ANNUAL REPORT
ON
THE DEVELOPMENT OF SENSORS & TECHNIQUES FOR IN SITU WATER QUALITY MONITORING

NASA GRANT NSG-3002

June 13, 1975 - June 12, 1976

Chung-Chiun Liu
University of Pittsburgh

(NASA-CR-148169) THE DEVELOPMENT OF SENSORS AND TECHNIQUES FOR IN SITU WATER QUALITY MONITORING Annual Report, 13 Jun. 1975 - 12 Jun. 1976 (Pittsburgh Univ.) 42 p HC $4.00 CSCL 13E G3/45

N76-26717

Unclas
43635

ANNUAL REPORT

on

THE DEVELOPMENT OF SENSORS AND TECHNIQUES FOR IN SITU WATER QUALITY MONITORING

NASA GRANT NSG-3002**

June 13, 1975 - June 12, 1976

Submitted By

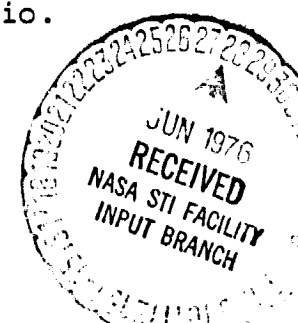
Chung-Chiun Liu*

Chemical and Petroleum Engineering Department

University of Pittsburgh
Pittsburgh, Pennsylvania 15261

*Principal Investigator, and to whom to address all correspondence.

**The NASA Technical Officer for this grant is Dr. J. Stuart Fordyce, Lewis Research Center, NASA, Cleveland, Ohio.



INTRODUCTION

The Chemical and Petroleum Engineering Department at the University of Pittsburgh is carrying out a research program on the development of sensors and techniques for in situ water quality monitoring under the sponsorship of Lewis Research Center, NASA. The support is a continuation of a research grant (NSG-3002). This report describes the progress made over this period of time.

Philosophically, the water pollution control programs rely to a great extent on the reliable detection and identification. Perhaps, the most challenging and most meaningful type of instrumental analysis for the water quality characterization is that which involves in situ measurements. In situ measurements here, suggest an instrument which is used for continuous, unattended monitoring of the essential environmental parameters quantitatively. To a lesser extent, in situ measurements also imply a mobile and portable instrument which is capable of detecting and identifying the pollutants in a quantitative manner. Fundamentally, in situ measurements require good sensitivity, durability, reliability, minimum power consumption and small sample volume, etc. Moreover, in situ measurements imply unattended, long term, continuous monitoring and recording of one or more meaningful parameters over a time period considered representative of valuable information.

At present, while commercial water quality monitoring instruments are available, most of them are designed for laboratory analytical purposes. There is a very limited amount of equipment available for in situ measurement of selected water quality parameters. Their performance, in

general, requires much improvement.

Hence, the objective of this research is to develop sensors which are capable of long-term, unattended, reliable, in situ monitoring. These sensors should require little power consumption and small sample volume. The proposed research will coordinate with the ongoing research effort related to water quality monitoring sensors in our laboratory as well as those at Lewis Research Center of NASA. We do not intend to repeat our ongoing research, nor compete with commercially available and proven sensors but rather to develop new types of sensors as well as to further refine our presently developing one.

Our research goal is to develop practical sensors for in situ monitoring. These sensors should be capable of long-term, unattended, continuous in situ applications. Innovative research efforts will be involved in developing these new sensors for water quality monitoring.

With this intention, we aim specifically in this research:

1. To continue the development of miniature chloride ion and ammonium ion sensors for in situ water quality monitoring.

2. To develop techniques for in situ monitoring of selected trace elements in water.

3. To investigate new approaches in monitoring biological oxygen demand (BOD). Based on the findings, instrumentation for the in situ monitoring of BOD will be developed.

4. To investigate and evaluate the measuring techniques of Adolesine Triphosphate (ATP) and total phosphates in water.

5. To investigate new approaches in monitoring total nitrate in water. Based on the findings, instrumentation for the in situ monitoring of total nitrate will be developed.

6. To investigate new techniques in monitoring phenol. Based on the findings, in situ monitoring of phenol in water will be developed.

7. To further refine our developing prototype in situ pO_2, pH and temperature monitoring unit. This phase of work will also include evaluation and comparison of commercial sensors.

Our research efforts have been devoted to achieving these research goals. However, the investigations of monitoring trace elements and BOD were not carried out. This was due to the adjustment of available manpower and resources. We devoted most of our efforts upon enzyme electrodes, i.e. phenol electrodes, nitrate electrodes, and ATP electrodes. Also, chloride ion electrodes were evaluated. In summary, we have gained adequate knowledge in all these phases of research, and feel the chloride ion electrode and the phenol electrode are most promising. More research efforts in the future will be devoted to the development of these two electrodes while other new research will be attempted. This year, we have one publication being accepted and published by Bioengineering and Biotechnology. We have made a preliminary patent disclosure on the phenol sensor. A technical note is being revised and two other articles will be prepared in the coming months.

The details of each phase of this research will be described as follows.

EXPERIMENTAL WORK

(1) Chloride Ion Electrode

In this phase of our research the development of a miniature chloride ion electrode for *in situ* water quality monitoring was continued. The formation of liquid membranes for the chloride-ion electrode was first undertaken. This was done by first heating the Ag/AgCl electrodes and then dip-coating the electrode into the liquid membrane material. When this procedure is followed a good adhesive characteristic of the membrane is obtained. Furthermore, we found that the poisoning of the electrode by sulfur ions up to an intensity of 0.03 mole shows no adverse effect on the performance of the electrode.

In summarizing this phase of research, we shall discuss the fabrication of the electrode, experimental results obtained, and the prototype sensor.

The Ag/AgCl electrode was made using a plunger type die which was placed between a flat-plate press. Reagent grade powdered silver chloride (Fisher Scientific Co., Pittsburgh, Pennsylvania) was used and a silver wire (99.5% purity, I. Miller Company, Cleveland, Ohio) in the diameter of 0.25 mm (10 mil) was used as the backbone as well as the lead wire for the electrode. The finished electrode would have an AgCl pellet in the size of 2 mm in diameter and 3 mm in length with the silver wire placed in the middle of the pellet and serving as the lead wire. The upper part of the pellet, with the lead wire attached, was sealed with epoxy resin providing the insulation. Both Araldite 6040 epoxy (Ciba-Geigy) and Epon 815 (Shell Co.) proved to be suitable. A heat shrinkable tubing in the diameter of 3 mm (Alpha Wire Co.) was used as a sleeve to provide complete insulation to the lead wire.

The formation of the chloride ion selective membrane was derived from the formula reported by James, et al.[1] with modifications in the reagent proportions. All the chemicals used in the membrane preparation are of reagent grade quality. Twenty ml of a 60 v/v% solution of Aliquat 336S (tricaprylyl methyl ammonium chloride) in 1-decanol was mixed with 200 ml of 1 M NaCl solution. After each of the 3 two-minute intervals of agitation, the inorganic and organic layers were allowed to separate. The organic phase, which was the Aliquat complex, was then decanted and stored for use in the preparation of the membrane.

Two methods were used in forming the membrane coating on the surface of the Ag/AgCl electrode. The first method involved the dipping of the electrode into an 8 w/v% polyvinyl chloride (PVC):cyclohexanone solution. Normally, three layers of coating would be applied for each electrode. A drying period of 30 minutes was required between each coating. The PVC coated electrode was then soaked in the Aliquat complex at room temperature for 24 hours. The electrode was then rinsed and ready to be used. In this preparation, the PVC coating acted as an inert matrix in which the chloride ions, from the Aliquat complex, was embedded.

The other method required a 10:1 v/v mixture of the prepared PVC solution and the Aliquat complex. The Ag/AgCl electrode was then dip coated with this mixture. Also, three coatings were applied to the electrode under normal conditions and the drying time between each coating was an hour.

Either of these two methods proved to work satisfactorily and did not show any marked difference in the electrode performance.

The electrode potential measurements were made using a Keithley 610C Electrometer and recorded on a Morsley 680 strip chart recorder. A prepared Ag/AgCl electrode was placed in a 0.1 M NaCl solution as reference, while another testing Ag/AgCl electrode was placed in the testing solution. A saturated KCl salt bridge was then used to complete the circuitry.

Initially, all the prepared Ag/AgCl electrodes were placed in a 0.1 M NaCl solution, and their electrode potential versus a standard Ag/AgCl reference electrode were measured. The measurements were continuously recorded on a strip-chart recorder for 1-3 days. Any drifting of the electrode would be noted and examined. For most of the electrodes, excellent stability of the electrode was exhibited indicating that the electrode prepared was good. This testing method was repeated, only this time, a prepared Ag/AgCl electrode was chosen randomly as the reference electrode. Again, any drifting in the electrode potential would be examined. As expected, a potential close to zero (0-5 millivolts) was observed.

The testing electrode was then placed in NaCl solutions of different concentrations. A complete dissociation of NaCl in the solution was assumed, and the electrode potential was measured as a function of Cl^- concentration.

Sodium sulfide, Na_2S was then added as the source of the sulfide ions. The electrode potentials were measured and compared with those recorded in sulfide-free NaCl solutions.

The response to chloride ion concentrations of the sintered Ag/AgCl electrode with and without the liquid membrane matrix was investigated. In the absence of any sulfide

ions, the measured electrode potentials of these electrodes exhibit a good linearity with respect to the logarithmic values of the chloride ion concentrations as predicted by the Nernst equation. A slope of 38-60 mv/$\Delta[Cl^-]$ was obtained for all the electrodes tested. The slope values remain the same for individual sintered Ag/AgCl electrodes with and without liquid membrane matrix. However, each electrode must be calibrated individually to determine this calibration constant. The constancy of the calibration constants for both the electrode with and without the liquid membrane matrix indicates that the membrane does not alter the electrode performance.

In the existence of sulfide ions, however, the electrodes without membranes show a severe deterioration in performance within a short time. Under this circumstance, the potential outputs of the electrode are widely scattered thus exhibiting a poor response to chloride ion concentration. This phenomenon is expected since sulfide ions can poison the Ag/AgCl electrode easily, forming silver sulfide.

An electrode with the liquid membrane matrix shows a good response to chloride ion concentration even in the presence of sulfide ions. Over the range of chloride ion concentration of 0.1-0.5 M, with sulfide ions in the concentration of 0.01-0.03 M added into the testing solutions, the calibration constant was not affected. Figure 1 shows a typical calibration curve for the membrane-coated electrode. The results demonstrate that the membrane matrix minimizes the sulfide ion interference.

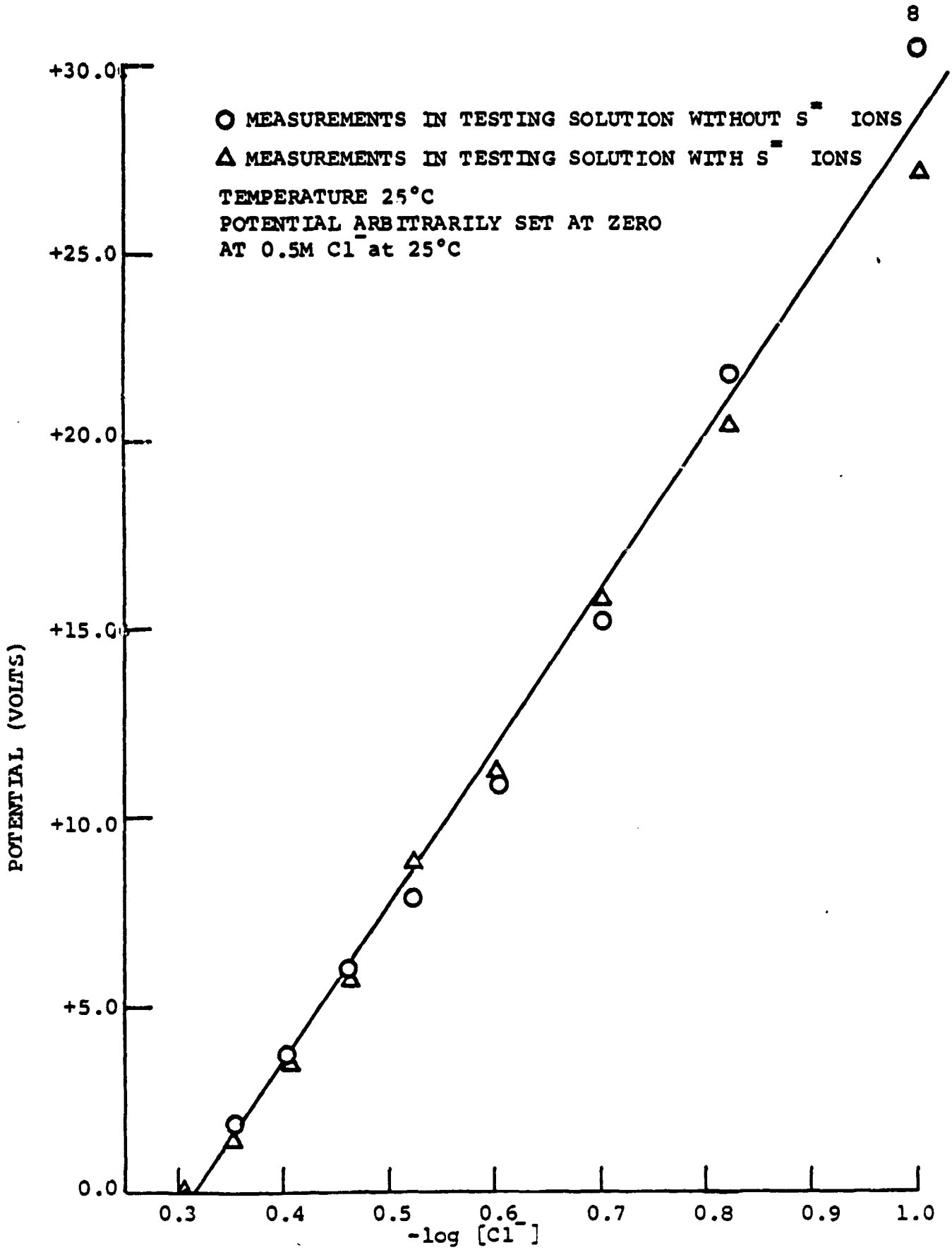


FIGURE 1: EXPERIMENTAL RESULTS OF THE ELECTRODE POTENTIAL MEASUREMENTS IN TESTING SOLUTIONS WITH AND WITHOUT SULFUR IONS.

The electrode with the membrane, normally, can last up to 20 days with repeated usage and showed no sign of deterioration in performance. The limitation of the operational life of the electrode can result from: (1) the deterioration of the liquid membrane matrix or (2) the deterioration of the Ag/AgCl electrode itself. In our studies, this limitation appears to be caused by the deterioration of the liquid membrane. The electrode required periodic calibration at an interval of no more than 72 hours. Hysteresis effects on the electrode were also investigated over the concentration and later the temperature, ranges, and the effects on the electrode in either case was negligible.

The thickness of the coating, as expected, affects the response time of the electrode. Response time for a three layer coating of the membrane matrix is 15-30 seconds while for an electrode with 5 coatings the response time increases to 90 seconds. The selectivity advantage of the thicker coating was observed and considered insignificant. Our results show that a three layer coating is adequate.

The temperature dependence of the electrode potential was evaluated over a temperature of 25-45°C. This temperature dependence was evaluated at three different chloride ion concentrations, i.e., 0.1, 0.3, and 0.5 M with sulfide ions of 0.01 M also added into each of the solutions. In all testing solutions, the temperature dependence of the electrode maintained its state of constancy. Average values of 0.46-0.60 mv/°K were obtained for all the electrodes tested. Again, each electrode required individual calibration. Hysteresis effects of temperature on the electrode

over the temperature range of 25-45°C were studied, and the effects were negligible.

Based upon these experimental results, a prototype sensor was then fabricated for evaluation. The prototype sensor had a physical appearance of a combination pH electrode. Both the liquid membrane coated Ag/AgCl and the reference Ag/AgCl electrode were housed inside a cylindrical tube with the working electrode exposed to the testing medium. Internal salt bridge was used to replace the salt bridge employed in our basic study. Several attempts in our own machine shop to fabricate this prototype failed. Precision Small parts of Bedford, Ohio was then contacted regarding the redesign and fabrication of the prototype of the chloride electrode. The overall length of the prototype is 3.8 cm (1.5 inches) and the overall diameter is 0.8 cm (5/16 inch). The lower part of the sensor has the appearance of saw-tooth which permits good flow pattern and protects the testing electrode simultaneously.

Preliminary experimental evaluations show the prototype to respond sluggishly to chloride ions. This is probably due to the use of Agar-gel and KCl solution salt bridge. We are now in the process of using only saturated KCl solution (without the Agar gel) as the salt bridge. This requires minor modification of the structure of the prototype.

Our future efforts will focus upon the experimental evaluation of this prototype sensor. The ranges for both chloride ions and sulfur ions will be extended to 1×10^{-5} M to 0.5 M. The long term applicability and durability of the sensor will also be investigated.

(2) Ammonium Ion Electrode

Our work in this research area involved a modification of the ammonium ion permeable membrane. Testing cells which were similar to dialysis cells were constructed and used for testing purposes.

One of the problems encountered was that the porosity of the membrane formed was too high. Also, the membrane exhibited a curling characteristic. A surfactant material was then added in forming the membrane which might eliminate this curling problem. In our study, we found stearic acid in the concentration level of 6 wt% appeared to be most suitable.

The evaluation of the membrane, as mentioned, employed testing cells similar to dialysis cells. Qualitatively, we used both pH electrode and pH paper to detect the NH_4^+ permeability through the membrane from one side of the half-cell to the other. On one side of the cell, NH_4OH solution in the concentration level up to 6 molar approximately 40 ml was placed in one of the half-cells. Distilled water was placed in another half-cell. The pH value of the distilled water side was monitored continuously by a Beckman pH electrode. Any pH change would be caused by the diffusion of the NH_4^+ ions. This was repeated except that NaOH was used instead of NH_4OH. Our results show that the membrane permits NH_4^+ to migrate through its wall easily as compared to Na^+ ions.

Quantitatively, the titration technique using EDTA (ethylendinitrilo) tetracetic acid suggested by Sadek and Reilly[2] was employed. Efforts were given in developing the calibration curve for quantifying the NH_4^+ ions selectivity of the membrane using this technique. The calibration curve was completed. Actual measurements on the selectivity of the NH_4^+ ions will have to be undertaken.

(3) Phenol Electrode

In this phase of research, the phenol oxidase assay technique was first experimentally verified. As suggested by the literature, L-tyrosine was used for the enzyme assay. The following series of reactions occur.

$$\text{Tyrosine} \xrightarrow[\text{phenol oxidase}]{O_2} \text{dihydroxyphenylalanine (DOPA)} \rightarrow \text{DOPA- quinone}$$

A Cary 14 double beam spectrophotometer was used to measure the change in absorbancy at 2800 Å versus time. A typical reaction employs:

L-tyrosine 0.00033 M

phenol oxidase in buffer 0.1 mg/0.2 ml buffer

buffer (phosphate) pH = 6.5, 0.17 M

Figure 2 shows the results of this assay. In this assay, one enzyme unit is defined as being equal to a change in absorbancy of 0.001 optical density/min at 25°C. In this work it is necessary to first determine the enzyme activity. The enzyme activity (interpreting the slope in Figure 2) is 30.7 eu (enzyme unit) which is ~ 50% of manufacturer suggested value.

Assay for (poly) phenol oxidase with phenol as substrate was then undertaken. Preliminary results show that a phenol concentration in the order of 10^{-5} M was detectable using this technique. The detection time required was about 8 minutes. A concentration level of 10^{-7} molar of phenol was detectable. This assay technique adheres to the following reactions:

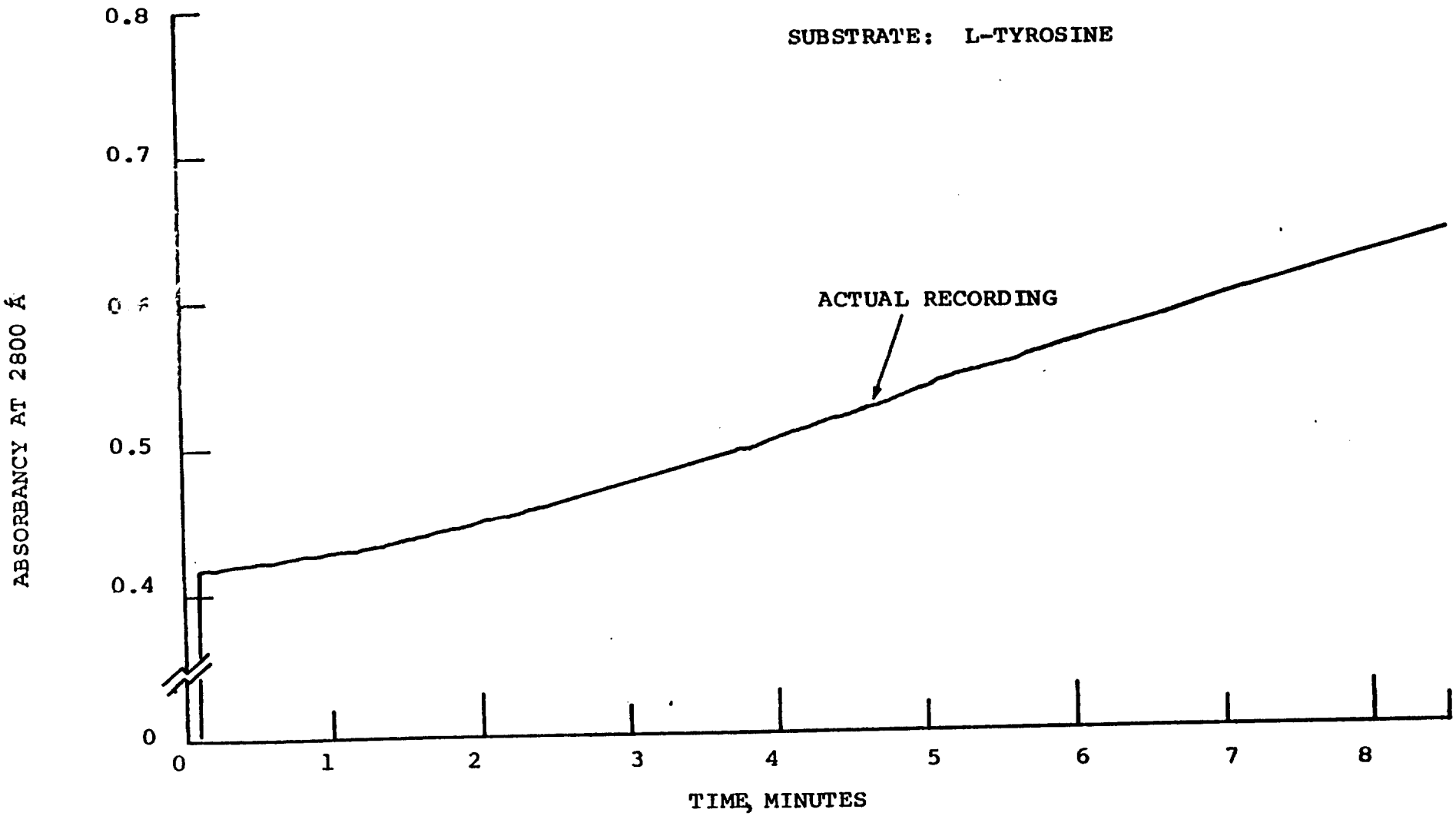
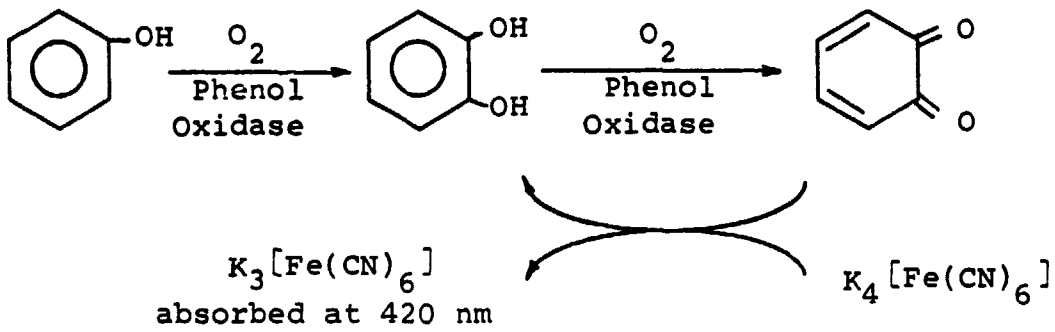


FIGURE 2: EXPERIMENTAL RESULT OF ASSAY FOR (POLY) PHENOL OXIDASE ACTIVITY



The stability of the enzyme, using the enzyme for actual environmental samples as well as for immobilization techniques were also investigated.

Environmental samples were used in this study to determine if these actual water samples would provide any interference to the phenol measurement. The three environmental samples used were: 1) Tap water, 2) River water (from Allegheny River, North Side), and 3) Reservoir water (Highland Park Reservoir). The procedure of this experiment was:

1. Assay the phenol in the water samples, and
2. Add a known amount of phenol, 10^{-4} M, in the water samples and then assay the phenol in the water samples.

Figure 3 shows the actual traces of the study. All assays were performed using phenol-ferrocyanide assay method, wherein the extent of oxidation of ferro-to ferrocyanide was monitored at 420 nm and used as an index of the extent of oxidation of phenol. All assays solution contain 0.02 M ferrocyanide in 0.25 M potassium phosphate buffer at pH 6.5.

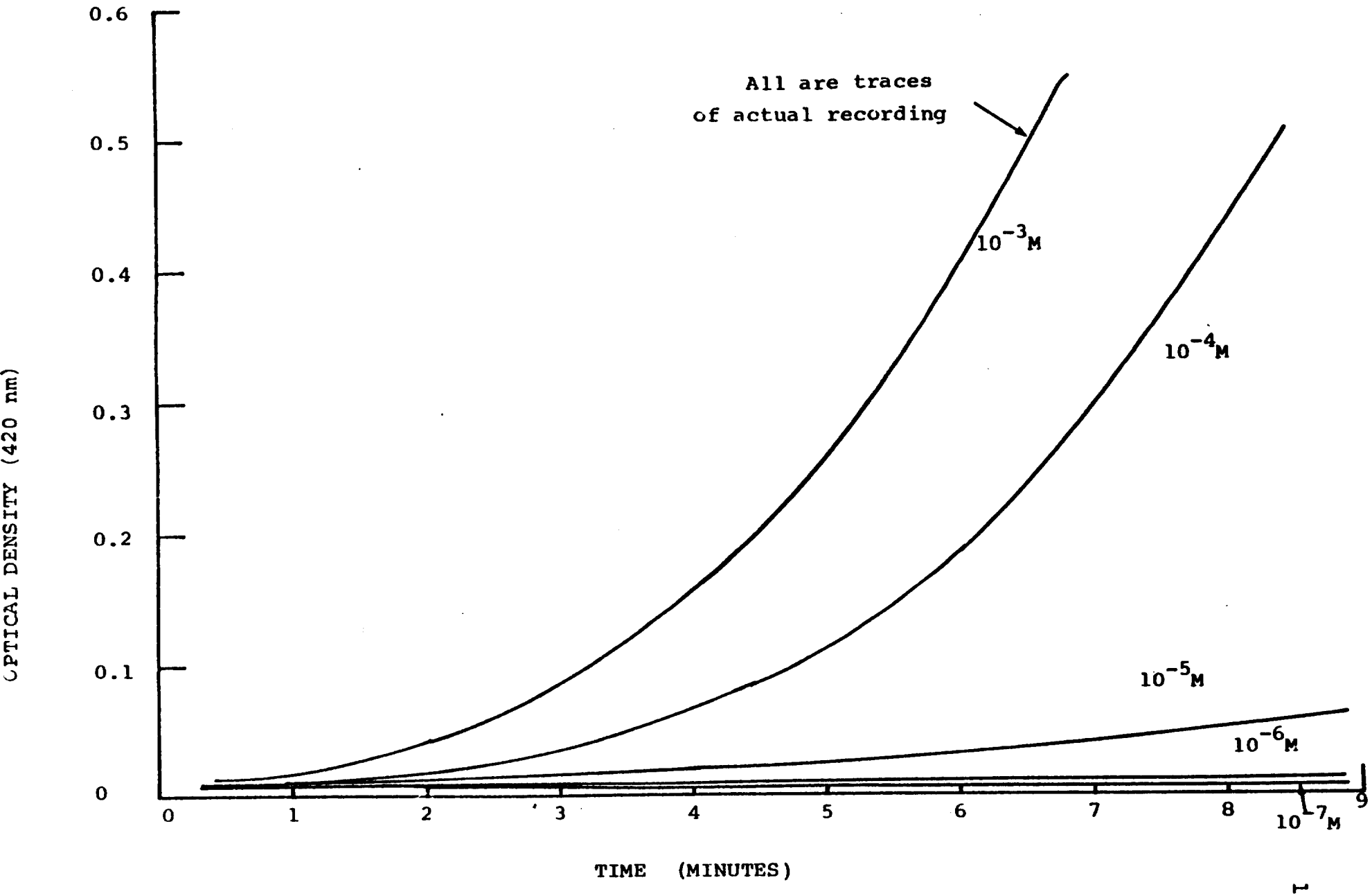


FIGURE 3 ASSAY OF PHENOL OXIDASE ACTIVITY WITH VARIOUS AMOUNTS OF PHENOL AT A CONSTANT ENZYME CONCENTRATION

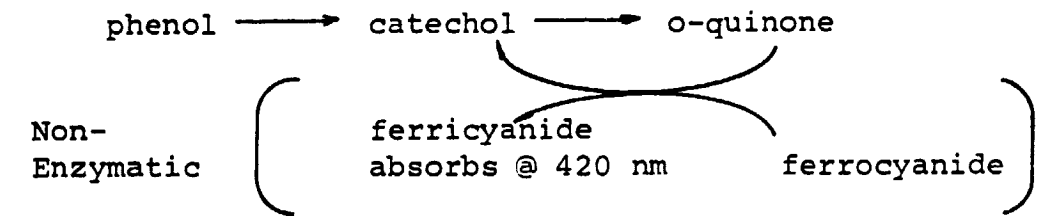
All assays utilize 100 µg of enzyme per 4.0 ml reaction mixture. The results indicate that none of the water samples contain detectable amounts of phenol. Furthermore, the contents of these samples do not affect the phenol assay technique used.

Efforts were devoted to immobilizing the enzyme, phenol oxidase, on a platinum matrix, and its long term catalytic activity was evaluated.

Phenol oxidase was immobilized in an acrylamide gel polymer essentially as described by Hicks and Updike.[3] The gel was formed in the presence of the enzyme and was cast around a platinum screen (1.50 x 1.50 x 0.11 cm). The screen served as a supporting matrix for the gel. The enzyme grids were stored in 0.15 M potassium phosphate buffer, pH 6.5 at 6°C when not in use.

The immobilized enzyme was assayed according to Sussman[4] by suspending the platinum grid in 10 ml of 0.25 M potassium phosphate buffer at pH 6.5 containing 10^{-3} M phenol and 0.02 M potassium ferrocyanine at room temperature. During the reaction the medium was constantly stirred and 100% oxygen was continuously bubbled through the medium.

The reaction sequence carried out by the enzyme was



The oxidation of ferro to ferricyanide was monitored spectrophotometrically at 420 nm.

The activity of the immobilized phenol oxidase was evaluated as follows:

(a) response of immobilized enzyme to different phenol concentrations

(b) activity of enzyme grid containing different amounts of enzyme

(c) ability to reuse the same enzyme

(i) single assays with nine day intervals between assays

(ii) four consecutive assays on the same enzyme grid on the same day with phenol concentration of 10^{-3} M

(iii) same as in (ii) with phenol concentration of 10^{-5} M

(d) activity of unused enzyme grids after storage

(e) the free enzyme in solution was tested for activity over a period of 19 days.

The preparation and storage of the enzyme grid has been described above. The assay methods for the enzyme grid and free enzyme will be described as follows:

(a) Enzyme Grids

Assays were performed at 22°C by suspending the enzyme grid in 10 ml of 0.25 M potassium phosphate buffer, pH 6.5 containing 0.02 M potassium ferricyanide and the appropriate concentration of phenol. The reaction medium was magnetically stirred and oxygenated (40 cc of 100% O_2/minute) for ten minutes prior to and during the entire course of the assay. At 5 minute intervals after immersion of the grid, 1.0 ml aliquots were removed from the incubation medium and their absorbancy at 4200 Å determined in a 1.00 cm cell in a Cary Model 14 dual beam spectrophotometer. The sample was returned to the reaction mixture after the assay (elapsed

time of 20-30 seconds).

(b) Free Enzyme in Solution

The enzyme in solution (1.4 mg/ml) was assayed as described above using 0.011 M L-tyrosine as substrate. Absorbancy readings at 2800 Å were taken at two minute intervals.

The results of this investigation can be described as follows.

(a) Response of Immobilized Enzyme to Different Concentrations of Phenol

Figure 4 shows the response of three identical enzyme grids to different concentrations of phenol (10^{-3}, 10^{-4}, and 10^{-5} M) in the incubation medium.

(b) Activity of Enzyme Grids Containing Different Amounts of Enzyme

Figure 5 shows the response of three enzyme grids, each with different amounts of enzyme but otherwise identical, to the same concentration of phenol (10^{-3} M).

(c) Ability to Reuse Same Enzyme Grid

(i) Figure 6 shows results of assays on a single enzyme grid. Assays were performed the day after the grid was constructed and at 9 day intervals thereafter using phenol at 10^{-3} M.

(ii) Figure 7 shows results of four consecutive assays performed on the same enzyme grid using phenol at 10^{-3} M. Grids were washed for 15 minutes between assays as described previously.

(iii) Figure 8 shows results of four consecutive assays performed on the same enzyme grid using phenol at 10^{-5} M. Grids were washed for 15 minutes between assays as described above.

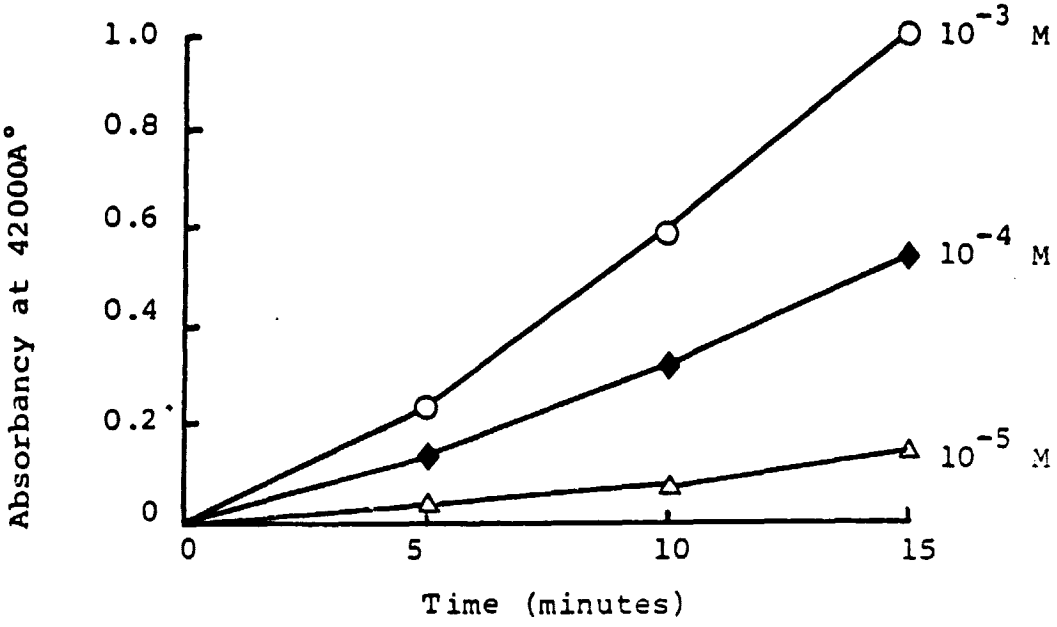


Figure 4: Experimental Results of Various Substrate Concentrations

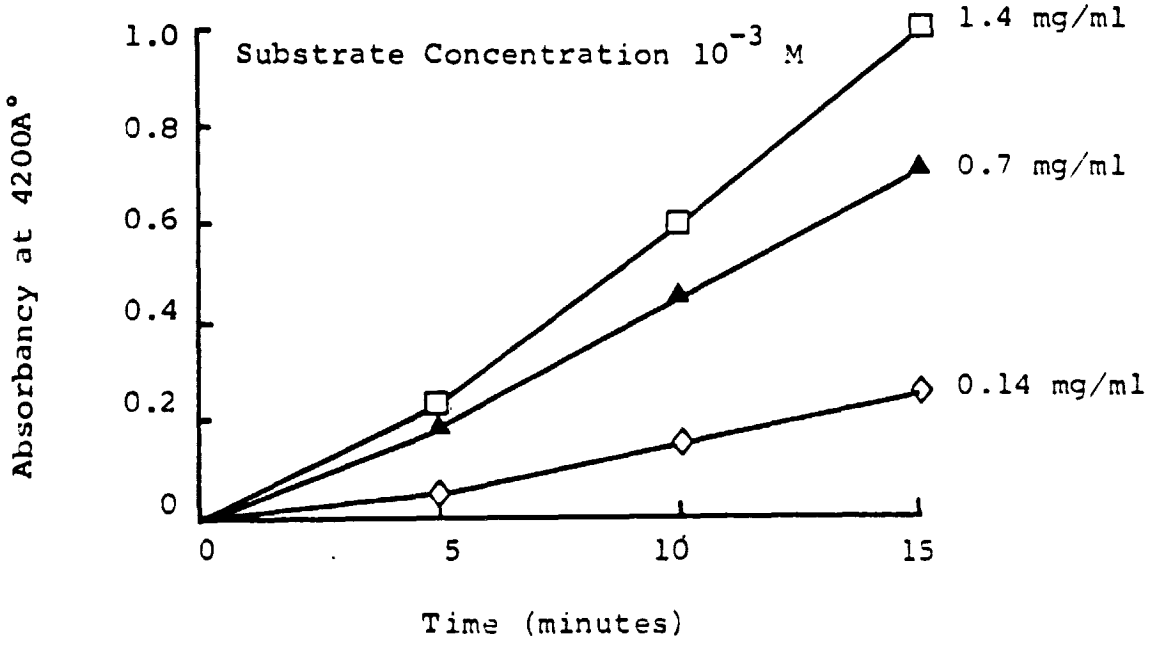


Figure 5: Experimental Results of Various Enzyme Loadings in the Grids

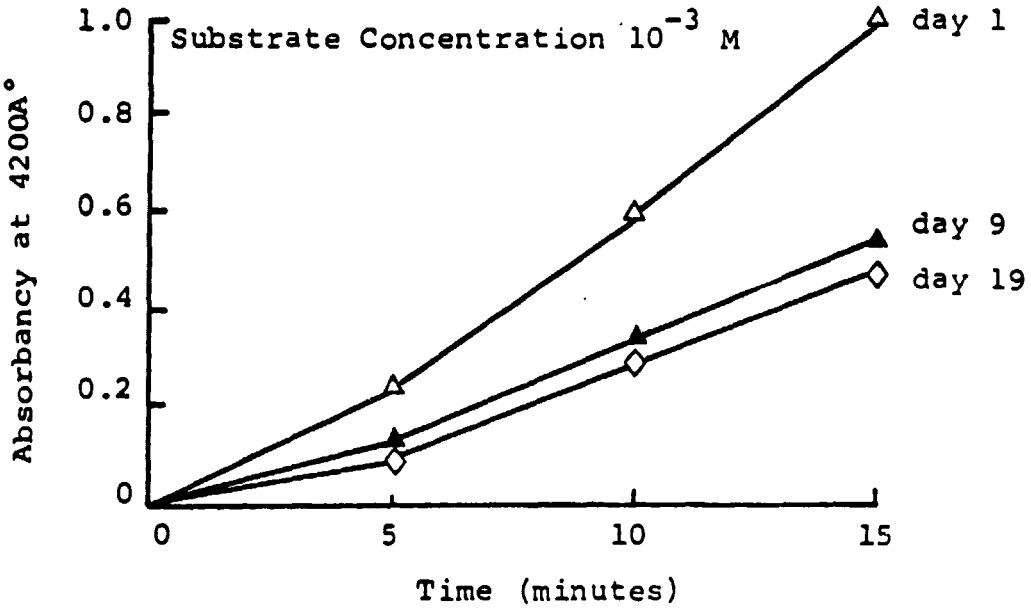


Figure 6: The Re-Use Ability of a Single Enzyme Grid Over a 19-Day Period of Time

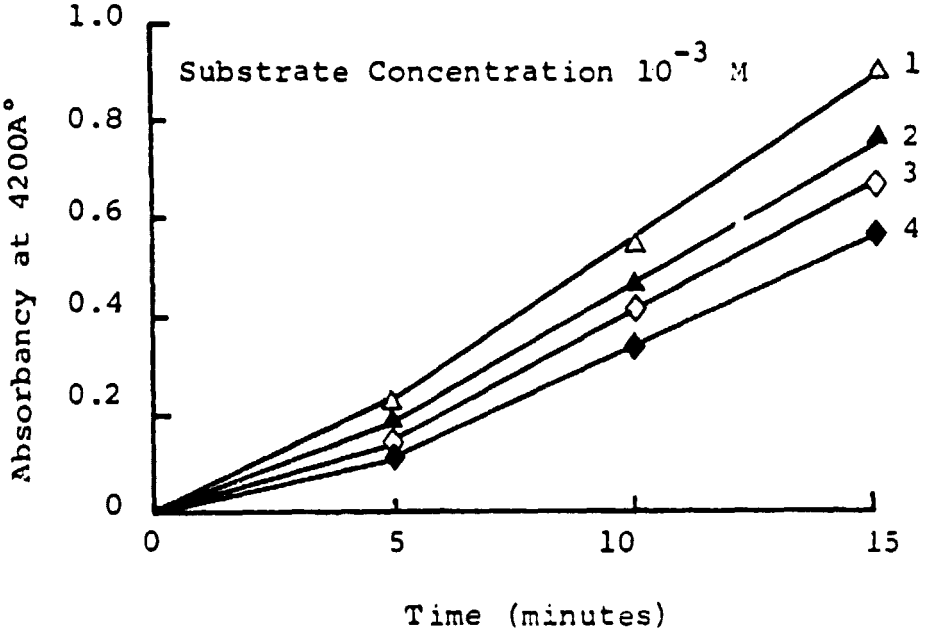


Figure 7: Consecutive Re-Use Ability of A Single Enzyme Grid Between 20-Minute Intervals

(iv) Figure 9 shows results of assays on three identical enzyme grids that were stored for different periods of time. Phenol concentration was 10^{-3} M.

(v) The free enzyme in solution (1.4 mg/ml of enzyme in 0.25 M potassium phosphate buffer at pH 6.5) was tested for activity over a period of 19 days. Figure 10 shows the percent of initial activity remaining after various time intervals. The concentration of enzyme in solution was chosen to be identical to its concentration in the enzyme grids.

The results of this phase of research indicate that phenol oxidase can be reproducibly trapped in the acrylamide gels. The enzyme is stable in the immobilized form as well as in free form over a period of 19 days. However, its stability at ambient temperature was not quantitatively determined.

Thus, in another study, phenol oxidase was immobilized in polyacrylamide gels as previously described. Five enzyme grids were employed in this study. One grid was assayed in the usual way on the first day. Two enzyme grids were then stored at 22°C in 0.25 M potassium phosphate buffer containing 0.01% sodium azide to inhibit growth of microorganisms. The remaining grids were stored in the absence of sodium azide. The enzyme grids were then assayed after five and thirteen days of storage at 22°C.

The results indicate preservation of 43% of the initial enzyme activity after thirteen days of storage at 22°C compared to 70% retention of activity when the grids are stored at 6°C.

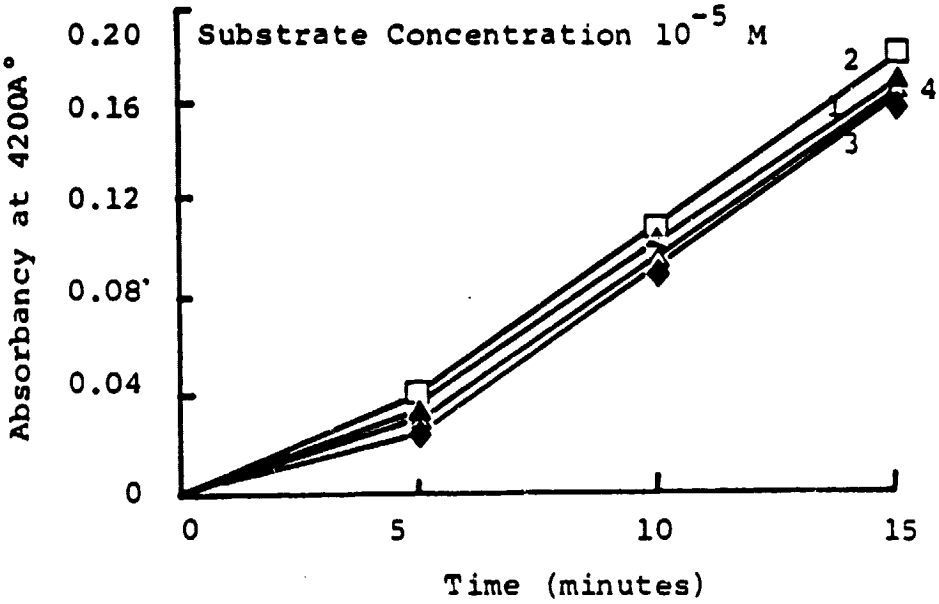


Figure 8: Consecutive Re-Use Ability of a Single Enzyme Grid Between 20-Minute Intervals at a Lower Substrate Concentration

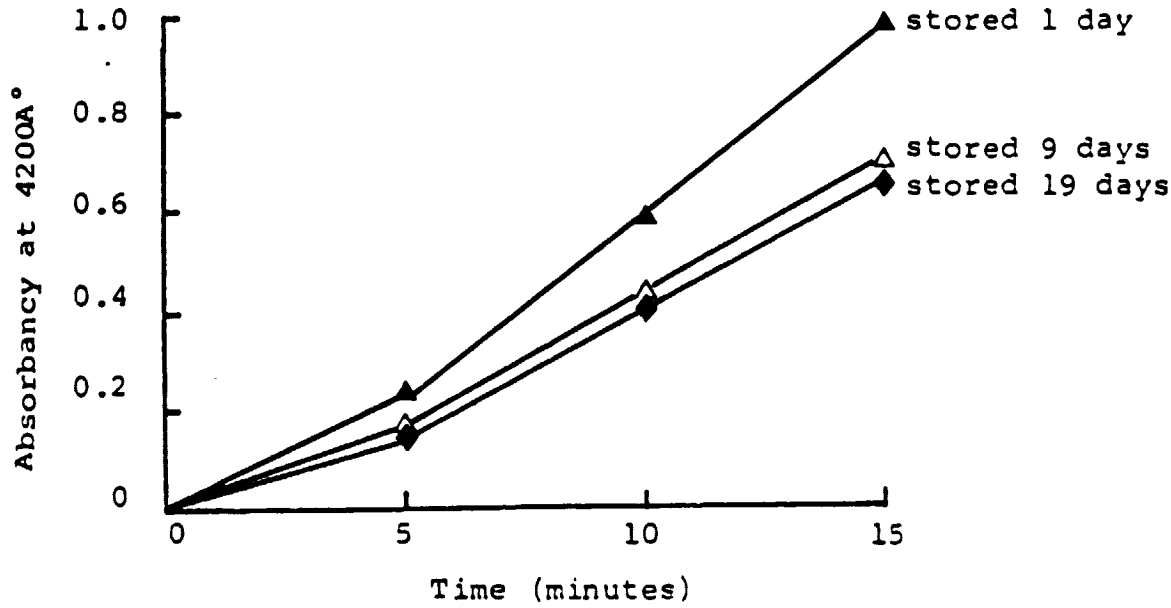


Figure 9: Stabilities of the Enzyme Grids Stored Over Different Lengths of Time

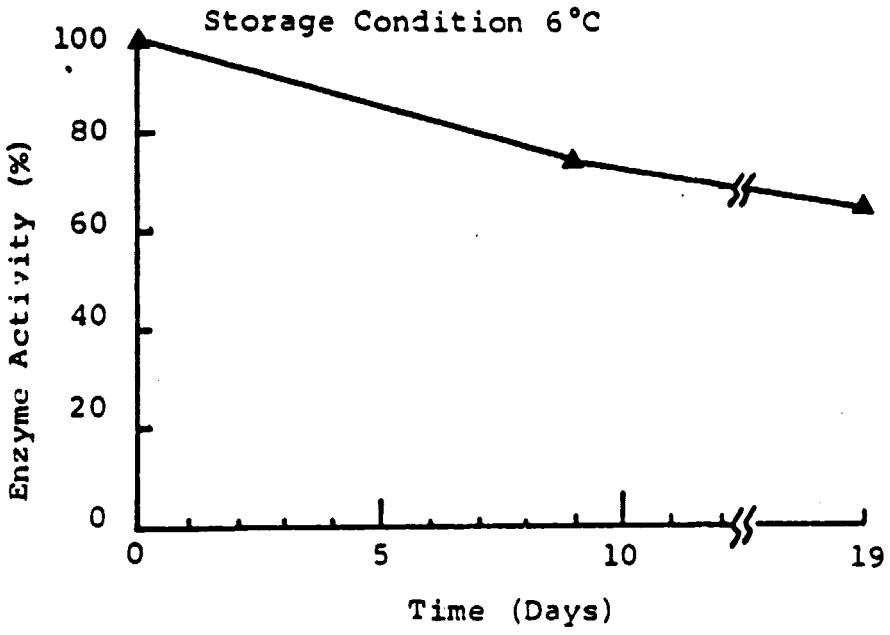


Figure 10: Change in Enzyme Activity Over a 19 Day Period of Time

The buffer solution containing the enzyme grid stored without sodium azide showed definite signs of microbial growth after thirteen days at 22°C. This could account for the lower enzyme activity found in this preparation after thirteen days.

In summary, the enzyme activity does decrease while stored at 22°C comparing to that at 6°C. However, it maintains sufficient enzyme activity to carry out the enzymatic reaction. Thus, if a sufficient amount of enzyme is immobilized initially then the electrode will function and last for a reasonably long period of time.

Based upon the results of the above investigation, the design of the electrochemical cell for measurement of phenol concentrations was then undertaken.

During the course of the enzymic oxidation of phenol to o-quinone by phenolase, electrons are generated according to the following postulated mechanism:

Anode: (1) $C_6H_6O + 1/2\ O_2 \longrightarrow C_6H_6O_2$
(phenol) (1,2 dihydroxyl benzene)

(2) $C_6H_6O_2 \longrightarrow C_6H_4O_2 + 2H^+ + 2e^-$
(o-quinone)

Employing the above reactions as an anode we propose to couple this half cell to an oxygen electrode that would act as cathode and the cathodic reaction is

(3) $1/2\ O_2 + 2e^- \longrightarrow O^=$

Summing the half cell reactions (1), (2) and (3):

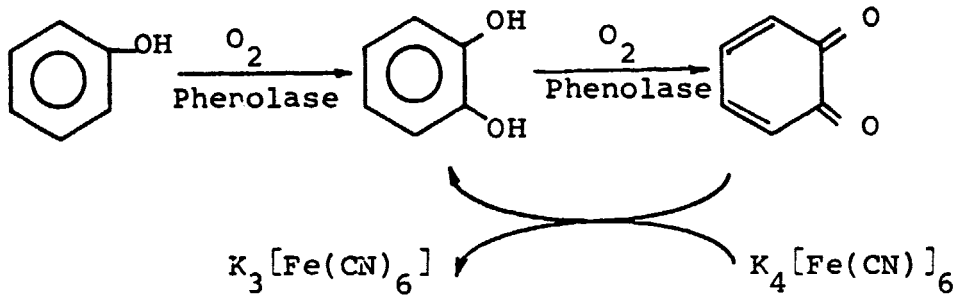
Overall: $C_6H_6O + O_2 \longrightarrow C_6H_4O_2 + \underbrace{2H^+ + O^=}_{H_2O}$

At thermodynamic equilibrium, the electrochemical potential generated by this cell can be used as a measurement of the quantity of phenol being oxidized. Our preliminary measurements show this postulation is qualitatively corrected.

Capitalizing on the technology of H_2-O_2 fuel cell, one realizes that platinum and palladium are the best oxygen reduction catalysts in the low temperature range. In addition, electrodes of a porous matrix provide good diffusional characteristics. Thus, a gold mesh screen loaded with platinum black particles was used as the cathodic material. Oxygen gas was bubbled in the half-cell throughout as the reactant for the cathodic reaction. The anodic half-cell also contains a continuous supply of oxygen as well as 40 ml of 0.25 M potassium phosphate buffer, pH 6.5. The anode is a piece of basket weave platinum screening (1.5 x 1.5 cm), that is coated with a polyacrylamide gel containing 140 enzyme units of phenol oxidase.

The lead wires of both the anode and the cathode are connected to a potentiometer or a (voltage) strip-chart recorder. In a typical run, the system was first equilibrated, in the absence of phenol and an open-circuit potential was developed in the order of ~ 100 mv. This potential resulted from the difference between the anode (the enzyme electrode) and the cathode (the gold/platinum black electrode). When a steady baseline potential is obtained, phenol in a predetermined amount was added into the anodic half-cell. A change in the open-circuit potential was established, and the steady state was achieved with 5-10 minutes of operation. This system was operated in the presence or absence of 0.02 M $K_4Fe(CN)_6$ in the anodic half-cell. $K_4Fe(CN)_6$ serves as a

coupled reacting agent, in which Fe^{+2} is oxidized to Fe^{+3}. This agrees with the overall general assay reaction mechanisms as described earlier



This coupled reaction facilitates the phenol oxidation reaction, but should not alter the electron-transfer in the anodic reaction. Figure 11 illustrates the experimental steady state potentials versus molarity of phenol. As expected, the use of $K_4Fe(CN)_6$ in the anodic half-cell enhances the potentiometric response in absolute value of the potential output. However, the slope of the system with and without $K_4Fe(CN)_6$ remains unchanged. The response of $\Delta mv/\Delta decade$ of phenol is ~ 25 mv at 22°C. This corresponds very well with the prediction of the Nernst equation for a two-electron transferred mechanism as described above.

Control experiments, wherein a gel coated platinum grid containing <u>no</u> enzyme was employed, indicated no change in potential upon the addition of phenol to the anodic half-cell. This observation clearly indicated that, in the absence of the enzyme, the enzymic oxidation reaction does not take place. Quantitatively, we hoped to increase the sensitivity of our measurement of phenol concentrations utilizing our immobilized enzyme-electrochemical method of detection. The major experimental difficulty we attempted

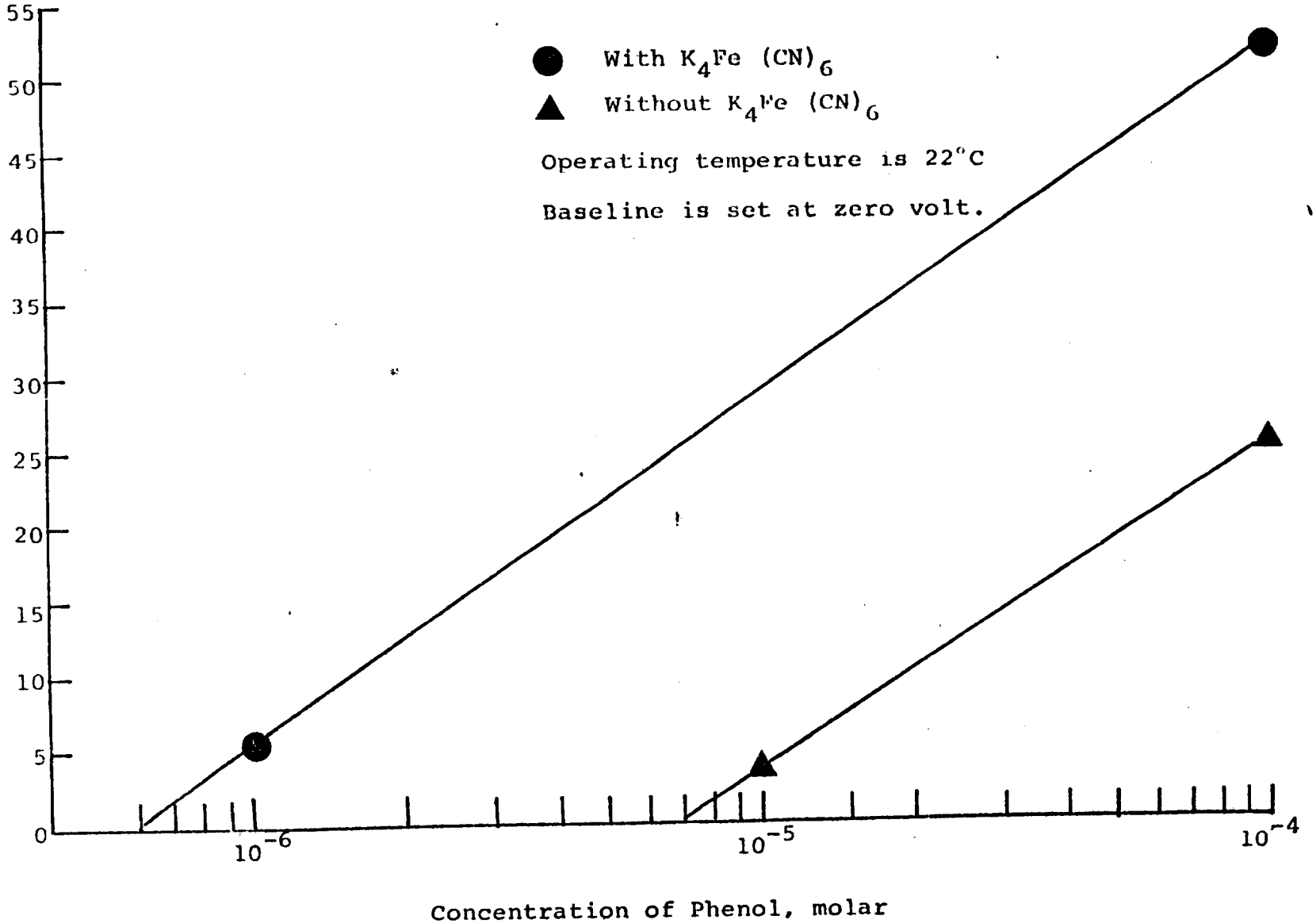


FIGURE 11: Relation Between the Measured Open-Circuit Potential and The Phenol Concentration

to overcome was the higher sensitivities required at the lower phenol concentrations. We realized: 1) the measuring equipment and 2) the bubbling of oxygen both contributed to the noise level in the measuring signal output. Deflector for the bubbling mechanism was used in order to minimize the bubbling effect of the oxygen on the signal.

Thus far, we have carefully evaluated the open circuit potential obtained with our immobilized enzyme electrode both in the presence and absence of $K_4Fe(CN)_6$ in the electrode chambers. Figures 12 and 13 show the results of these studies. In Figure 12, the open circuit potentials that were obtained five minutes after the introduction of phenol to the anode compartment are shown. Each experimental point was obtained with a fresh enzyme grid and with fresh 0.25 M potassium phosphate buffer (pH 6.5) plus 0.02 M $K_4Fe(CN)_6$ in the electrode compartments. Each compartment was oxygenated for seven minutes (75 cc O_2/min) prior to the introduction of phenol. Two experimental trials are shown here, while the second trial was performed a week later wherein all experimental conditions were faithfully reproduced.

The results of our experiments carried out in the absence of $K_4Fe(CN)_6$ in the electrode compartments are reproducible but scattering. Also, the range of the phenol detection is more limited in the absence of $K_4Fe(CN)_6$ compared to in the presence of $K_4Fe(CN)_6$. We intend to examine the reasons which may account for the reproducibility problems in the absence of $K_4Fe(CN)_6$. However, based on these results, we will proceed with our experimental plans with emphasis on the ones in the presence of $K_4Fe(CN)_6$. We feel the presence of $K_4Fe(CN)_6$ will not hinder our overall phenol

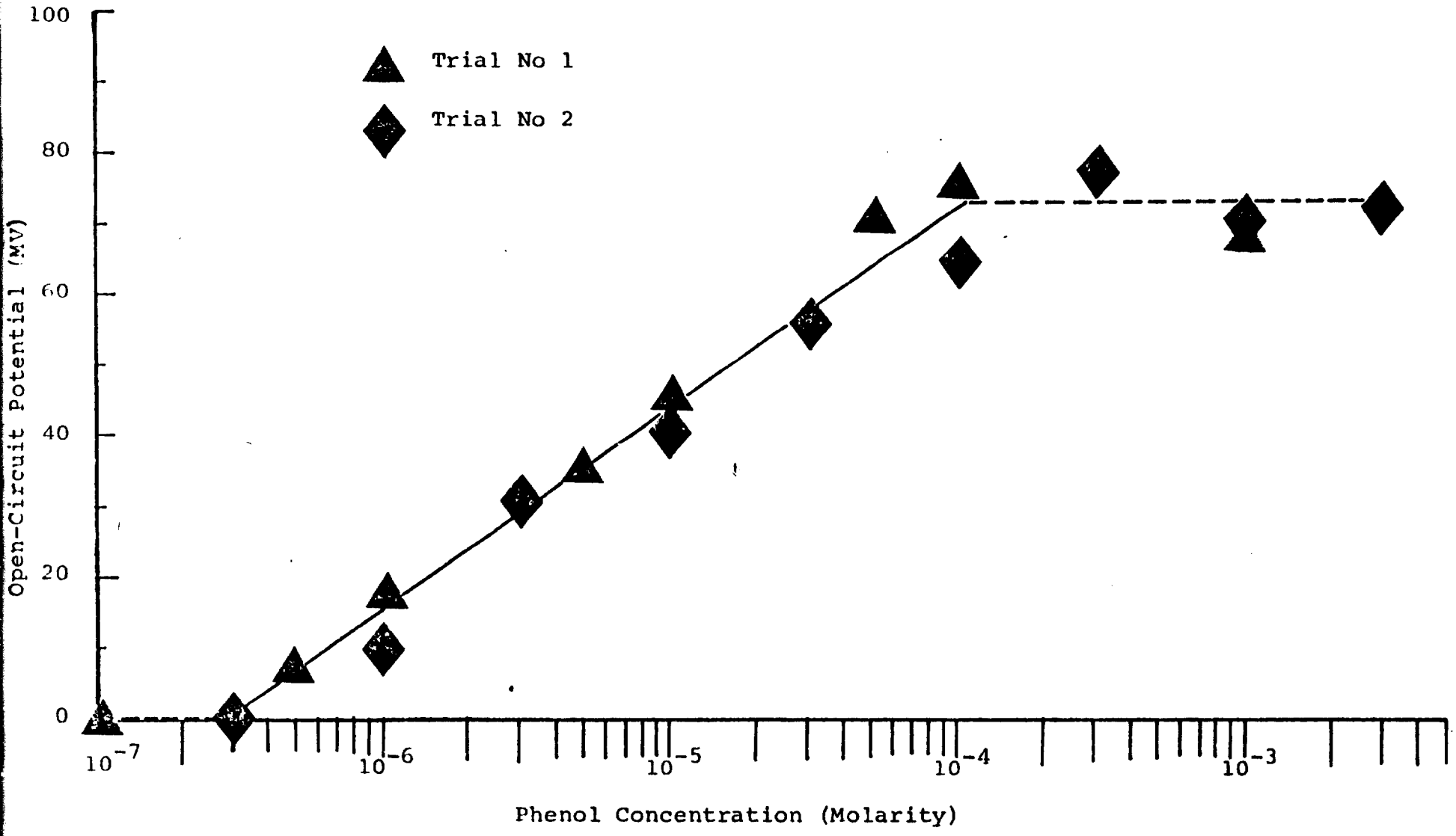


Figure 12: Open Circuit Potential of the Electrochemical Cell for Phenol Measurement In the Presence of $K_4Fe(CN)_6$

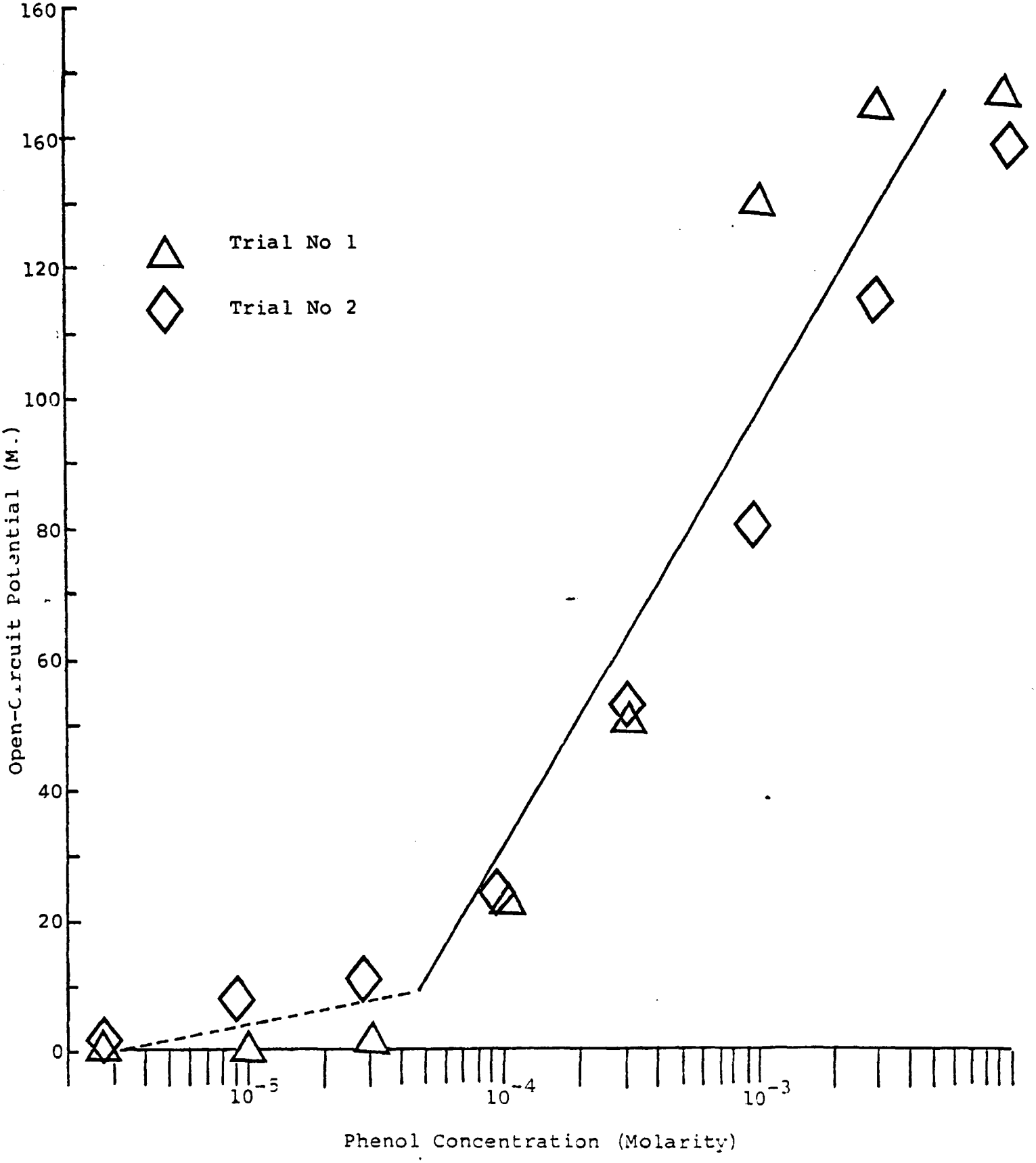


Figure 13: Open Circuit Potential of the Electrochemical Cell for Phenol Measurement in the Absence of $K_4Fe(CN)_6$

sensor development. The administration of the minute amount of $K_4Fe(CN)_6$ can be provided periodically in a flow-through sensor chamber.

Evaluation of the oxygen level on the electrochemical measurement of the phenol concentration are now being undertaken. We intend to continue this study in order to quantify the influence of oxygen concentration on this overall reaction.

(4) Nitrite Electrode

The essential literature research was first completed. The limitation and the range of detection using our proposed enzymatic means was then determined.

The enzyme assay for nitrate reductase was established. The conversion of NO_3^- to NO_2^- was detected as described by Lowe and Evans.[3] The enzymatic reaction was carried out in the presence of methyl viologen and sodium dithionite. The NO_2^- was then diazotized in the presence of sulfanilamide and N-(1-napthyl) ethylene diamine HCl. The reaction product was then quantified spectrophotometrically.

Figure 14 shows the linear relationship between the NO_2^- produced and the number of units of the enzyme employed. This result indicates that nitrite concentration in the order of 0.6 ppM is detectable.

The stability of the enzymes and detecting the nitrate level as well as the required detecting time were all investigated. Figure 15 shows a time course for the conversion of nitrate to nitrite was complete. In the study of the stability, nitrate reductase maintained much of its activity over 28 days when refrigerated at 4°C.

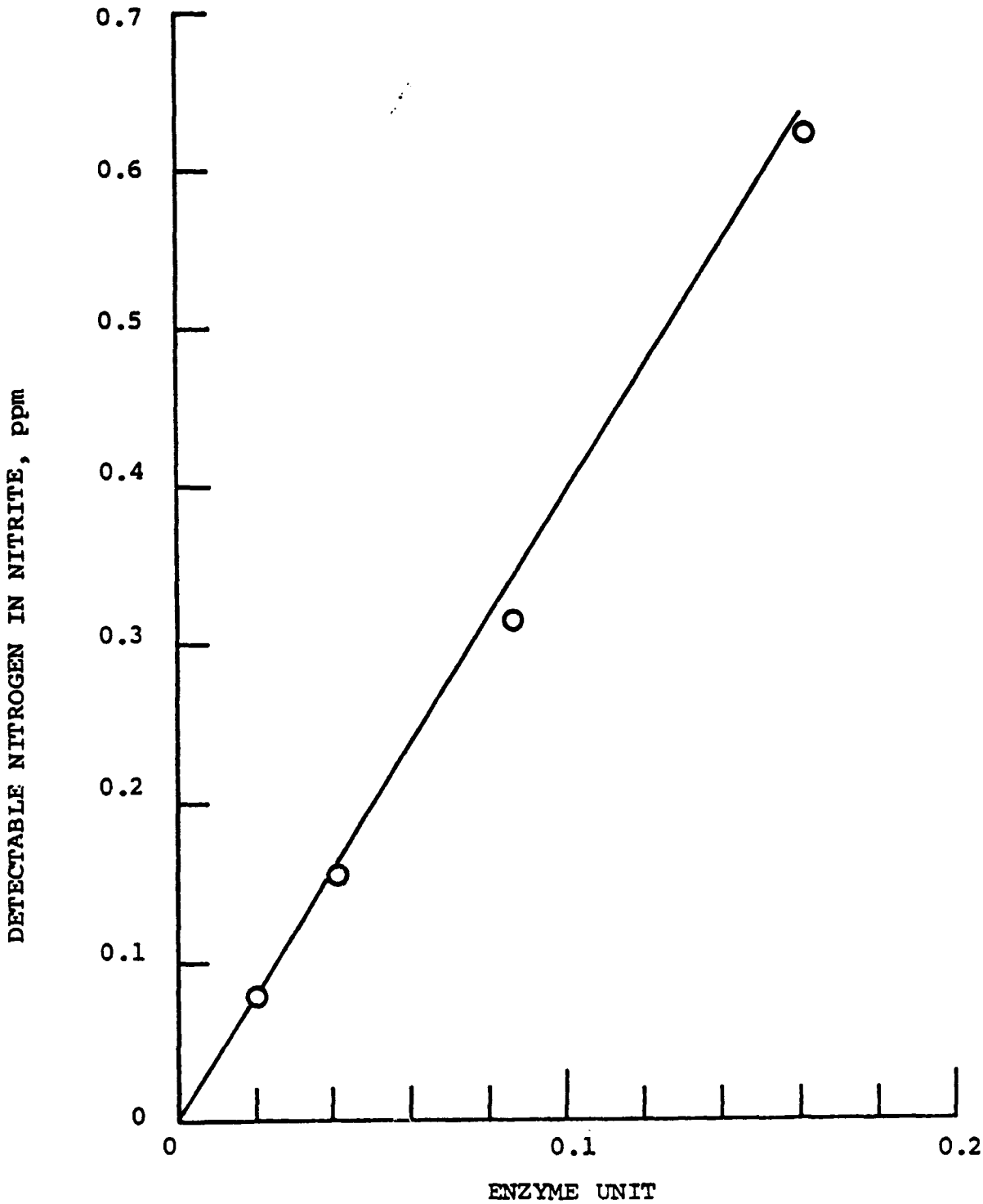


FIGURE 14: RELATIONSHIP BETWEEN NITRATE REDUCTASE AND AMOUNT OF NITRATE CONVERTED TO NITRITE IN 10 MINUTES.

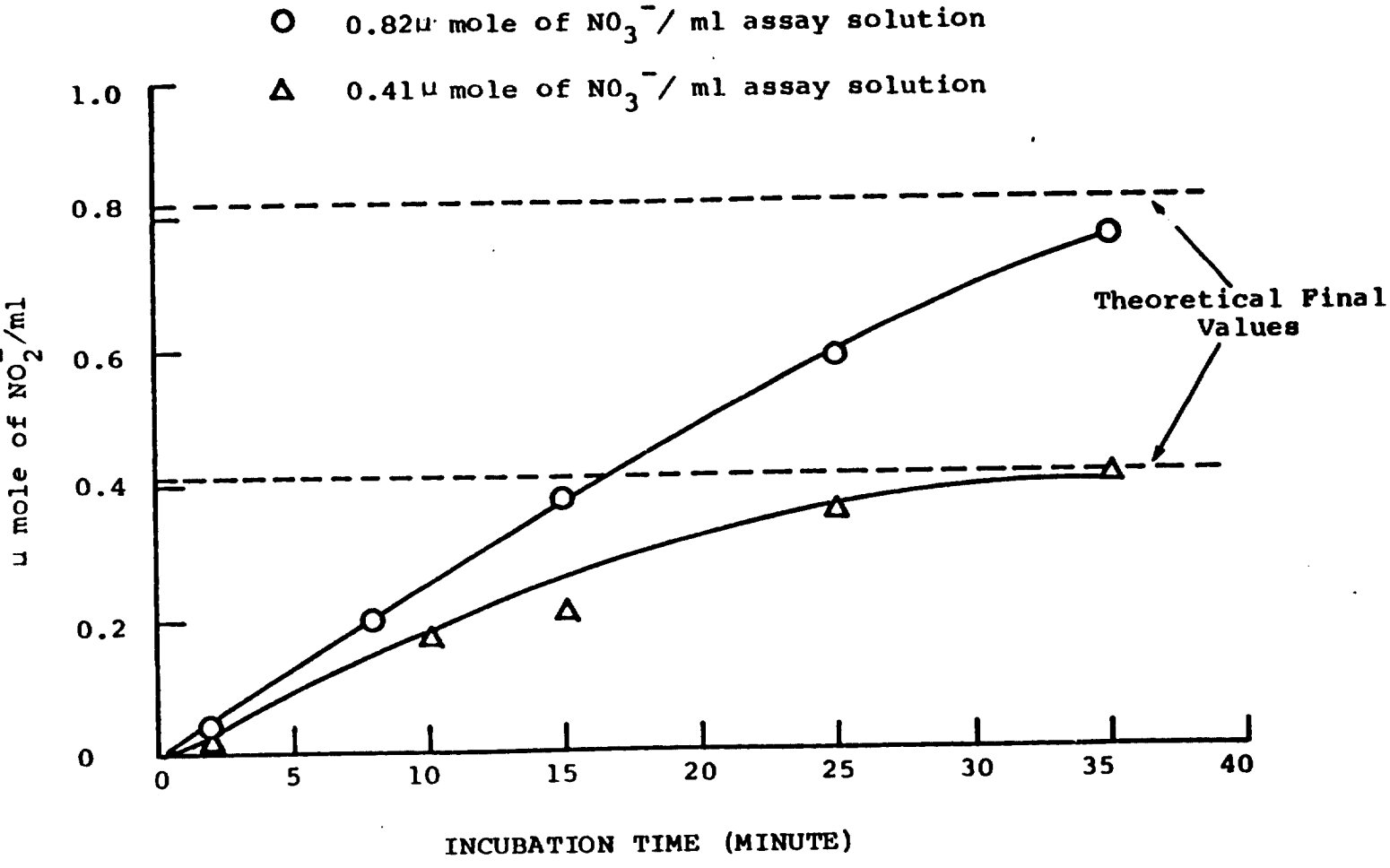


FIGURE 15: TIME COURSE FOR STOICHIOMETRIC CONVERSION OF $NO_3^- \longrightarrow NO_2^-$ BY NITRATE REDUCTASE

Environmental samples identical to those used in phenol electrodes were also used in this study. All assays were performed in the usual manner. Environmental samples were added to constitute 20% (v/v) of solution to be tested for NO_2^- or NO_3^-. The experimental results are summarized as follows.

A. Test of Environmental Samples for Pressure of NO_2^-

	nano moles NO_2^- per ml test solution
Reference (distilled H_2O)	0.0
River Water	10.8
Reservoir Water	9.6
Tap Water	9.7

B. Test of Samples for Presence of NO_3^-

	nano moles NO_3^- per ml test solution
Reference (distilled water)	0.0
River Water	52.0
Reservoir Water	38.4
Tap Water	24.3

C. Test of Samples for Interference with Usual Assay

	nano moles NO_3^- per ml test solution
Reference (known amount NO_3^-)	858
River Water	763
Reservoir Water	791
Tap Water	830

According to Test C, the NO_3^- amount in Test B should be cumulative to the known amount NO_3^- (858 nano moles). Howwver, there is a 10-15% decrease in the total amount of NO_3^-

assayed. This may be due to: 1) experimental errors and 2) the samples may contain slightly inhibited substance(s) that are inhibitory to the enzyme assay.

Similar to phenol electrodes, efforts were devoted to immobilizing nitrate reductase on a platinum matrix. Nitrate reductase was immobilized in polyacrylamide gel essentially as described above for (poly) phenol oxidase. The immobilized enzyme was assayed for activity by incubation of the enzyme grid in a solution of 0.1 M potassium phosphate buffer, pH 7.0 containing the usual quantities of methyl viologen and sodium dithionite. The beaker containing the enzyme grid was maintained at 30° in a water bath. At proper time intervals, samples were removed from the incubation medium and assayed for the presence of nitrite by diazotization with sulfanilamide and N-(1-naphtyl) ethylenediamine hydrochloride.

The stability of nitrate reductase was also studied both in solution and in the immobilized form (i.e. trapped in 7% acrylamide gels). Free enzyme in the amount of 0.17 enzyme units was stored in 1.0 ml of buffer solution (0.15 M potassium phosphate, pH 7.0) for 18 days either at + 6°C or - 10°C. The similar study was carried out with the immobilized enzyme. In these studies, 0.035 enzyme units were entrapped in a 7% acrylamide gel (250 mg) that was cast around a 1.5 x 1.5 cm basket weaved platinum screen. The enzyme grids were stored in 0.15 M potassium phosphate buffer pH 7.0 at either + 6°C or + 23°C for up to 9 days. The results of these storage studies show that the enzyme is more stable at 6°C in the free form as compared to the immobilized form. Furthermore, the enzyme is more stable when maintained at 6°C in the immobilized form than at 23°C.

The performance characteristics of immobilized nitrate reductase were also studied in relation to the ability to reuse the immobilized enzyme. The results of this study indicate that although the immobilized enzyme may be reused, a loss in activity occurs which is probably due to the assay procedure.

Nitrate reductase entrapped in 7% acrylamide gels showed only 10% of the predicted enzyme activity. In addition, the entrapped enzyme lost activity at a relatively fast rate at both + 6°C and + 23°C.

The experiments performed with the immobilized enzyme employed low amounts of enzyme which required a more sophisticated spectrophotometer in order to measure differences between successively small absorbance changes (e.g. differences on the order of 0.005 to 0.03 OD units). Use of 10 fold greater amounts of enzyme would be very expensive (i.e. 0.5 enzyme units cost $20.00).

We have completed a detailed evaluation of the nitrate reductase as a mean in developing for a nitrite electrode. While the experimental results show scientific merits of this approach, the high cost of the enzyme makes this approach impractical. Thus, we intend to devote no further effort on this development.

(5) ATP Electrode

The development of an ATP electrode was based upon the enzymatic reaction of adenosine phosphatase. A commercial photometer - Chem-Glo manufactured by Aminco was used for standard calibration. Figure 16 shows the calibration curve of the assay. Calibration was performed in the ATP concentration range of 20-100 nano molar. Detection at the

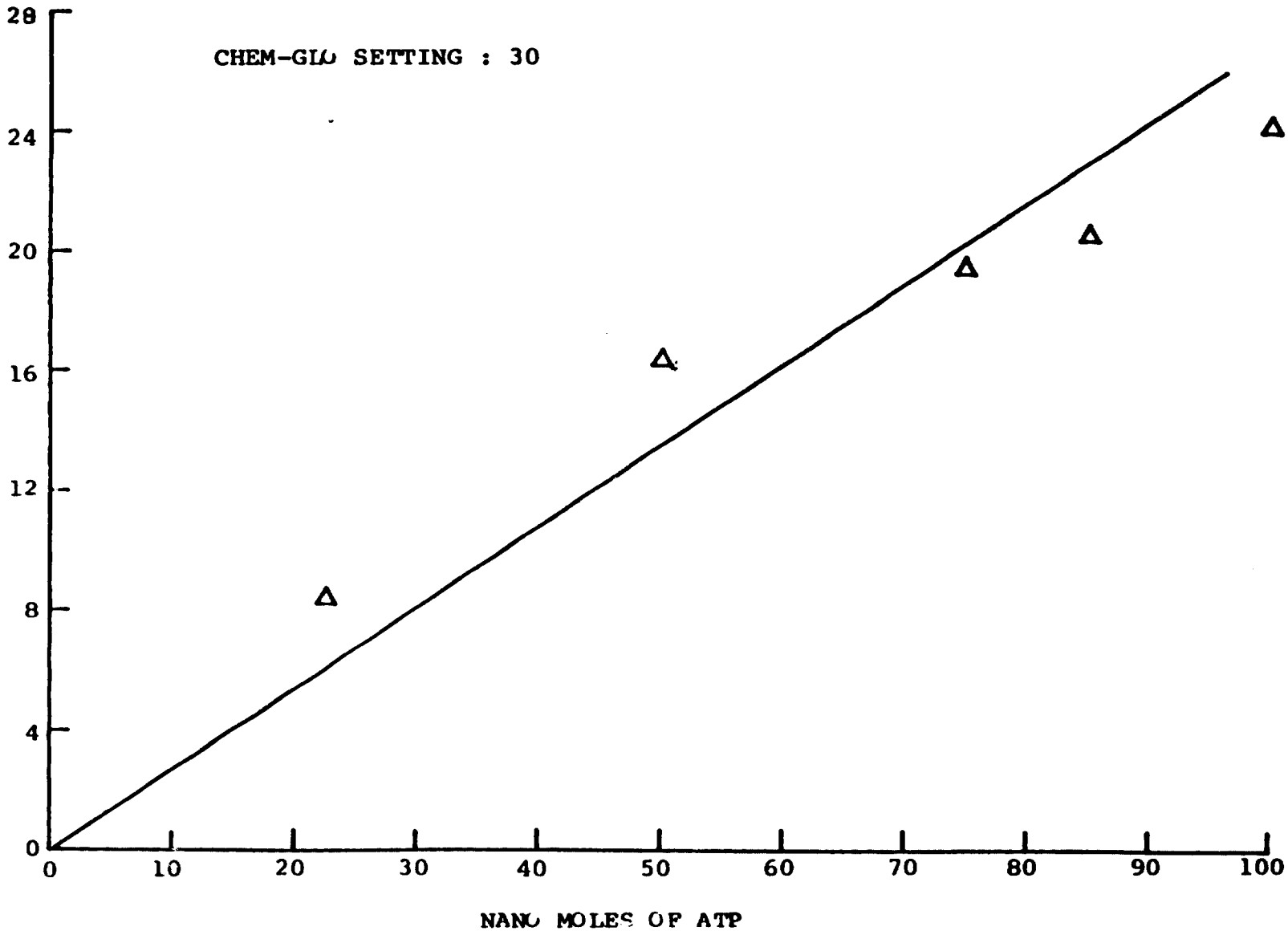


FIGURE 16: CALIBRATION CURVE OF ATP DETECTION PHOTOMETER

levels of 0-20 nano molars ATP was attempted because this level was more realistic in actual environmental conditions. The stability of the enzyme was also investigated.

The environmental samples used in the phenol and nitrite studies were used here. The assay solution contains:

0.500 ml	diluted firefly extract
0.24 ml	ATP (various concentration) solution in 0.04 M Tris/Borate Buffer
0.002 ml	environmental sample

Figure 17 shows the experimental results. All the samples are in triplicate and the references are in duplicate. The readings are quite scattered.

The minute amount sample used was suspected to be the source of the poor reproducibility of the measurements. The additional limited measurements did not yield conclusive results.

In summary, the limitation in the detection level and the poor reproducibility of this technique discouraged us in further pursuit of this work. Research efforts will be diverted to more profitable investigation, such as the phenol sensor.

SUMMARY

The research on *in situ* water quality monitoring has been undertaken. Experimental results obtained are promising and useful with respect to their scientific merits. For practical reasonings, chloride ion electrodes and phenol electrodes are attractive. We hope to continue our research efforts in these developments as well as to explore monitoring techniques for other water quality parameters. The results obtained in this research will be helpful in pursuing future research in this area.

REFERENCES

(1) James, M., Carmack, G. and Freiser, H., Analytical Chemistry 44, 856 (1972).

(2) Sadek, F.S. and Reilley, C.N., Analytical Chemistry 31, 494 (1959).

(3) Hicks, G.P. and Updike, S.J., Analytical Chemistry 38, 726 (1966).

(4) Sussman, A.S., Arch. Biochem. Biophys. 95, 407 (1961).

(5) Lowe, R.H. and Evans, H.J., Biochem. Biophys. Acta 85, 377 (1964).