an assay that was insensitive compared to that obtained by the use of a secondary antibody labeled with alkaline phosphatase. The POD electrochemical assay was based on the detection of the decrease in NADH and $H_2O_2$ concentration:

$$NADH + H^+ + H_2O_2 \xrightarrow{POD} 2H_2O + NAD^+$$

At the working electrode NADH and $H_2O_2$ were oxidized:

$$NADH + H^+ \longrightarrow NAD^+ + 2H^+ + 2e^-$$

$$H_2O_2 \longrightarrow O_2 + 2H^+ + 2e^-$$

The greatest analytical sensitivity was obtained at low substrate concentration ($\mu$M), but this limited the rate of the enzyme reaction. At higher substrate concentrations less sensitive instrument settings were used and the analytical signal was reduced. Fifty microliters of aOA/POD (DSP-Check Kit) were detected in a total volume of 3.35 ml sodium phosphate buffer (pH 6.0, 0.1 M)/KCl (0.1 M). However after reacting 50 $\mu$l of the same antibody solution in a microtiterplate well coated with immobilized OA for 10 minutes, and after washing away soluble antibody, the bound antibody could not be detected electrochemically using the POD label. The bound antibody was readily detected using the spectrophotometric procedure or electrochemically using a secondary antibody labeled with alkaline phosphatase (AP).

### 2. Use of a Peroxidase Label for the Spectrophotometric ELISA of Okadaic Acid

Figure 1 shows the absorbance change observed with calibration standards in the range 4-400 ppb OA. Between 4 and 40 ppb OA there was a gradual decrease in absorbance followed by a rapid decrease in the range 40-100 ppb. Between 100 and 400 ppb there was a slow change in absorbance. This inverse relationship between sample OA and $A_{492}$ was observed because at relatively low concentrations of OA more of the antibody bound to the immobilized antigen and this resulted in a relatively high $A_{492}$ value; conversely at high concentrations of sample OA (100-400 ppb) only a small amount of antibody was bound to the immobilized

antigen and this resulted in a low $A_{492}$ value. The assay had a useful analytical range of approximately 4-100 ppb OA.

### 3. Development of an Okadaic Acid Immunoassay with Electrochemical Detection Via Alkaline Phosphatase

This electrochemical assay required the use of a secondary antibody labeled with AP. The primary antibody (aOA/POD) was a mouse IgG protein and two anti-mouse IgG antibodies labeled with AP were evaluated (aMIgG(H+L)/AP and aMIgG(γ)/AP). AMIgG(H+L)/AP was specific for the binding region of mouse IgG where H and L chains are bound together whereas aMIgG(γ)/AP recognized the γ heavy chain at the opposite end of the molecule. It was not possible to conjugate AP directly to aOA as the antibody was only available as a dilute solution of aOA/POD in the DSP-Check Kits. Hence the assay was developed with a secondary antibody labeled with AP.

**a. Effect of incubation time on the amount of aOA/POD bound to immobilized okadaic acid:** Figure 2 shows the effect of incubation time of aOA/POD in OA-coated microtiter wells. The standard colorimetric procedure was used (section C.2.b) except that the incubation time was varied between 10 minutes and 17 hours. The amount of aOA/POD bound to immobilized OA increased rapidly as the incubation time was increased from 10 o 40 minutes (Fig 2). After 60 minutes incubation the amount of bound antibody was very similar to the amount bound after 40 minutes suggesting that the binding rate was slowing. After 17 hours incubation more antibody had been bound ($A_{492}$ = 1.48) but the

gain was too small to justify the inconvenience of using an overnight incubation. An incubation time of 30 minutes was adopted as standard because it resulted in a useful $A_{492}$ value in a convenient time.

**b. Effect of pH and buffer type on enzyme activity of aMIgG(H+L)/AP and aMIgG(γ)/AP:** The effect of pH on the enzyme activity of aMIgG(H+L)/AP was measured separately in borate buffer or carbonate buffer. Figure 3 shows the effect of pH on the activity of aMIgG(H+L)/AP in borate buffer. The AP activity rapidly increased as the pH was increased from 8.5 to 9.0 pH units and reached a maximum at approximately pH 10.0. In carbonate buffer the AP activity peaked at approximately pH 10.4 (Figure 4).

The peak enzyme activities of aMIgG(H+L)/AP in borate buffer (pH 10.0) and carbonate buffer (pH 10.40) were compared under parallel experimental conditions using a final antibody concentration of 17 μg/L. The enzyme activity of the antibody was far greater in carbonate buffer, yielding a maximum slope value of 2.14±0.94 nA/min (n=2), whereas in borate buffer the maximum slope was 0.36±0.02 nA/min (n=2). The effect of pH on aMIgG(γ)/AP in carbonate buffer was studied (Figure 5) using a final antibody concentration of 17 μg/L. The enzyme activity peaked at approximately pH 10.1, with a maximum slope value of 18.4 nA/min.

**c. Optimization of phenyl phosphate concentration and magnesium**

chloride concentration for the maximum enzyme activity of aMIgG(γ)/AP: The variation of the enzyme activity of aMIgG(γ)/AP with phenyl phosphate concentration is shown in Figure 6. The AP activity rapidly increased as the substrate concentration was increased from 0-1 mM and then more slowly reached a plateau at approximately 4 mM phenyl phosphate. Subsequent experiments were carried out at a phenyl phosphate concentration of 5 mM. $MgCl_2 \cdot 6H_2O$ was included in the reaction mixture as a stimulator of AP activity. With no added $MgCl_2 \cdot 6H_2O$ there was some enzyme activity which rapidly increased to a plateau value as the concentration of magnesium salt was increased to approximately 4 mM (Fig. 7). Subsequent experiments were carried out with 5 mM $MgCl_2 \cdot 6H_2O$.

d. Effect of secondary antibody type and concentration on the magnitude of the analytical signal: The effects of the concentration of aMIgG(γ)/AP and aMIgG(H+L)/AP on the magnitude of the analytical signal were investigated. Table 2 shows that as the concentration of aMIgG(γ)/AP was varied from 1 mg/L to 10 mg/L the maximum slope more than doubled.

TABLE 2. EFFECT OF SECONDARY ANTIBODY CONCENTRATION ON THE MAXIMUM SLOPE RESPONSE IN THE OKADAIC ACID ELECTROCHEMICAL ELISA.

| ANTIBODY | ANTIBODY CONCENTRATION (mg/L) | MAXIMUM SLOPE RESPONSE (nA/min) |
|---|---|---|
| aMIgG(γ)/AP | 1 | 16.8 |
| aMIgG(γ)/AP | 10 | 39.1 |
| aMIgG(γ)/AP | 100 | 33.1 |

| | | |
|---|---|---|
| aMIgG(H+L)/AP | 1 | 13.5 |
| aMIgG(H+L)/AP | 10 | 15.3 |

However on increasing the aMIgG(γ)/AP concentration to 100 mg/L the maximum slope response was slightly less than obtained with a concentration of 10 mg/L. 10 mg/L was chosen as the optimum concentration for this antibody. The response from aMIgG(H+L)/AP over the range 1-100 mg/L was measured. There was a small increase in response as the antibody concentration was increased from 1-10 mg/L, although neither response matched that obtained from 10 mg/L aMIgG(γ)/AP. One hundred mg/L aIgG(H+L)/AP produced a huge response (781 nA/min) probably due mainly to non-specific adsorption. For calibration, 10 mg/L aMIgG(γ)/AP was used.

**e. Okadaic acid electrochemical ELISA calibration.** The OA electrochemical ELISA was carried out as described in section 2f using 10 mg/L aMIgG(γ)/AP (Fig. 8). The final washing procedure prior to assay was necessary because the control, which contained no primary antibody, gave a large response (135 nA/min) which suggested that there was substantial nonspecific adsorption of the secondary antibody. The additional washing procedure successfully eliminated nonspecifically adsorbed secondary antibody and this resulted in zero slope at high OA concentration. The assay was carried out firstly in order of increasing OA concentration (4, 40, 400 ppb OA) and then in order of decreasing concentration (100, 10, 1 ppb OA) so that any time dependant changes would be evident. There were no apparent systematic errors from this source and a sigmoidal curve was

obtained that was similar to that obtained by use of the spectrophotometric method (Fig. 1). The electrochemical ELISA showed response changes over the range 1 - ~200 ppb with a linear range from approximately 5-50 ppb.

**f. Analysis of okadaic acid in real samples.** There was insufficient time to assay real samples with the electrochemical ELISA, however, the DSP-Check Kit includes a simple reproducible procedure for extracting OA from shellfish. The toxin is extracted with 90% MeOH after homogenization of the soft tissue in a blender, and the crude extract is assayed. Using this procedure the recovery efficiency is high (90-107%) and sample preparation is rapid, approximately 30 minutes.

**g. Stability of components of ELISA for okadaic acid:** ELISA enzyme substrates were prepared fresh each day to prevent high background readings. The peroxidase substrate OPD clearly autohydrolyzed, as aqueous solutions developed a yellow color over several hours; the stability of phenyl phosphate to autohydrolysis was not measured. The components most likely to deteriorate were the solutions of antibody. Controls were carried in all experiments in order to compensate for any change in the immunological or enzyme activity of antibody-enzyme conjugates. Antibody solutions were kept at 4 °C when in use and stored at -20 °C when not in regular use. Anti-OA/POD solution lost some activity after multiple freeze/thaw cycles as shown in Table 3.

TABLE 3. STABILITY OF aOA/POD SOLUTION

| $A_{492}$ | Storage Time (d) | No. freeze/ thaw cycles |
|---|---|---|
| 0.622 | 0 | 0 |
| 0.824 | 1 | 1 |
| 0.556 | 3 | 2 |
| 0.539 | 6 | 3 |

The secondary antibodies aMIgG(H+L)/AP and aMIgG(γ)/AP clearly lost considerable activity when stored as TS solutions at 4 °C and -20 °C. For instance aMIgG(γ)/AP gave a response of 18.4 nA when freshly prepared but after a month of continuous use the activity had reduced to 2.6 nA/min. Fifty percent glycerol was tested as a stabilizer but it remained sufficiently adsorbed to the microtiter plates to give a large interferant signal. In Phase II a rigorous survey of stabilizers such as antibacterial agents (eg. $NaN_3$) and BSA will be tested in order to minimize variations in activity of antibody solutions. For commercial applications of this ELISA technology, the antibodies would be stored at 4°C in a lyophilized form rather than in solution.

**g. Technical feasibility of ELISA for toxins using electrochemical detection.** The present work has demonstrated that ELISA with electrochemical detection can be used for the rapid detection of okadaic acid at low ppb concentrations. Traditionally mouse bioassays and high-pressure-liquid chromatography (HPLC) have been used to detect toxic levels of toxins. The mouse assay is expensive due to the necessity of keeping a large number of mice and the requirement for highly

trained personnel to carry out and interpret the assays under laboratory conditions. HPLC requires very expensive instrumentation, highly trained personnel and a laboratory environment. In sharp contrast, the ELISA format is very simple and rapid and can be used in-field by personnel with only basic scientific training.

In the work reported here the ELISA was performed in the wells of microtiter plates on which antigen-protein conjugate had been adsorbed. Preactivated membranes are now available that rapidly (1 minute), covalently bind buffered solutions of proteins such as antibodies and enzymes that contain free amino groups. For instance Immobilon membrane (Millipore Corporation, Bedford, Massachusetts) and Immunodyne (Pall Biosupport Division, New York, New York) have been used in recent years to produce a variety of biosensors (e.g.: 5,6). In Phase II it is envisioned that bioprobes will be developed either by immobilization of antigen-BSA to preactivated membranes, which will then be mounted on a base electrode (Pt or C), or by adsorption of antigen-BSA onto the base of a disposable microcell. In an ongoing NIH Phase I project at Universal Sensors microbiosensors (Pt and C) for the neuroregulator glutamate are being developed. This technology will allow extremely inexpensive disposable micro immunosensors to be developed as the working electrode will be approximately 25 μm in diameter and only minute amounts of reagents will be required. The electrochemical immunoprobes will be incorporated into an inexpensive flow-injection-analysis (FIA) system

incorporating disposable microcells (50 µl) that contain lyophilized buffer components and disposable electrodes. Samples such as extracts of seafood, blood or urine will hydrate the lyophilized components and after incubation (approximately 10 minutes), a washing step will remove excess sample. Addition of buffered phenyl phosphate solution for the alkaline phosphatase antibody label will then rapidly generate a dynamic response in approximately 2 minutes. Modern microchip technology, for instance, that incorporated in the ABD detector developed by Universal Sensors, Inc., will measure the dynamic response and display it as a concentration. The use of a cartridge containing several microelectrochemical cells will allow very rapid multi-toxin analysis. Further samples can be rapidly assayed simply by inserting new cartridges.

Recently an ELISA stick test has been developed for the assay of ciguatoxin in fish (7). Sample preparation was extremely simple; a bamboo stick was pushed into the dead fish and antigen was attached via a Liquid Paper (The Gillette Co., Rockville, MD) layer on the tip of the stick. The stick was then placed in anti-ciguatoxin antibody, excess antibody removed by washing and the peroxidase label developed to yield the concentration of antigen. The usefulness of this procedure will be assessed in Phase II for seafood contaminated by toxins such as saxitoxin and brevetoxin. An assay for ciguatoxin will only be developed if the toxin becomes commercially available; purification of ciguatoxin is extremely arduous and at present no pure samples

are available for sale.

The electrochemical competitive ELISA for OA required a secondary antibody to effect electrochemical detection via AP. The analysis time could have been shortened firstly, by using an anti-OA antibody labeled with AP, and secondly, by reduction of the incubation time of the primary antibody from 30 minutes to 10 minutes. This would yield a total analysis time, including sample preparation, of approximately 60 minutes.

Toxin antibodies are currently available at Universal Sensors to saxitoxin (Dr. Chanh, Southwest Foundation for Biomedical Research), ricin (Sigma), and brevetoxin (Dr. Poli, USAMRIID). Conjugation of alkaline phosphatase to these antibodies will be performed in Phase II in-house using alkaline phosphatase pre-activated for covalent coupling (Biomol, Hamburg, Germany). These antibodies will be used for initial work in Phase II. However, for commercial production of microtoxinimmunosensors, Universal Sensors requires a long-term, supply of purified, high affinity, labeled antibodies, which have binding constants that vary very little from batch to batch. An excellent way of achieving this is to have monoclonal antibodies custom produced and this can now be carried out inexpensively (see Phase II proposal). In this way rapid one step ELISA assays will be developed for a variety of low molecular weight toxins in complex samples such as fish tissue, urine and blood.

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Figure 1. ABSORBANCE VERSUS OKADAIC ACID CONCENTRATION

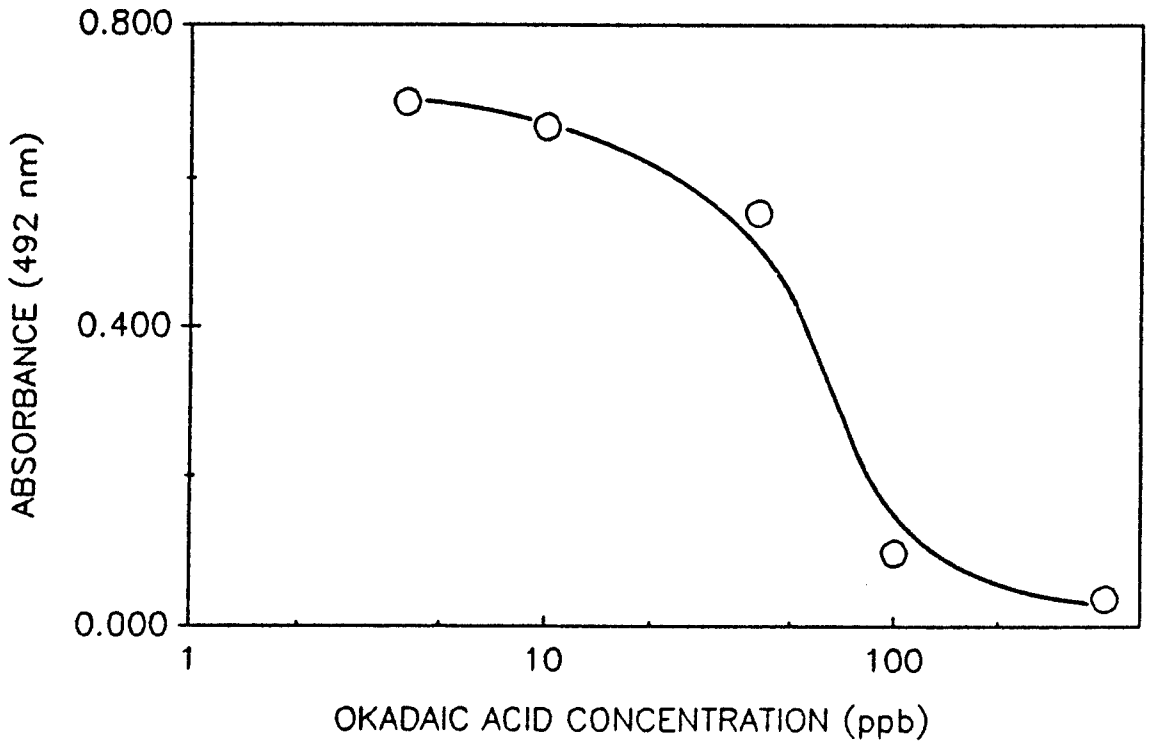

Figure 2. VARIATION OF AMOUNT OF aOA/POD BOUND VERSUS INCUBATION TIME

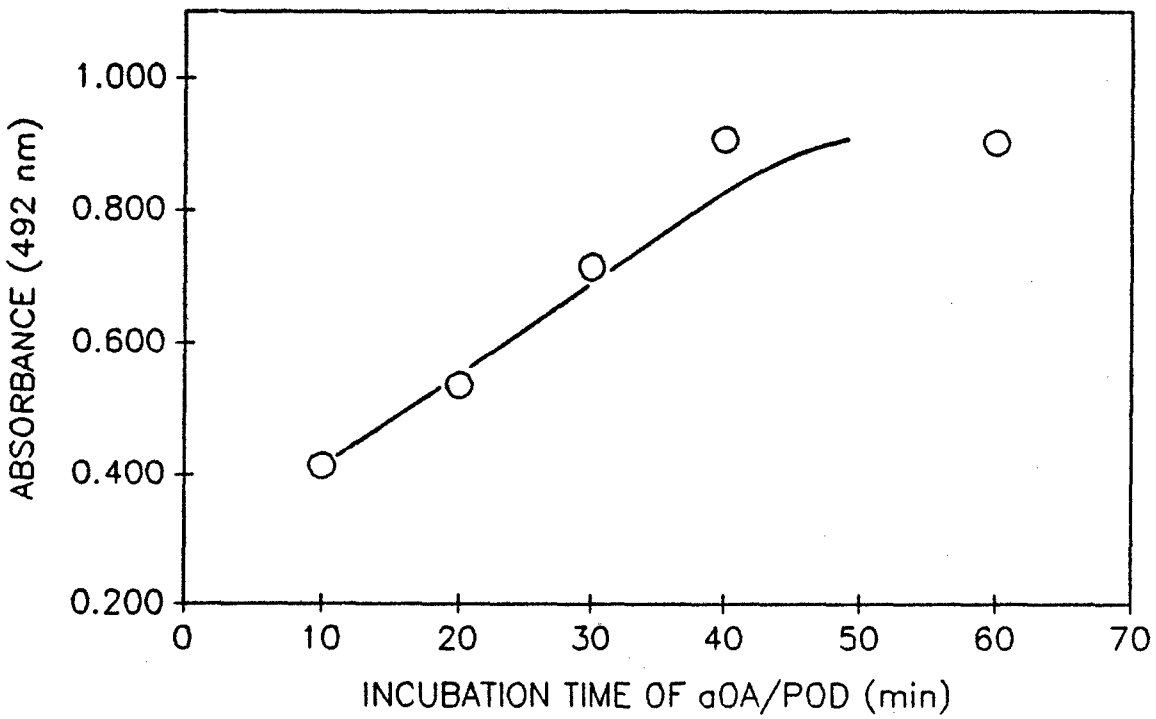

Figure 3. EFFECT OF pH ON ENZYME ACTIVITY OF aMIgG(H+L)/AP IN BORATE BUFFER

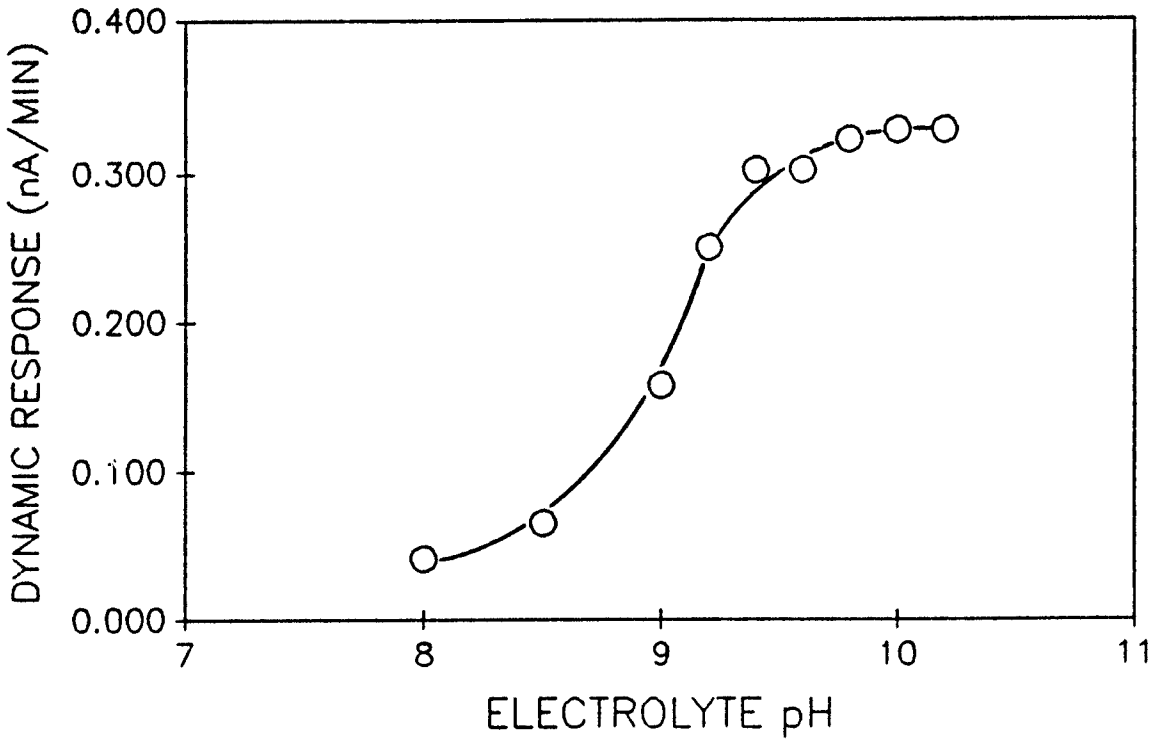

Figure 4. EFFECT OF pH ON ENZYME ACTIVITY OF aMIgG(H+L)/AP IN CARBONATE BUFFER

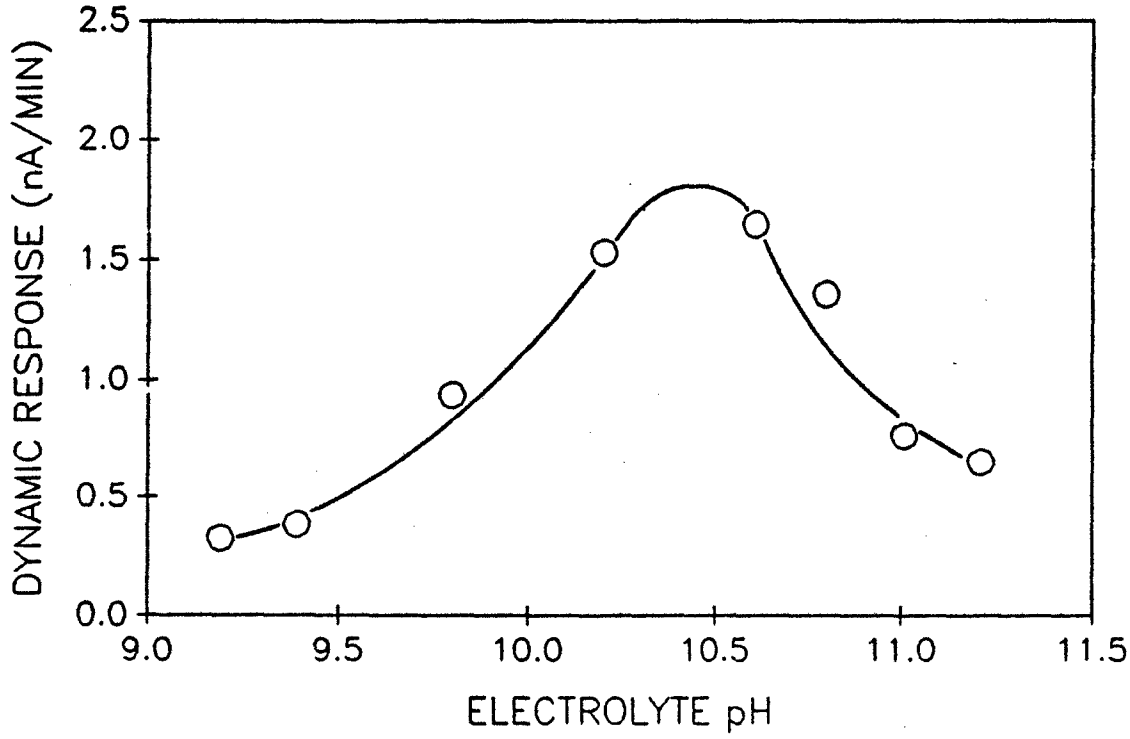

Figure 5. EFFECT OF pH ON ENZYME ACTIVITY OF aMIgG(T)/AP IN CARBONATE BUFFER

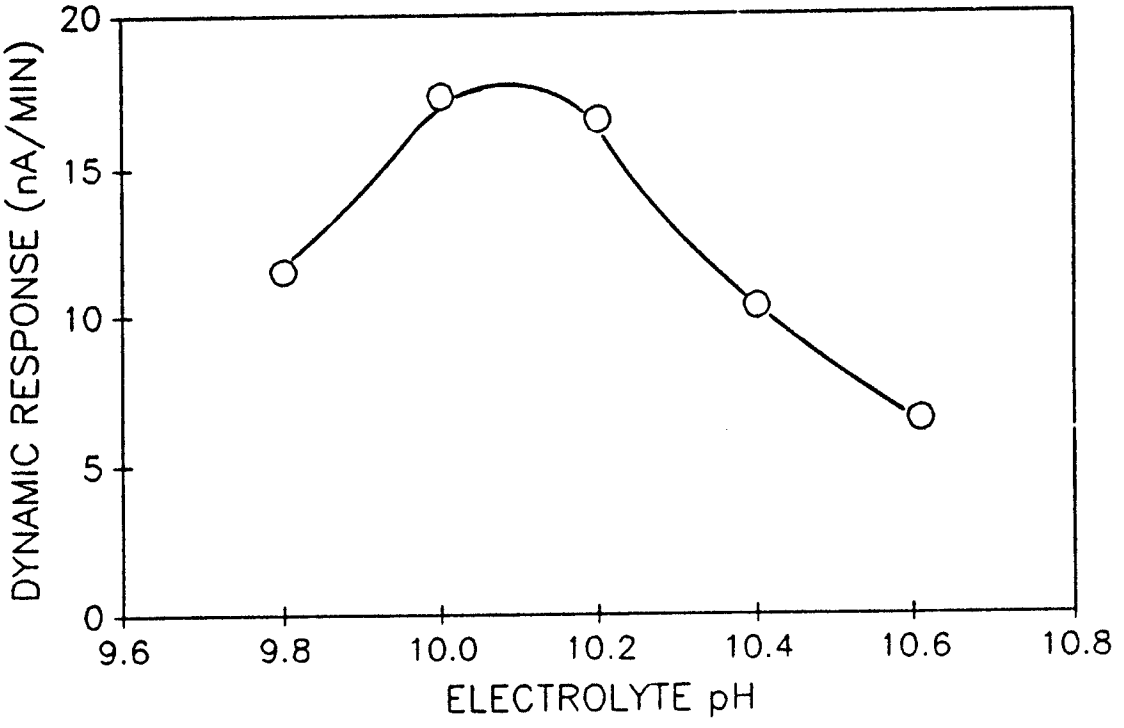

Figure 6. EFFECT OF PHENYL PHOSPHATE CONCENTRATION ON ENZYME ACTIVITY OF aMIgG(T)/AP

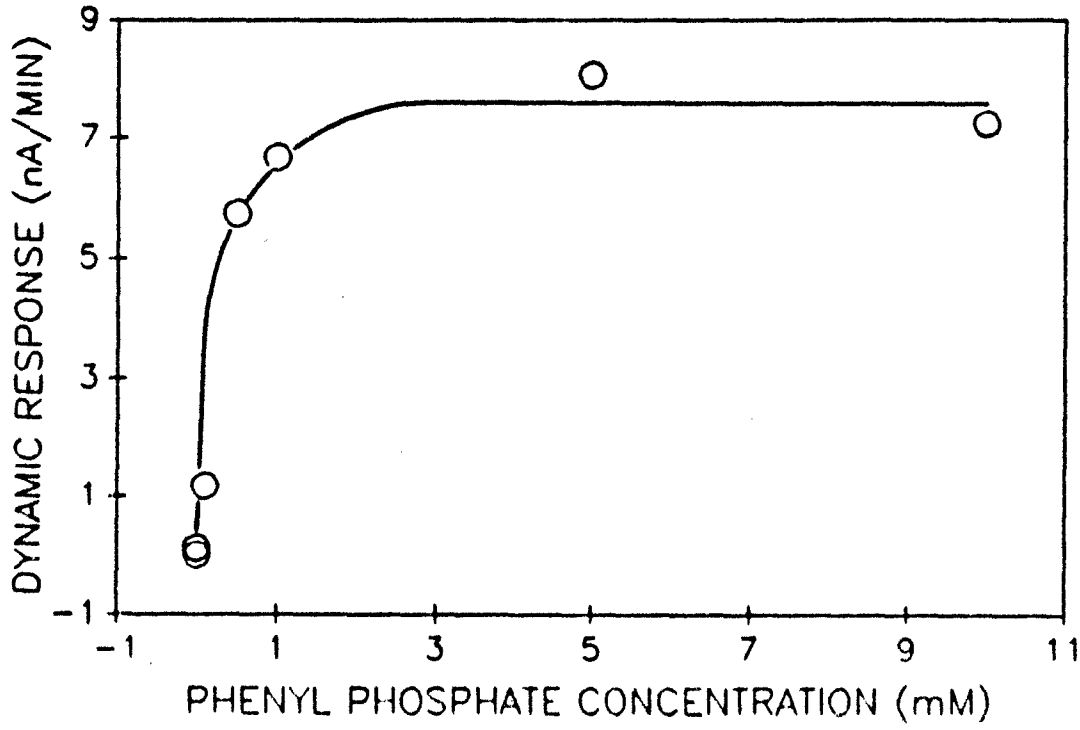

Figure 7. EFFECT OF $MgCl_2 \cdot 6H_2O$ CONCENTRATION ON ENZYME ACTIVITY OF aMIgG(T)/AP

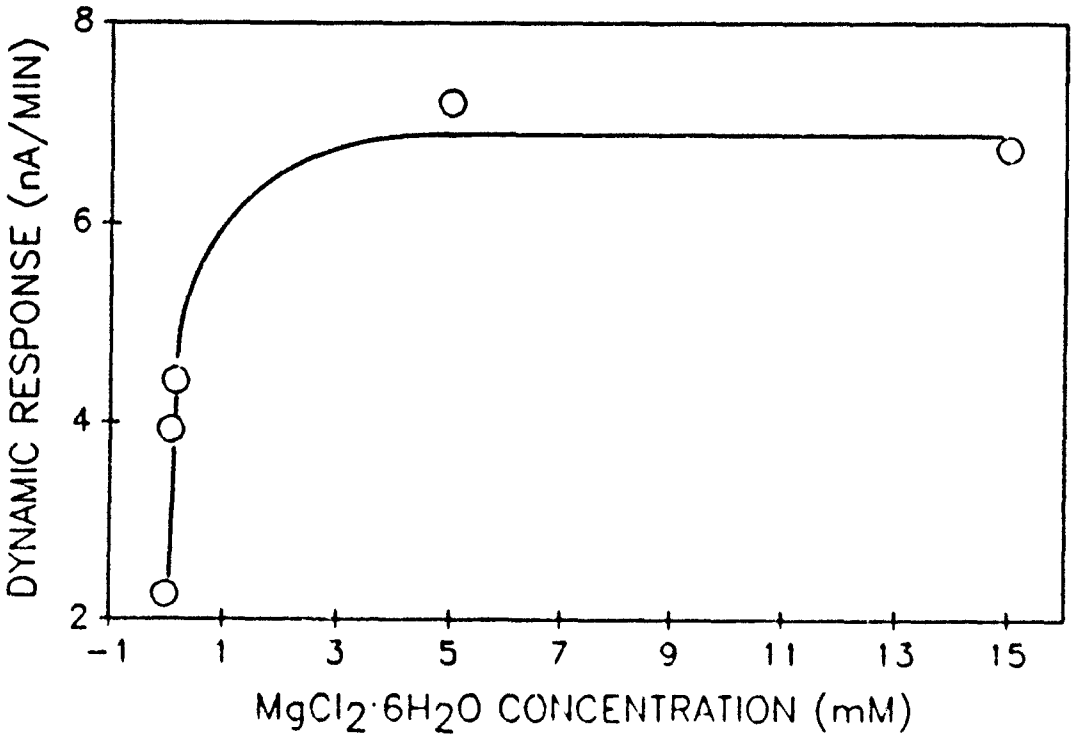

Figure 8. MAXIMUM SLOPE VERSUS OKADAIC ACID CONCENTRATION