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A PROTEIN COATED PIEZOELECTRIC CRYSTAL DETECTOR

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Aberdeen Proving Ground, Maryland 21010-5423

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A research study was to be conducted to develop a piezoelectric crystal monitor for chemical agents using immobilized enzymes (cholinesterases) and an organophosphorus antibody as coatings. A monitor was to be designed that is small, simple to use, of low cost, and based on analytical methodology of suitable accuracy and sensitivity.

The initial evaluation in Phase 1 was to be performed using an organophosphorus chemical warfare simulant (diisopropyl methyl phosphonate) to ascertain the feasibility of the concept for military applications. The monitors were to be evaluated as to sensitivity, selectivity, response time, linear range, reproducibility, and lifetime.

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PREFACE

The work described in this report was authorized under Contract DAAA15-85-C-0060. This work was started in October 1985 and completed in March 1986.

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A PROTEIN COATED PIEZOELECTRIC CRYSTAL DETECTOR

1. TECHNICAL OBJECTIVE

A research study was to be conducted to develop a piezoelectric crystal monitor for chemical agents using immobilized enzymes (cholinesterases) and an organophosphorus antibody as coatings. A monitor was to be designed that is small, simple to use, of low cost, and based on analytical methodology of suitable accuracy and sensitivity.

The initial evaluation in Phase I was to be performed using an organophosphorus CW agent simulant (diisopropyl methyl phosphonate) to ascertain the feasibility of the concept for military applications. The monitors were to be evaluated as to sensitivity, selectivity, response time, linear range, reproducibility and lifetime.

2. SUMMARY OF FINDINGS

All of the technical objectives of Phase I have been met.

Enzymes and a specific antibody were tested as selective protein adsorbent coatings for utilization in piezoelectric quartz crystal detection systems. Results of this study indicate a selective and reversible response of the coated detector to certain organophosphorus compounds. A general response to several organophosphorus compounds was elicited from the enzyme

coatings. A more specific response to a particular organophosphorus compound was found using an antibody coating. Immobilization of the protein adsorbents directly onto the electrode of the crystal increases the average coating lifetime and considerably enhances response reproducibility and stability. Responses are fast (120 seconds), completely reversible, sensitive (ppb) and highly selective, indicating the viability of the use of protein coatings for gas phase detection of certain organophosphorus compounds.

3. INTRODUCTION

The class of chemical weapons of most concern are nerve gases or organophosphorus anti-cholinesterase compounds. These are colorless and odorless, fast-acting and highly toxic. A detector for these compounds must be self-contained, able to operate under many different environmental conditions, and small enough to be carried by the soldier. Furthermore it should provide for easy transduction of the recognition event into an electrical signal, ideally use a biomaterial for total specificity of response, provide a 10^6 amplification of signal, and require no liquid flow or reagents.

We have shown that a piezoelectric crystal monitor can be developed into such a device that can be made small and rugged enough to fulfill the

demanding requirements of such a monitor, yet specific enough through the use of protein coatings representing the ultimate in selectivity and sensitivity.

4. EXPERIMENTAL SET-UP

A schematic diagram of the continuous sample generation system is shown in Figure 1. The carrier gas is controlled with flow meters obtained from Cole Parmer Instrument Company. The gas can be directed using a 4-way valve so that either the sample gas or the pure carrier gas flows through the detector cell.

The sample generation is accomplished with gas dispersion tubes. Carrier gas is bubbled through the liquid sample generating a fixed concentration based on the vapor pressure of the sample. By varying the flow rate of the carrier gas and the temperature of the liquid sample, the vapor pressure and the amount of sample generated in the vapor phase can be increased. The concentration generated is calculated using the following equation[27]:

$$C_{ppm} = 1 \times 10^{6} \frac{Pn}{P}$$

where P_n is the partial pressure and P is the total pressure at that particular temperature. The concentrations of sample generated in this study are summarized in Table 1.

The entire system was first purged for at least 24 hours, with the organophosphorus compound of interest, before any response measurements were made. This ensures the saturation of the carrier gas with the vapor generated, and that the system contains only the substrate of interest. The vapor saturated carrier gas was diluted with the pure carrier gas before

introduction to the sample chamber so that a calibration of the coating response could be constructed.

Gas chromatography was used to confirm the vapor phase concentrations generated by the evaporation method used in this study. The vapor samples were dispersed through a flask containing 25 mL of isooctane chilled in a water bath at -5 to 0°C. After a specific collection time, the contents of the flask were transfered to a volumetric flask and aliquots were injected into a Perkin-Elmer Sigma 1B chromatographic system. A 5 m x 0.25 cm fused silica WCOT capillary column, coated with 5% methyl phenyl SE52 (Quadrex Corporation, Albany), was used with a nitrogen phosphorus detector.

For interferences studies, the atmospheric gases tested were generated by introducing the pure gases, from lecture bottles, into a dynamic flow system. A syringe pump (Sage Instruments, Model 341) was used in order to control the injection flow rate. The concentrations generated were calculated from the following equation:

$$Ca = \frac{10^6 \times q_a}{q_d}$$

where C_a = concentration in ppm

q_a = sample flow rate

q_d = diluent flow rate

TABLE 1

Headspace Concentrations of Various Interferent Pesticides at 30°C and 20°C

PESTICIDE	VP(mmHg)	Temp.	Concentration (ppm)
Parathion	2.7×10^{-5}	30°C	3.6×10^{-2}
Malathion	4.0×10^{-5}	30°C	5.3×10^{-2}
p-Nitrotoluene	2.7×10^{-1}	30°C	357.

PESTICIDE	VP(mmHg)	Temp.	Concentration (ppm)
Parathion	5.7×10^{-6}	20°C	7.5×10^{-3}
Methyl Parathion	9.7×10^{-3}	20°C	1.3×10^{-1}
Disulfoton	1.8×10^{-4}	20°C	2.4×10^{-1}
Ethion	1.5×10^{-6}	25°C	2.1×10^{-2}

5. INSTRUMENTATION

AT-cut, 9MHz piezoelectric quartz crystals were used for this study. The crystals, obtained from International Crystals, Oklahoma City, OK, had primarily gold coated, 4.9 mm electrodes. A low frequency transistor oscillator, type OT-13, was used to drive the crystal. A regulated power supply, 1-30 VC, model IP-2728 from Heathkit, was used to power the oscillator and was kept constant at 9 VDC. A Systron Donnor Frequency Counter, Model 6241A, was used for the frequency measurements of the piezoelectric crystal. Relative humdity measurements were made with a hygrometer, # HI 8964, obtained from Universal Sensors, Inc. (New Orleans, Louisiana).

The system is shown in Figure 2.

6. RESULTS AND DISCUSSION

6.1 Enzyme Coatings

In a preliminary study, it was found that diisopropyl methyl phosphonate (DIMP) could be detected in the gaseous state, based on its inhibition of cholinesterase, providing the enzyme is bound with glutaraldehyde. In an extension of this study, various cholinesterases were tested as coatings on a piezoelectric crystal, previously immobilized with different binding agents: glutaraldehyde, bovine serum albumin, diazo coupling and carbodiimide.

Acetyl cholinesterase from the electric eel (E.C. 3.1.1.7), butyryl cholinesterase from horse (E.C. 3.1.1.8) and acetyl cholinesterase from human erythrocytes (Type VIII) were tested as coatings for organophosphorus detection. The system used for the testing is the same as the one used for the antibody study and is discussed in detail in the section on apparatus.

Eel cholinesterase, when immobilized with glutaraldehyde, exhibited a large response to 4 ppm of DIMP for repeated exposures. The responses were usually complete in 30 seconds, but required 4-6 minutes for baseline recovery. A coefficient of variation of 5.6% was observed for 6 repeated exposures. A slight upper shift in baseline was also noted, thus suggesting incomplete recovery, even after six minutes. The response curve is shown in Figure 3.

Horse serum cholinesterase was tested using 50 ppb concentrations of malathion. Using a 24,457 Hz coating, responses of approximately 250 Hz were observed. The response was complete within 30 seconds; however, approximately 10 minutes was required for complete recovery. A coefficient of variation of 5.5% was observed for seven exposures to 50 ppb malathion as can be seen in Figure 4. The response was then tested for possible atmospheric pollutants, the results of which are shown in Tables 2 and 3. No significant interferences were noted, other than from 100 ppm of nitrogen dioxide, a concentration which, itself, is hazardous to ones health and is highly unlikely to be commonly found.

The effect of moisture on a cholinesterase coated crystal is shown in Figure 5. It was found that as the humidity increases, the response of the crystal also increases in a linear fashion. The responses are reversible, but at high humidity there is an increase in recovery time. The increase in sensitivity with increasing humidity is probably due to an increase in sensitivity of the hydrated enzyme. The reversibility of the reaction of the enzyme with the organophosphorus compounds is probably the cause of the increase in recovery time with increasing humidity. At low moisture levels, the reversible [EI] complex forms, which is irreversibly converted to an aged

TABLE 2

Effect of Atmospheric Pollutants on Various Enzyme Coatings

Crystal Coating	100 ppm CO	100 ppm SO_2	100 ppm NH_3	100 ppm N_2O
	$-\Delta F$ response (Hz)			
Butyryl Cholinesterase (horse)	16	19	18	—
Acetyl Cholinesterase (electric eel)	14	18	22	15
Butyryl Cholinesterase (Immobilized)	16	27	15	14
Acetyl Cholinesterase (Immobilized)	10	25	12	8

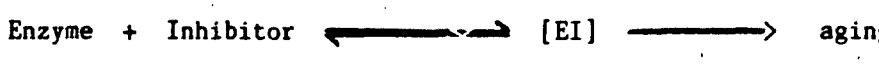
TABLE 3

Response of Cholinesterase Coated Crystal to Interferences in the Presence of Malathion

Interferent	Conc. (ppm)	ΔF Response, Hz
None[a]	----	26
SO_2	100	26
NH_3	100	26
CO	10	29
	100	35
NO_2	10	26
	100	76

[a] Response to 10 ppb Malathion alone.

complex in the presence of moisture. A generalized sample reaction is shown below:



Since plots of ΔF vs. organophosphorus compound concentrations at different humidites show similar slopes, calibration plots for particular humidities can be constructed, and a more useful detector developed.

The stability of the glutaraldehyde bound cholinesterase coating was also studied. A coated crystal remained active for 40 days while exhibiting little variation in the ΔF response to 50 ppb malathion over the tested time period.

Calibration curves for the enzyme coatings were constructed using both DIMP and malathion. The responses were linear from 4.0 ppb to 4.0 ppm DIMP and 5-50 ppb malathion. A correlation coefficient of 0.998 was observed. The limit of detection has been determined to be 180 ng of DIMP and 4 ng of malathion.

The cholinesterase coatings were also screened for response to parathion, carbamates and organochlorine compounds. The reactivity in the gaseous phase to the various pesticides is highest to the G agent structure (DIMP) and followed the order of reactivity of cholinesterase to pesticides in solution (see Table 4). Note that the response of the carbamate Sevin appears to be lower than a chlorinated compound, i.e. Lindane, because of a difference in the V.P. of the compound.

Organophosphorus $>$ Carbamate $\approx$ Chlorinated Compounds

All of the enzyme coatings were tested in the free state or immobilized with bovine serum albumin and glutaraldehyde. In general, the response was greater with the free enzyme coating, but was more reproducible with the immobilized enzyme coating.

The response of the free enzyme coatings to 50 ppb malathion is shown in Figure 6. The change in frequency due to malathion exposure is plotted against the amount of enzyme placed on the crystal. Similar responses were obtained from eel acetyl cholinesterase and horse butyryl cholinesterase. Much smaller responses were seen with the human erythrocyte cholinesterase. The response to malathion for immobilized coatings is shown in Figure 7. In general, a higher sensitivity using large coating weights is attained with the human cholinesterase.

The coating procedure for both the free enzyme and the immobilized enzyme is outlined below:

Free enzyme coatings - A 2.5% enzyme solution is prepared using distilled H_2O. This solution can be placed directly onto the electrode of the piezoelectric crystal using a microsyringe. The enzyme solution is then carefully spread across only the surface of the electrode using a glass pipette with a closed rounded end. The resulting coatings were allowed to dry for at least one hour in a desiccator before testing for response to organophosphorus pesticides.

Immobilized enzyme coatings - A 5.0% solution of Bovine Serum Albumin (BSA) is prepared using 0.1 M phosphate buffer, pH 6.5. A mixture of BSA-ENZ (2:1) is then prepared. This solution is applied to the electrode of the piezoelectric crystal using a microsyringe.

TABLE 4

Response of Various Enzyme Coatings to Organophosphorus, Carbamate and Chlorinated Pesticides

	Response, ΔF					
Coating	Parathion	Malathion	DIMP	Sevin	Lindane	Ethion
ACh (EE), Sol.	88	76	--	35	76	64
ACh (EE), Imm.	57	61	180	24	41	21
ACh (Human), Sol.	71	45	378	--	15	32
ACh (Human), Imm.	52	45	123	26	51	38
BuCh, Imm.	85	62	--	30	44	38

ACh = acetylcholinesterase, EE = electric eel; Sol = Soluble; Imm = Attached via glutaraldehyde.

Parathion = 36 ppb; Malathion = 50 ppb; Ethion = 21 ppb; DIMP = 40 ppb; Lindane = 12 ppb; Sevin = saturated vapor.

A 50% solution of glutaraldehyde in phosphate buffer (0.1 M, pH = 6.5) is now added. It was found experimentally that the optimum BSA:ENZ: GLUT. ratio is 2:1:2. The immobilized enzyme coatings were allowed to dry for 1 hour. At this time, the coatings can be washed of excess glutaraldehyde using 0.1M phosphate buffer, and allowed to dry for 1 hour in a desiccator before testing for response to organophosphorus compounds.

6.2 Antibody Coatings.

Parathion antibodies, obtained from Dr. Ralph Mumma of the Pesticide Research Laboratory in Pennsylvania, were evaluated as coatings for a piezoelectric crystal detector in both the free state, and immobilized. The results of this study illustrate selective and reversible responses to the complimentary antigen, parathion, at ppb levels.

In this study, piezoelectric crystals were coated with parathion antibody, which binds with parathion by a direct reaction in the vapor saturated carrier gas. A decrease in frequency results, which is proportional to the concentration of parathion in the gas phase.

The original antibody solution used was diluted at the Pesticide Research Laboratory 1:5000 (v/v) with a general diluent consisting of 8.77 gms sodium chloride, 1.80 gms disodium hydrogen phosphate, 0.458 gm sodium dihydrogen phosphate, 0.40 ml Tween-20 and 0.020 gms sodium azide, dissolved in one liter of deionized water and adjusted to a pH of 7.1. This solution was used without further modification or treatment for crystal coating.

In coating the crystal with the free antibody solution, equal amounts were applied to both sides of the crystal (.2-1 μl) using a microliter syringe. The solution was then spread evenly across the surface of the

electrode using the blunt end of a glass rod. The coated crystals were allowed to dry in a desiccator for at least one hour before use. The amount of antibody deposited onto the crystal was determined by taking the frequency difference before and after coating. The amount of free antibody applied was varied to obtain the maximum response and also to study the effect of coating amount on response.

The optimum flow rate of this system was found to be 100 mL/min. The highest responses were obtained with very slow flow rates, but the corresponding recovery times were very long, usually 15 minutes or more. At high flow rates, smaller responses were observed with faster recovery times.

The results in Figure 8 illustrate a typical crystal response to parathion. An increase in crystal response was observed with an increase in the amount of antibody on the crystal. Very large amounts of antibody coatings exhibited erratic fluctuations in frequency and considerably slower recovery times. In addition, the response to 35 ppb parathion as a function of carrier gas is shown in Table 5. The frequency changes obtained using He as the carrier gas were generally larger than those attained with other gases, e.g. nitrogen. The inertness of helium may be the possible reason for this observation. None of the commonly available gases (e.g. N_2, He, O_2, or air) appear to inhibit the antigen/antibody interactions. A calibration curve for the antibody coating is shown in Figure 9. Linearity was obtained for concentrations from 2-35 ppb parathion, with a standard deviation of only 6%.

The binding affinity between an antigen or hapten and its specific antibody, compared to similar or structurally related molecules, can be used as a good measure of the degree of specificity and selectivity. In this study, the frequency response obtained with a known concentration of

TABLE 5

Response to Parathion, 35 ppb, as a Function of Coating and Amount and Carrier Gas, at 30°C

Volume of Antibody Coating Added (µl)	Response in N_2	Response in He	Response in moist air	Response in dry air
0.10	61.00	88.33	96.33	80.00
0.30	168.00	266.00	278.00	264.33
0.40	208.00	247.33	320.00	275.00
0.50	304.00	319.33	387.67	308.67
r	0.88	0.98	1.00	0.99
b	3.64	3.95	4.98	4.04

r = Correlation Coefficient

b = Slope

interferant, compared to the response of parathion and structurally related molecules, was used as a measure of the specificity of parathion antibody. Most of the organophosphorus compounds tested showed some response to the antibody coatings, however, only at much higher concentrations than parathion. The results are summarized in Table 6. Usually 3 to 20 times more interferent than parathion was required to produce a comparable change in frequency. Methyl parathion, the closest analog of parathion tested, differing by only one $-CH_2$ group, required almost five times more (158 ppb) substrate in the gas phase to produce a similar response when using the parathion antibody.

To investigate whether non-specific adsorption of these pesticides occurs on protein molecules, crystals were coated with bovine serum albumin, and human immoglobulin G (IgG). Very small irreversible responses (< 5Hz) to parathion were observed with either compound as coating material. The results obtained so far indicate that the temporary adsorption of hapten, in the gas phase, onto the antibody coated crystal is due to antigen/antibody association and dissociation and not to non-specific adsorption of the pesticides by the antibody molecules.

The effect of temperature on the free antibody coatings was also tested. As the temperature was increased from 20 - 30^{o}C, there was a corresponding increase in the observed frequency change. This is probably due to an increase in the amount of substrate in the carrier gas because of the increased vapor pressure of the compound. The results in Figure 10 illustrate this point. The choice of working temperature may be very critical for the purpose of obtaining accurate calibration curves.

Similar to the results found for enzyme coatings, an increase in response to the sample for antibody coatings with increasing humidity is

TABLE 6

Amount of Pesticide Causing 50% Inhibition of Parathion Binding to Antiserum in Solution and Amount (ppb) Needed to Produce a -400 Hz Change in the Gas Phase

Pesticide	Amt. (µgs) * in solution	Amt. (ppb) in gas phase
Parathion	50	36
Malathion	70,000	106
Paraoxon	1,850	--
Methyl Parathion	15,000	158
p-Nitrophenol	10,000	680
Disulfoton	—	560
Ethion	—	102

* Ercegovich et al. J. Agric. Food Chem. **1981**, 29, 559-563.

observed. In order to show the effects of water vapor on response, the difference in response in dry and moist air is plotted in Figure 11. It is possible to make accurate measurements in the presence of moisture (10-80% r.H) providing the humidity is not high enough (>80%) to cause condensation on the surface of the crystal.

The lifetime of these coatings is about seven days. Antigen/antibody interactions are sensitive to temperature and pH, as well as variations in the reaction medium. The responses were completely reversible and within an experimental reproducibility of 6%. The results are illustrated in Figure 12.

Parathion antibodies were chemically immobilized using glutaraldehyde coupling to bovine serum albumin (BSA). The antibody was directly immobilized onto the crystal, and its response to parathion and other compounds, as well as its stability, was studied.

The response of the immobilized antibody was lower than of the soluble antibody, as shown in Figure 13. All responses were very reproducible, and the crystal showed good stability for up to two months, with only a small decrease in sensitivity.

The response of the crystal to other interferences was also tested. The results, shown below in Table 7, indicate good selectivity of the parathion antibody crystal to parathion. Much higher concentrations of other organophosphates are required to give any response. The very high levels of such compounds as CO, SO_2, and NH_3, that are necessary to illicit a response, are hazardous, and would adversely affect the individual even at considerably lower (non-response) levels.

TABLE 7

Effect of Diverse Substances on the Response of a Parathion Coated Crystal

Compound	Concentration	Response, ΔF, Hz
Parathion	36 ppb	108
Malathion	53 ppb	94
Ethion	197 ppb	79
DIMP	4000 ppb	235
Carbon Monoxide	100 ppm	11
Sulfur Dioxide	100 ppm	19
Ammonia	100 ppm	14

Finally, Figure 14 shows a calibration plot for parathion, using 0.4 l of antibody immobilized with BSA and glutaraldehyde. Linearity is observed from 1-35 ppb. The responses are completely reversible and are 95% complete in less than 10 seconds.

7. CONCLUSIONS

It has been successfully demonstrated that protein coatings (eg. the enzyme cholinesterase or the parthion antibody) can be used for the gas phase determination of organophosphorus compounds. This work represents the first demonstration of this concept. The responses are fast (< 10 sec.), completely reversible, highly selective, very sensitive (ppb levels are assayable), and the coatings have long lifetimes.

This study should be continued into Phase II.

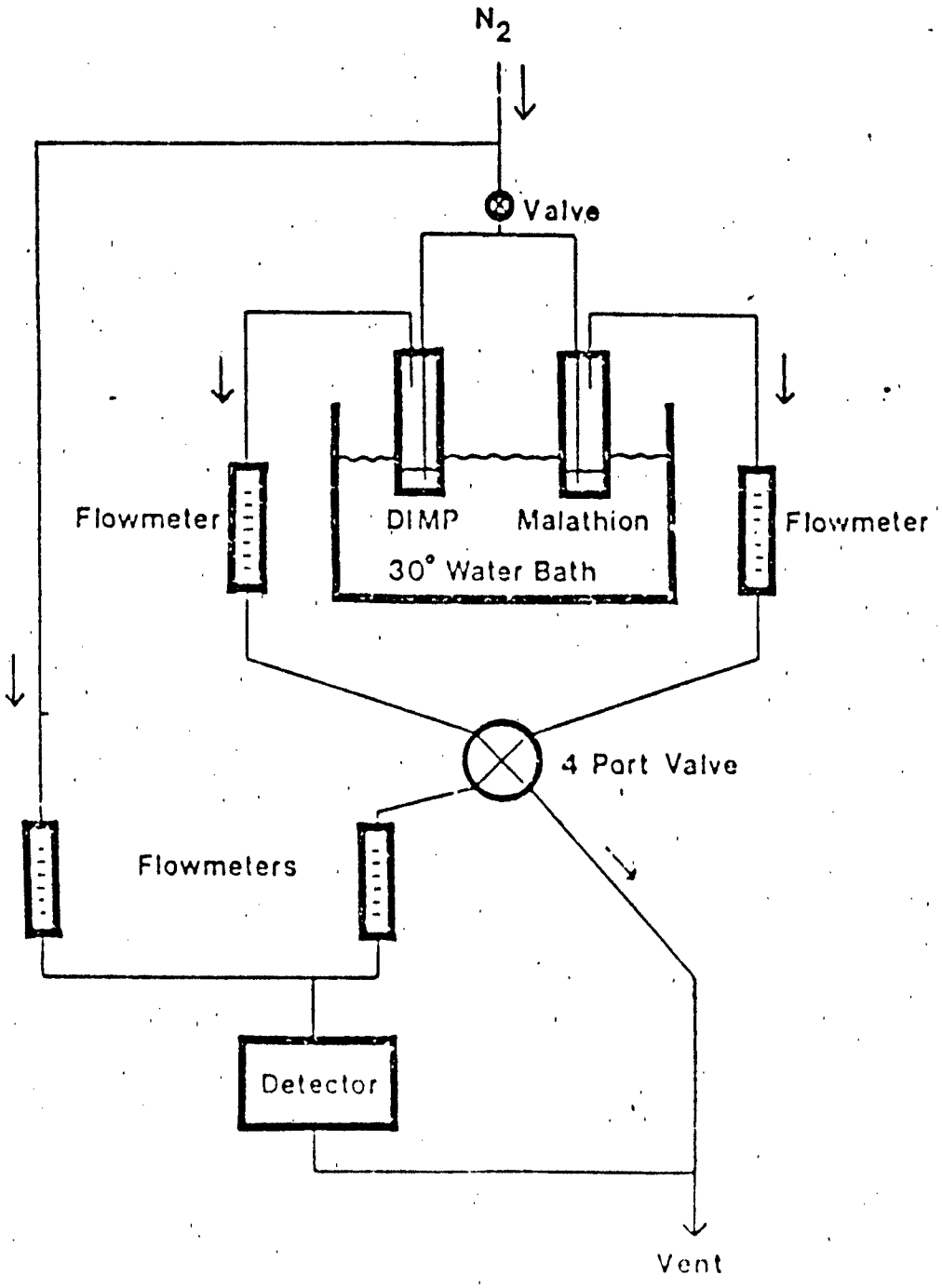


Figure 1. Schematic diagram of the continuous sample generation system

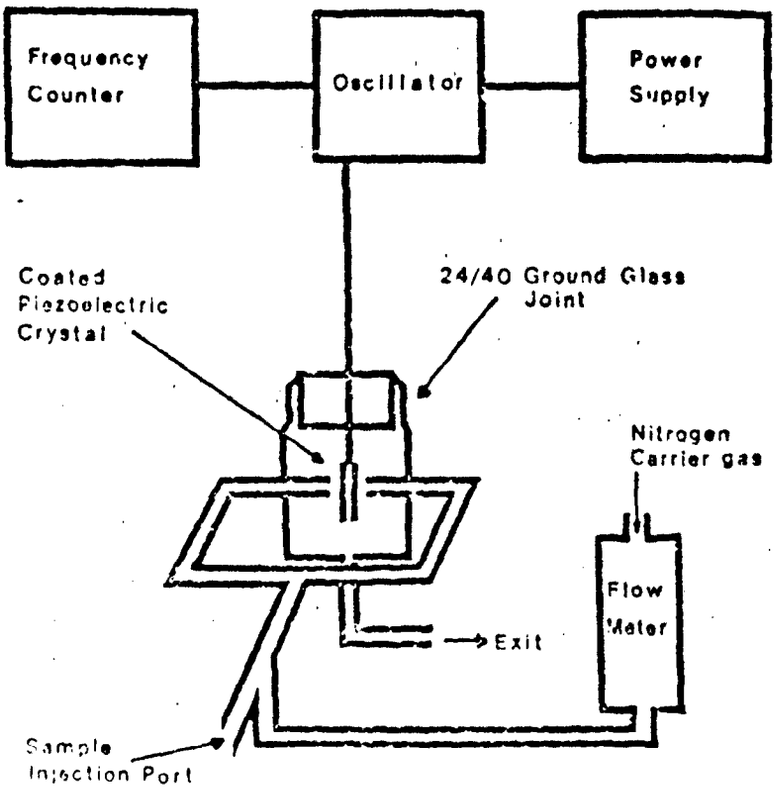


Figure 2. Schematic Diagram of the piezoelectric quartz crystal detector.

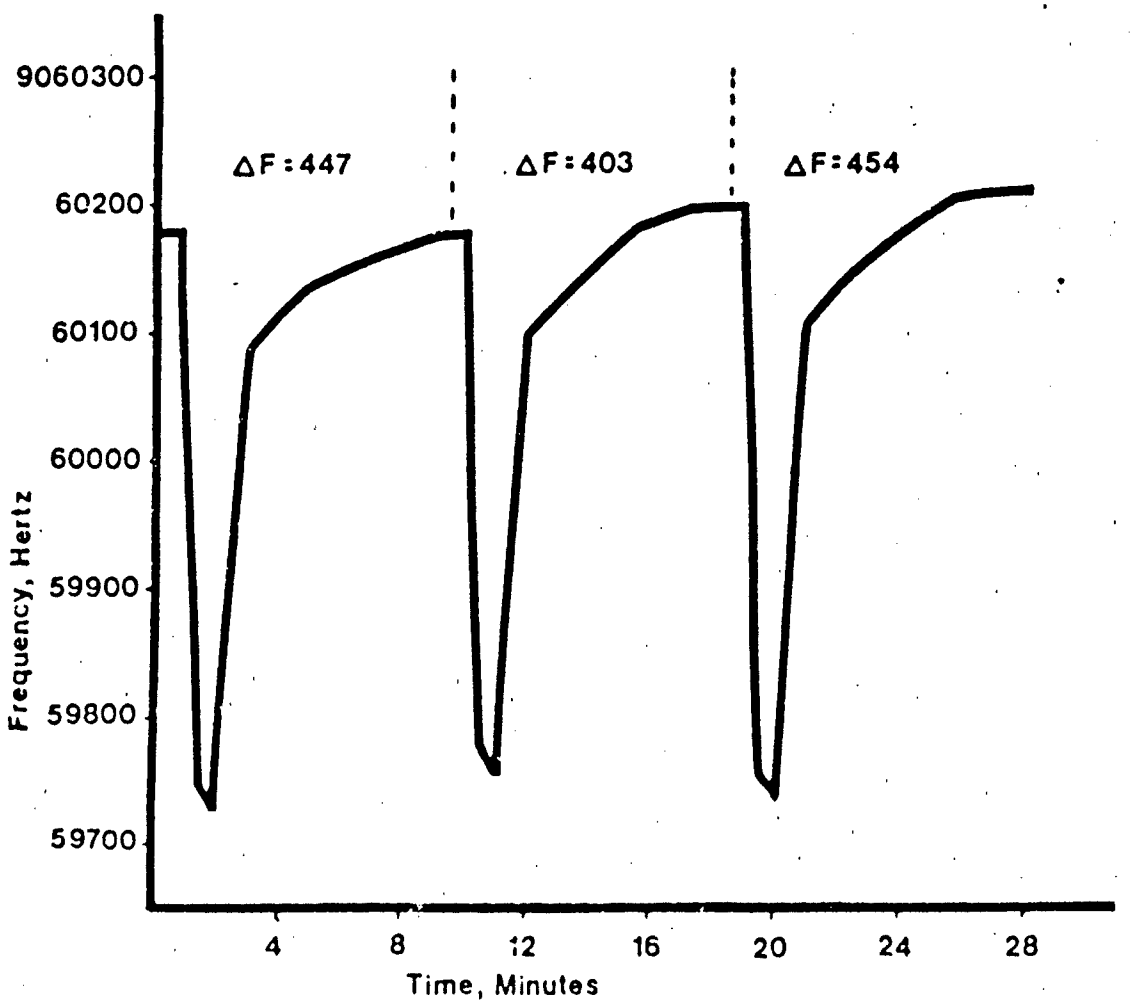


Figure 3. Response curve for repeated exposures to DIMP. 0.5 min exposures to 4 ppm DIMP.

Coating: 8057 Hz

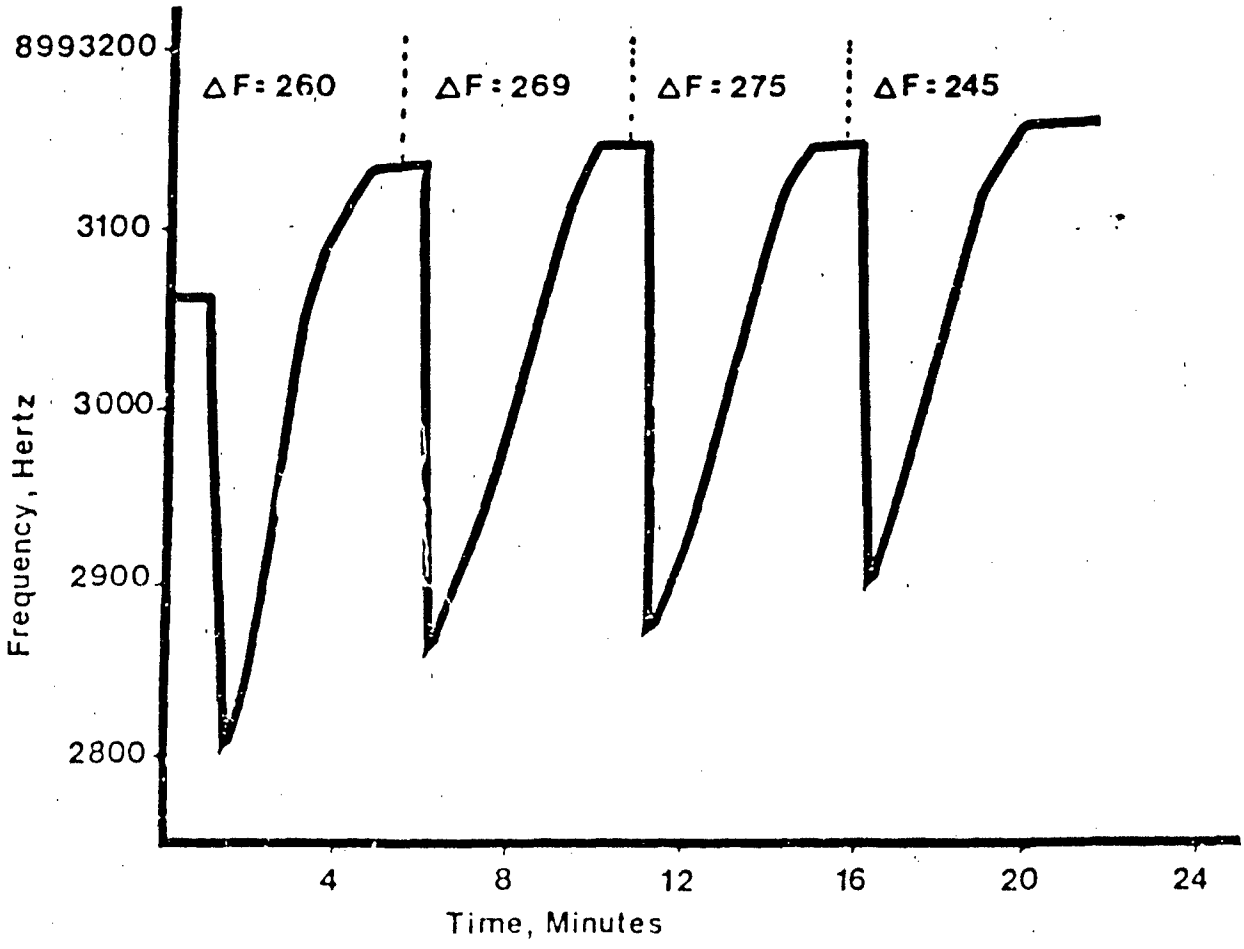


FIGURE 4. Response Curve for Repeated
0.5 min exposures to 50 ppb of Malathion.
Coating = 24,457 Hz

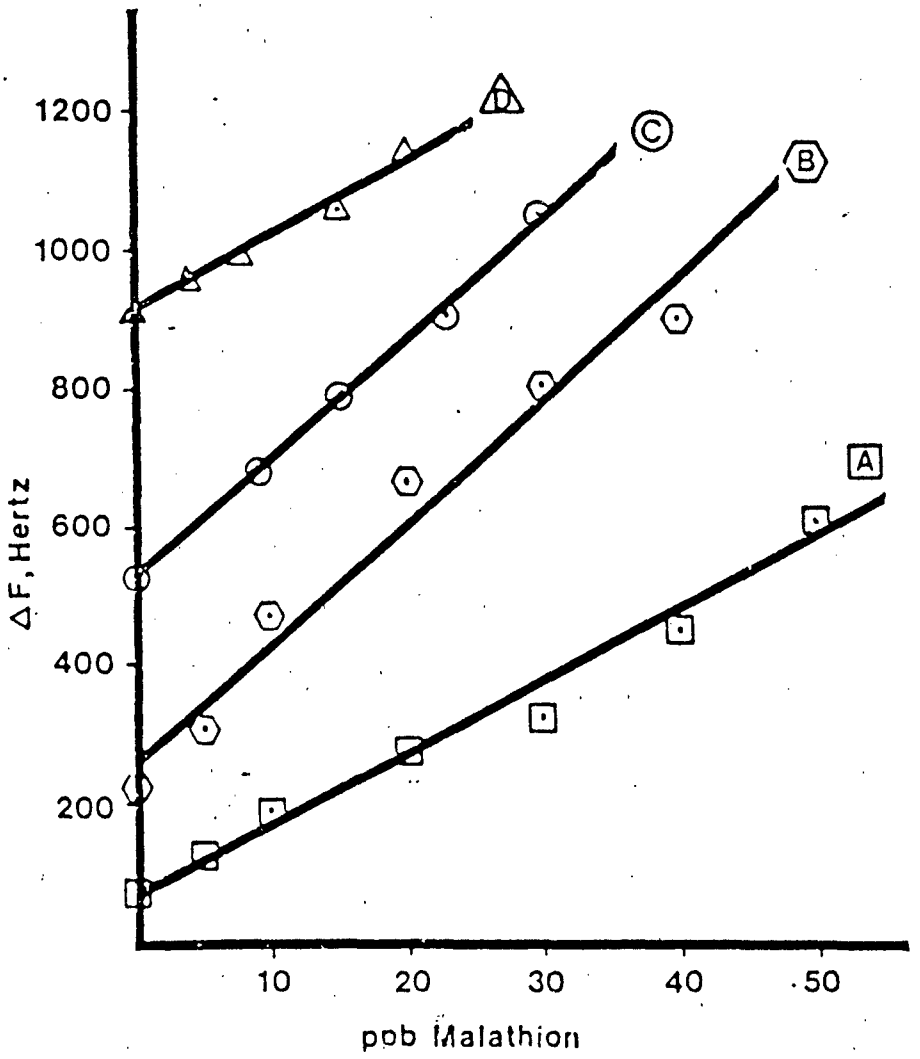


Figure 5. Effect of Humidity on the Detector Response to Malathion

A: 0% humidity; 24, 457 Hz coating
B: 20% humidity; 18, 943 Hz coating
C: 40% humidity; 20, 010 Hz coating
D: 60% humidity; 19, 185 Hz coating
0.5 min exposures

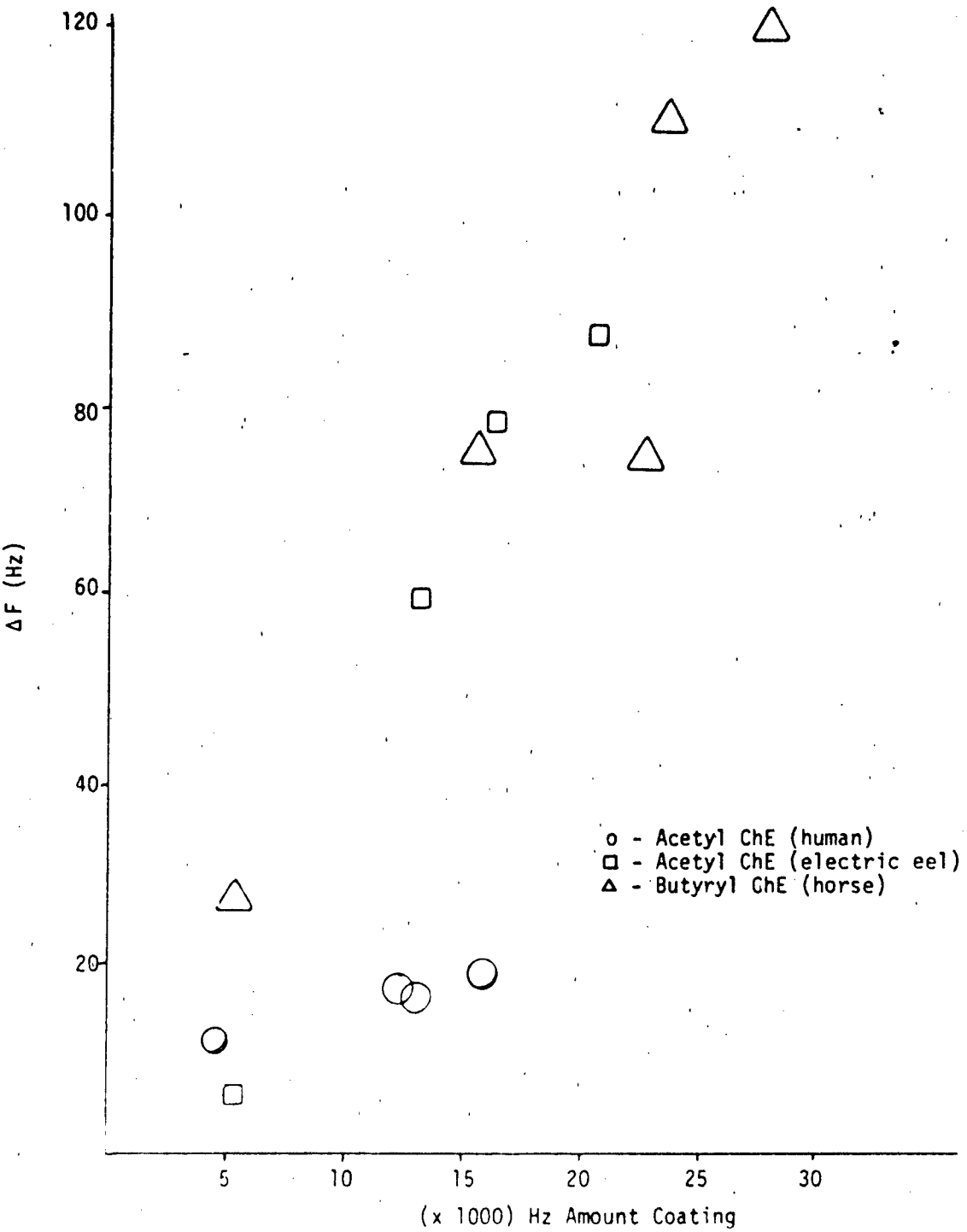


Figure 6. Response of Free Enzyme Coating to 50 ppb Malathion

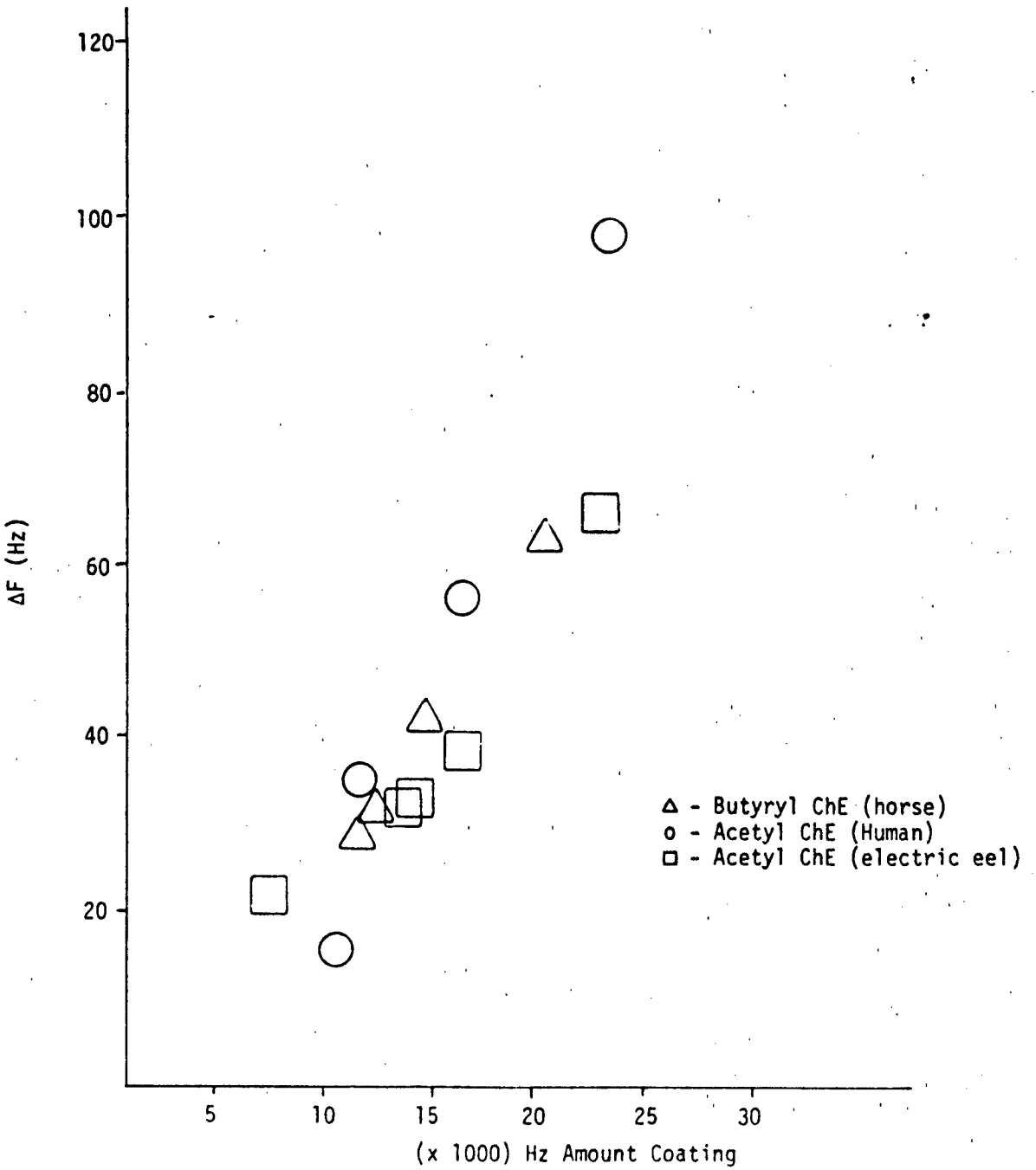


Figure 7. Response of Immobilized Enzyme Coatings to 50 ppb Malathion

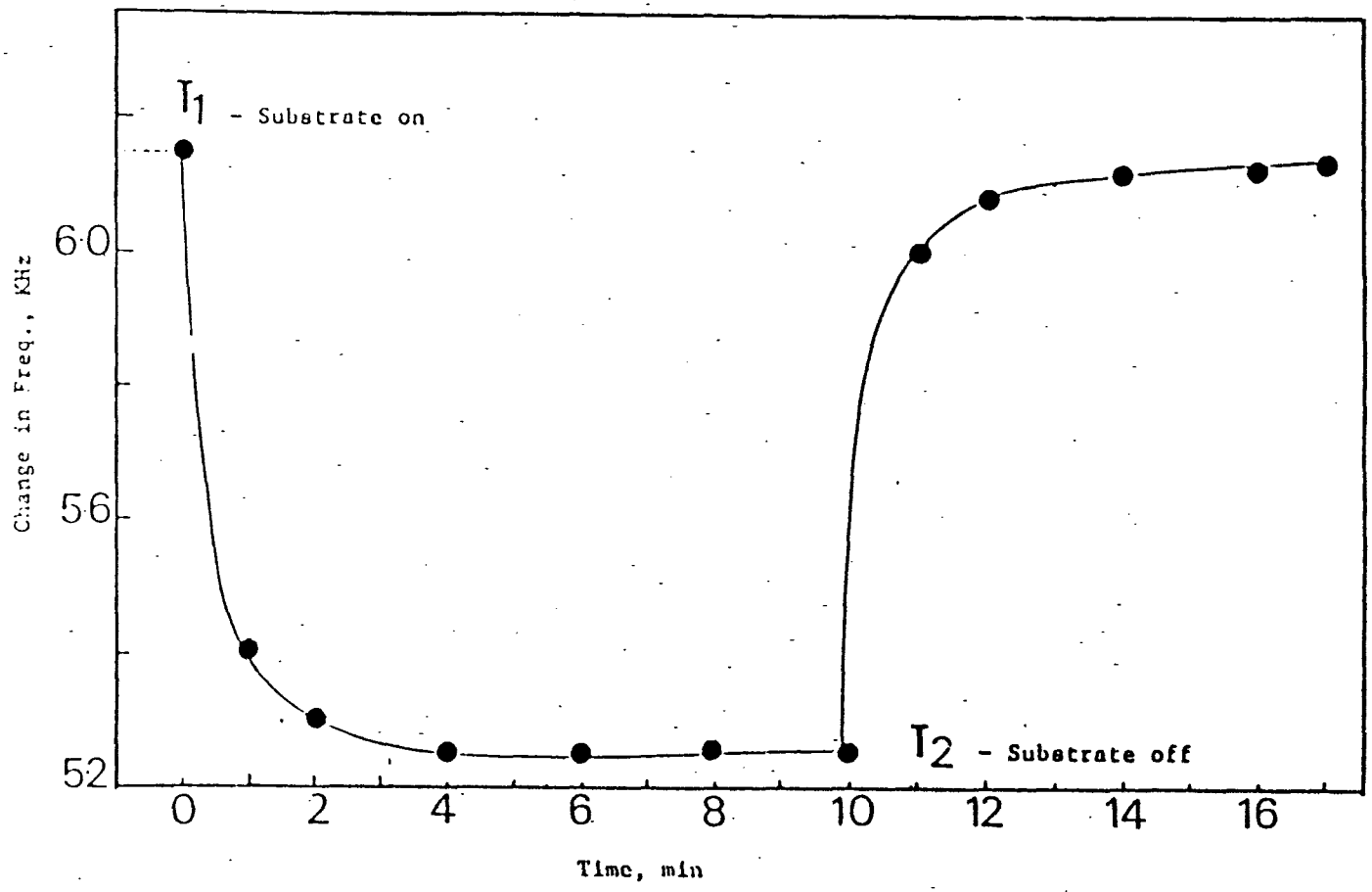


Figure 8. Typical Piezoelectric Crystal Response to Parathion Saturated Carrier gas, 35 ppb, at 30°C.

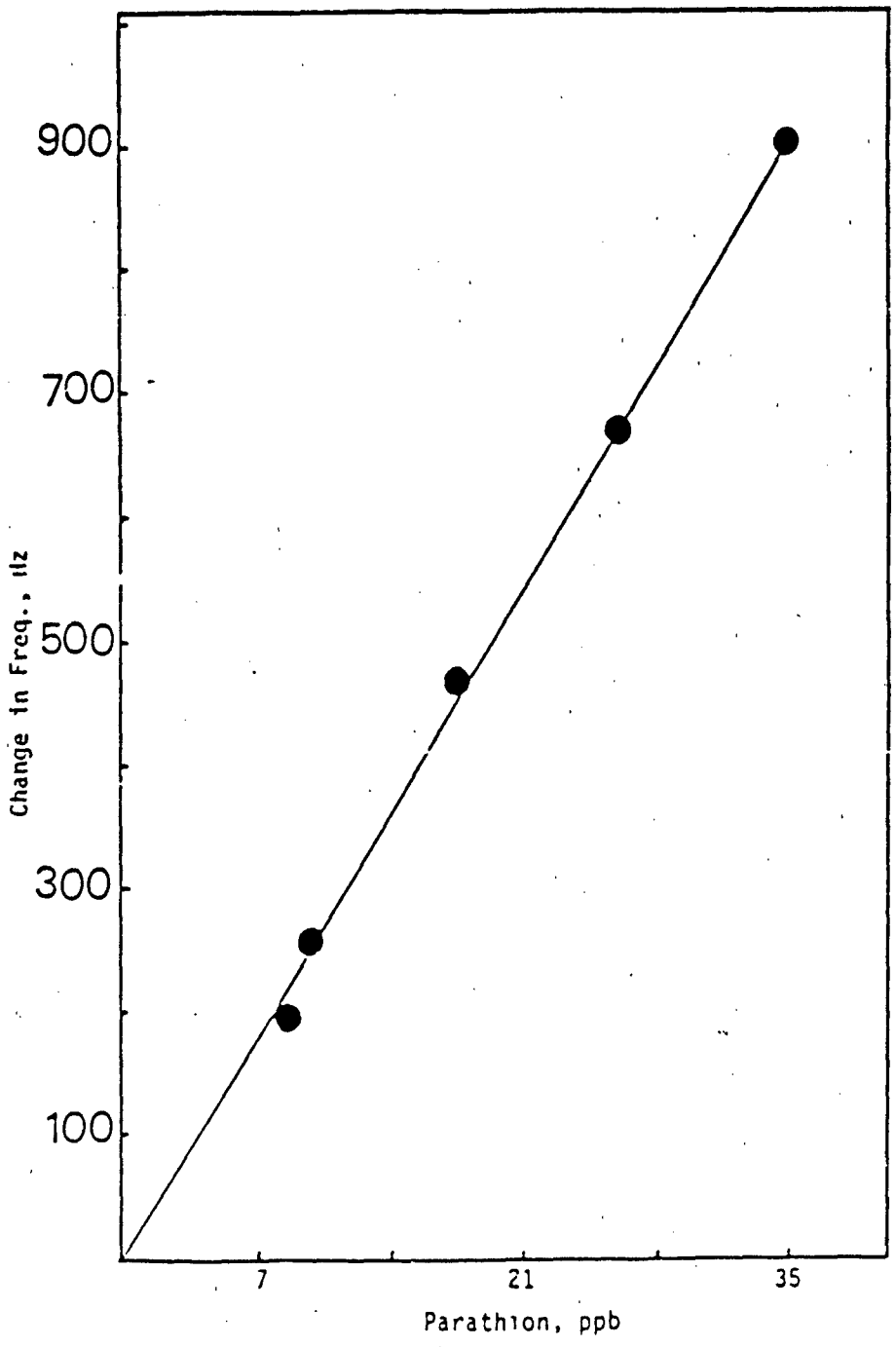


Figure 9. Calibration Curve for the Antibody Coated Crystal at 30°C.

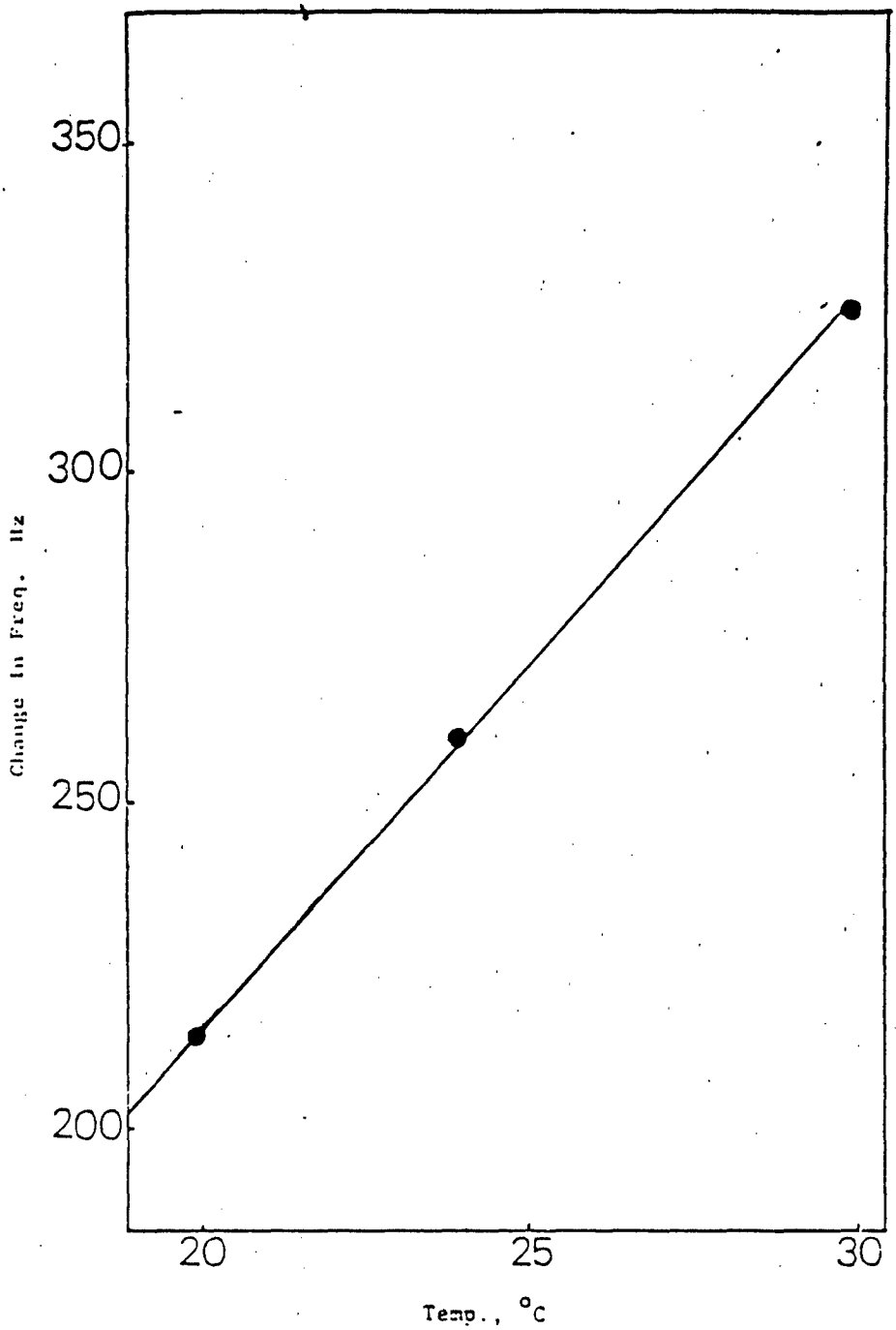


Figure 10. Response of the Antibody Coated Crystal as a Function of Temperature

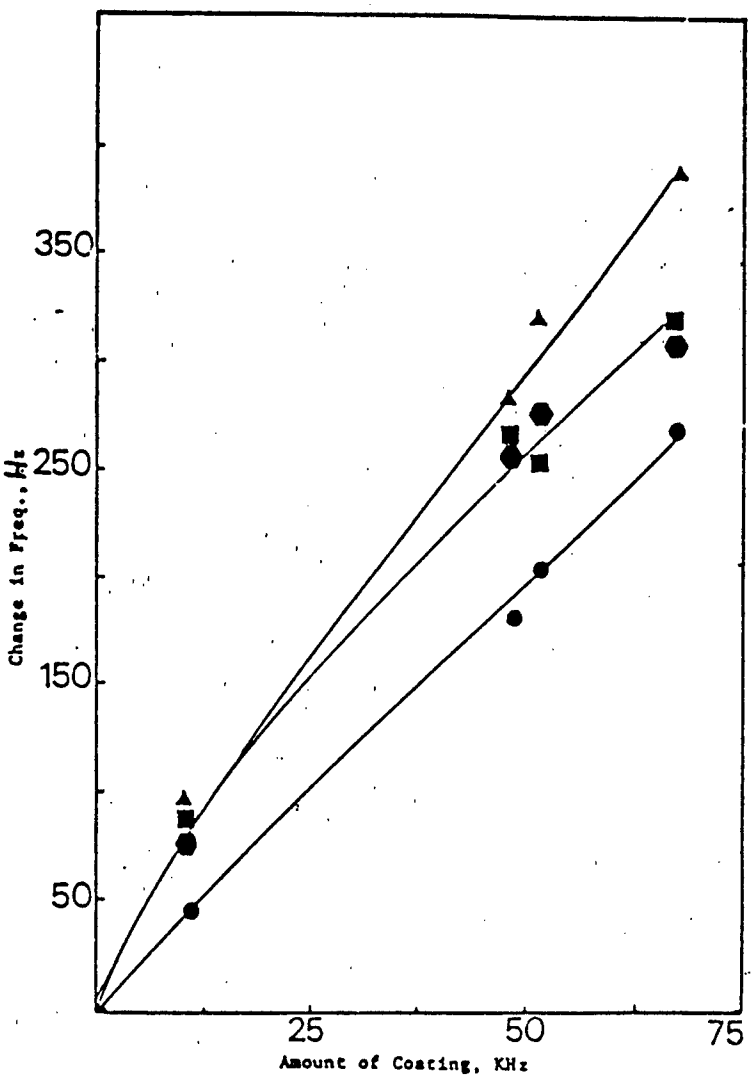


Figure 11. Crystal Response as a Function of the Amount of Antibody on the Crystal in Different Carrier Casses

35 ppb, at 30°C

▲ Wet compressed air
■ Dry compressed air
● Nitrogen
◆ Helium

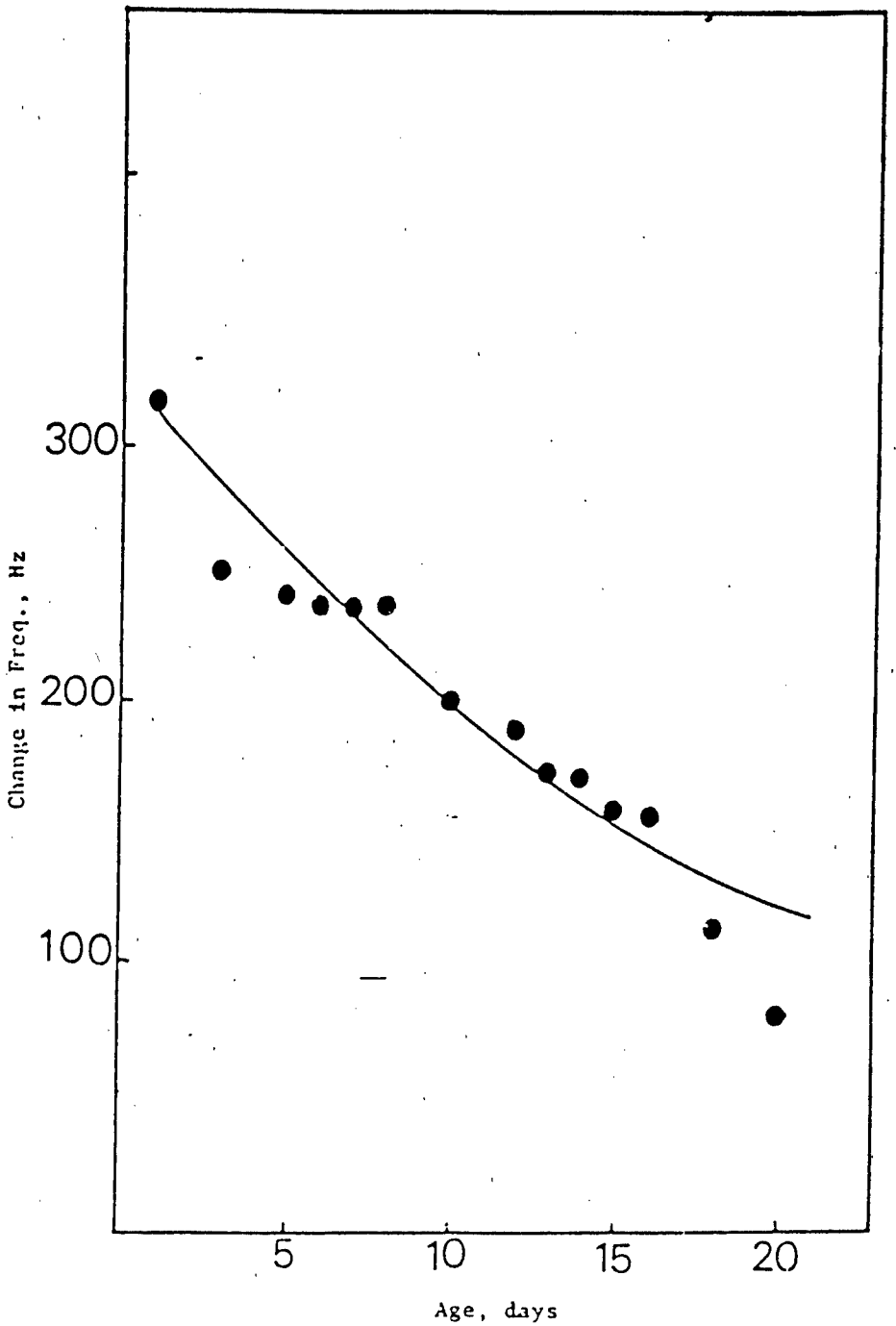


Figure 12. Long Term Stability Studies on the Response of the Coating with Age.

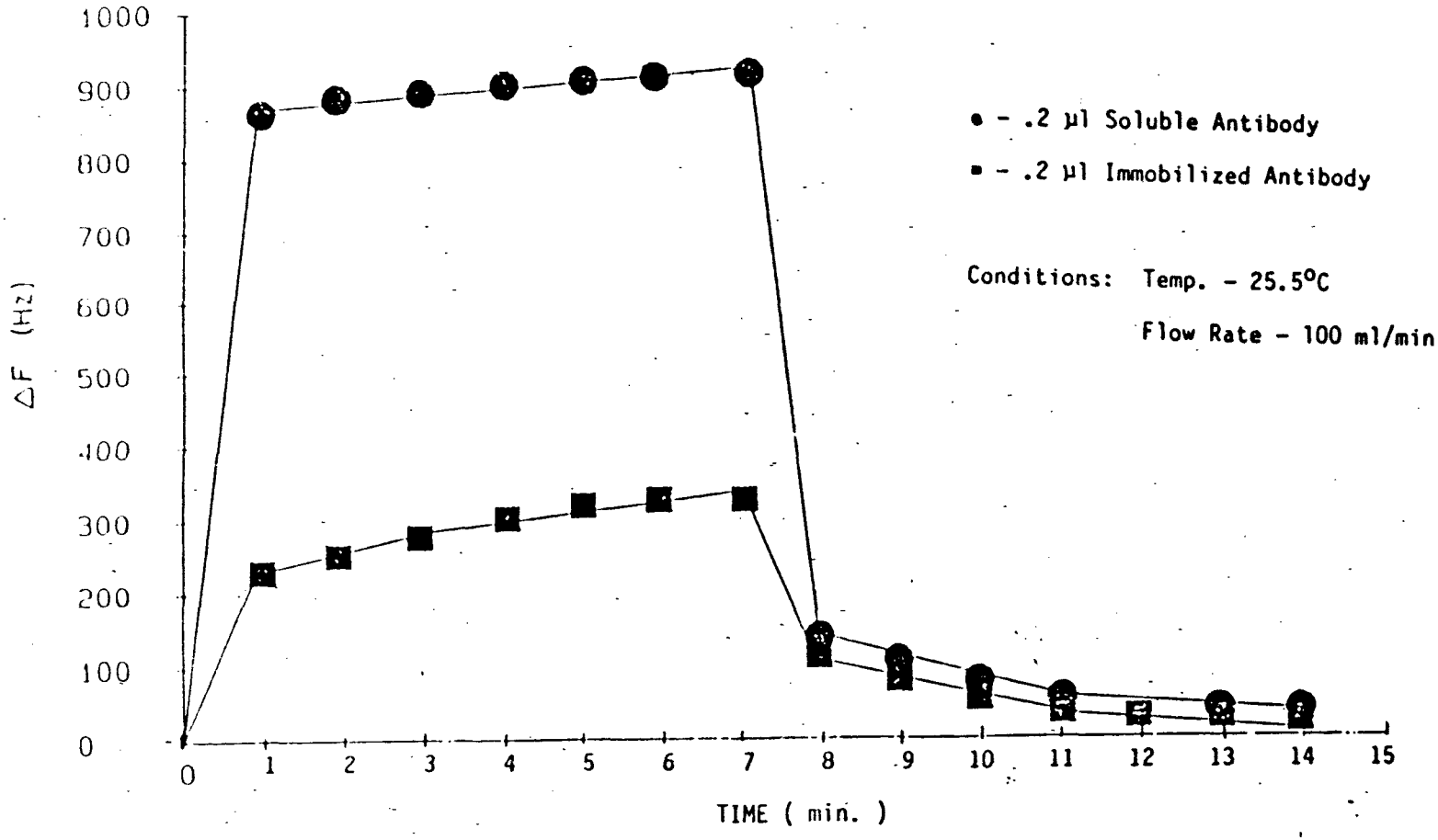


Figure 13. Response to 36 ppb Parathion

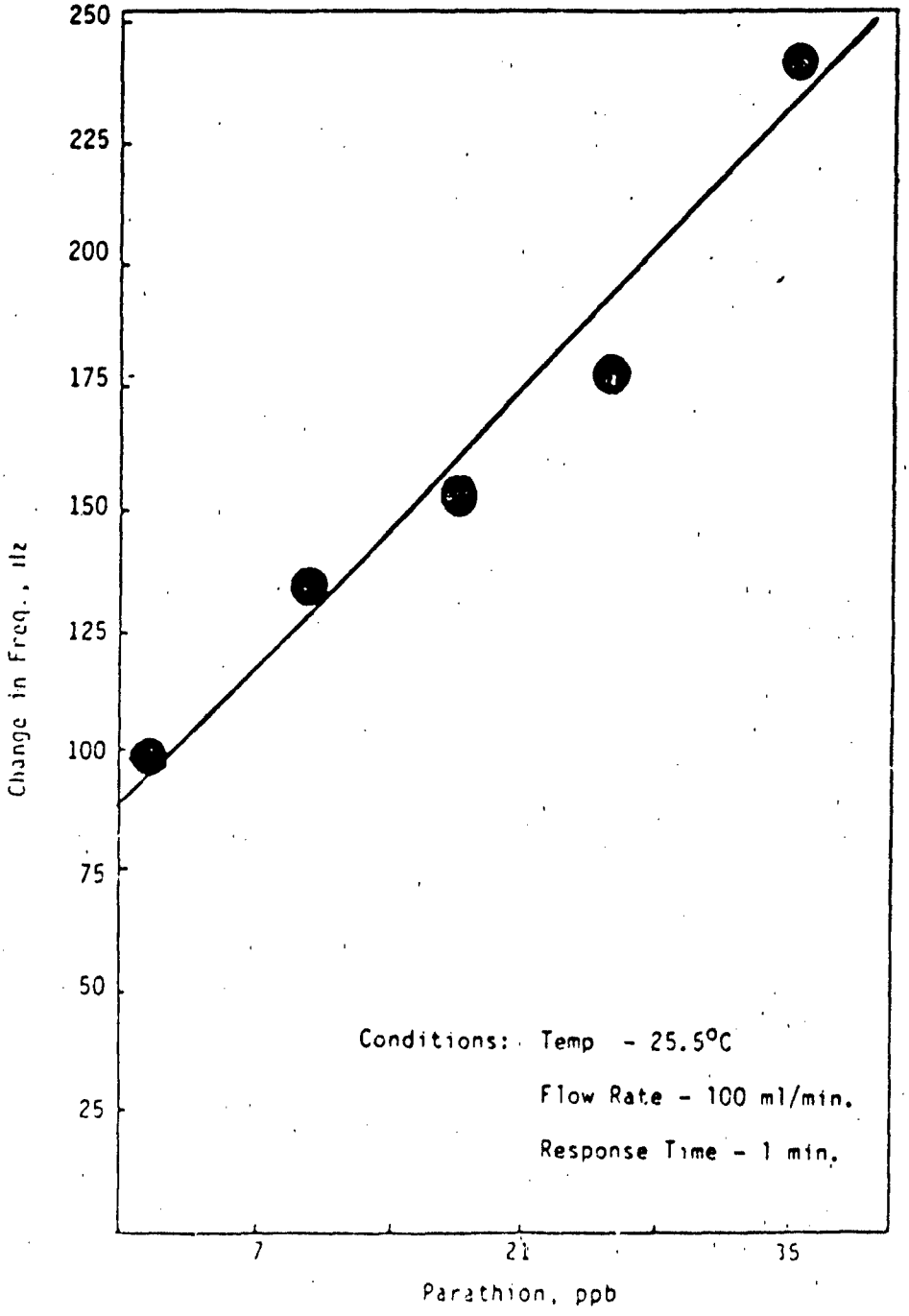


Figure 14. Calibration Curve For Immobilized Antibody