Parathion Antibodies on Piezoelectric Crystals

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Abstract: Piezoelectric quartz crystals have been coated with antibodies against parathion and used for the assay of parathion in the gas phase. Preliminary results indicate a selective and reversible response to the complimentary antigen, parathion, at ppb levels. The possibility of using biologically active material, like an antibody, as a piezoelectric crystal coating for the selective detection of complimentary antigens in the gas phase is discussed. Limitations to sensitivity and selectivity with possible improvements of the technique are also discussed. The potential ability to rapidly obtain qualitative as well as quantitative information on a sample through such an immunochemical assay makes this a very attractive alternative to conventional analytical techniques requiring prolonged pretreatment.

Most established conventional methods used in pesticide analysis require extensive sample preparation before analysis on very expensive and complex equipment. The more recent techniques, like radioimmunoassay (RIA),[1] enzyme-immunoassay,[2] and chromatographic methods,[3,4] though very selective and sensitive, still require a fair amount of sample preparation and even more expensive instrumentation.

The advent of "bioassays" and the "theoretical" capability of specific antibodies to recognize and combine with nanogram levels of complimentary antigens[5-8] in a mixture of similar molecules with little or no separation, makes this approach very attractive. In the past enzyme-immunoassay and radioimmunoassay have been very popular with clinical chemists and toxicologists. The cost of instrumentation and the potential health hazards inherent in RIA, in particular, have continued to initiate fundamental research in an effort to develop simpler, cheaper, and equally sensitive alternatives.

In a theoretical study at NIH,[9] it was concluded that solid-phase assays were indeed more sensitive than solution assays and capable of detecting picomolar amounts of the complimentary antibody. A number of solid supports[10] have been used for the antigen and/or antibody immobilization. Plastic carriers like polystyrene, polycarbonates, and silicone rubber, as well as discs and tubes, have also been used. These materials, particularly the plastics, are good because most proteins adsorb onto their surfaces. This is probably due to hydrophobic interaction between nonpolar protein structures and the nonpolar plastic matrices. Covalent attachment of antibodies and/or antigens using various bifunctional reagents is also well documented.[11,12]

The use of coated piezoelectric quartz crystals for the assay of various environmental contaminants is very well known.[13-20] Piezoelectricity, and the piezoelectric crystal phenomenon, was described in detail by Sauerbrey.[21] King[22] later adapted this principle for analytical use. In this technique the electrode surface is coated with material that selectively adsorbs only one compound. When such a coated crystal is exposed to the particular substance of interest, adsorption occurs, causing a frequency change, which can be used to determine the amount of material adsorbed according to the Sauerbrey equation[21]

$$\Delta F = -K\Delta m$$

where Δm is the amount of material adsorbed on the crystal, and ΔF is the change in frequency caused by the adsorbed mass. K is a constant that includes the size, thickness, and surface area of the electrode covered by the adsorbed material. The adsorption process on most crystals developed has been neither very selective nor very specific, probably because a specific and selective coating material is difficult to find.

Biologically active materials, like antibodies and enzymes, that are very selective and specific can enhance the selectivity of piezoelectric crystals many fold. A number of workers have, in fact, attempted to use biologically active molecules as a coating material on the surface of quartz crystals.[23-26] In all of these studies, however, the reactions were carried out in the liquid phase, followed by measurement of the frequency change in the gas phase.

In this study piezoelectric crystals were coated with antibodies against parathion. When the coated antibody binds with parathion by a direct reaction in the gas phase, the mass change on the crystal generates a frequency shift proportional to the concentration of the pesticide. This use of antibodies and antigen/antibody interaction for the direct assay of complimentary gas-phase antigens has not, to the best of our knowledge, been reported. This

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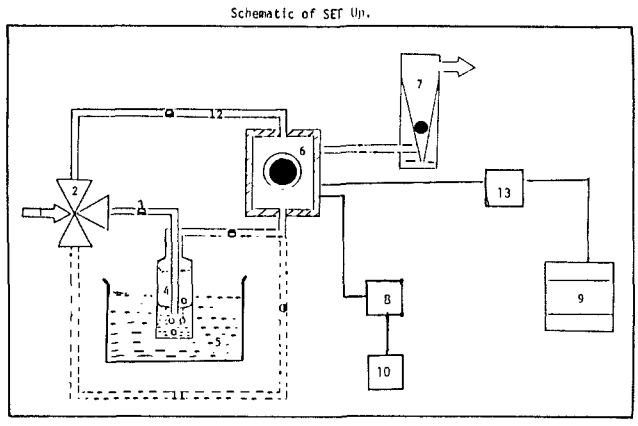

Figure 1. Schematic of experimental setup: (1) carrier gas, (2) 4-port valve, (3) one-way valve, (4) liquid sample, (5) constant-temperature bath, (6) antibody-coated crystal, (7) flow meter, (8) oscillator, (9) digital read out, (10) power supply, (11) diluent gas stream, (12) background gas stream, and (13) frequency counter.

paper reports preliminary results that demonstrate the feasibility of this approach for measurement of reactions that occur directly in the gas phase. Factors that may limit the sensitivity and selectivity of this technique and situations in which this technique might be useful are discussed.

Parathion was chosen for this study because of its wide use for mosquito control and because of the availability of its "specific" antibodies.[1] The fact that parathion can be vaporized also makes this pesticide a particularly good candidate for such a study.

Experimental Section

Instrumentation. All the piezoelectric crystals used were 9 MHz quartz, 4.9 mm in diameter, and AT-cut. They were purchased from International Crystal Co. Oklahoma City, OK. Most of the crystals had gold coated metal electrodes, but a few were silver coated. None of the crystals with silver electrodes responded to exposures to parathion saturated vapors after they were coated with the antibody. A low frequency transistor oscillator, type OT-13 from International Crystal Co., was used to drive the crystal. Power supply to the oscillator was provided by a regulated power supply, 1-30V DC, Model IP-2728 from Heathkit, set at 9 V. A Systron Donner frequency counter, Model 6241A, was used to measure the frequency of the vibrating crystal. Relative humidity measurements were made with a hygrometer, No. HI 8964, obtained from Universal Sensors Inc. (New Orleans, LA). The design of the detector cell is similar to that used by Karmakar and Guilbault.[17] Figure 1 is a schematic diagram of the experimental setup. The flow rate of the carrier gas was monitored with flow meters obtained from Cole Palmer Instrumental Co.

Reagents. Parathion (*O,O*-diethyl-*O*-(*p*-nitrophenyl) phosphorothioate) (98%) and methyl parathion (*O,O*-dimethyl-*O*(*p*-nitrophenyl) phosphorothioate) (80%) in xylene were obtained from Monsanto Chemical Co. (St. Louis, MO). Malathion (diethyl [(dimethyoxyphosphinothioyl)thio]butandiate (96% (w/w)) was a gift from American Cyanamid Co. (NJ). Ethion (*S,S'*-methylene bis[*O,O*-diethyl] phosphorodithioate), (88.8%) was a gift from FMC Chemicals (NJ), and Di-system (*O,O*-diethy *S*-[2-(ethylthio)ethyl] phosphorodithioate) (97%) was a gift from Mobay Chemical Corp. (MO). *o*-Nitrotoluene was obtained from ICN Biochemicals (Cleveland, OH). Human immunoglobulin G (IgG) in lyophilized form was obtained from Sigma Chemical Co. (St. Louis, MO) and dissolved in phosphate buffered saline, pH 7.4. Antibody against parathion was a gift from Dr. Mumma at the Pesticide Research Laboratory in Pennsylvania State University. This preparation, diluted 1:5000 (v/v) with general diluent (8.77 g of sodium chloride, 1.80 g of disodium hydrogen phosphate, 0.458 g of sodium dihydrogen phosphate, 0.40 mL of Tween-20, and 0.020 g of sodium azide, dissolved in 1 L of deionized water and adjusted to a pH of 7.1), was used without further modification or treatment. The carrier gas was generally nitrogen, but helium, compressed dry air, and compressed wet air were also used. All the chemicals were reagent grade.

Procedure

Coatings. Both sides of the crystal were coated with the same amount of antibody. This was applied onto the crystal using a 1 μL syringe and was evenly spread out with the syringe needle, or the blunt end of a glass rod, to cover only the electrode area.

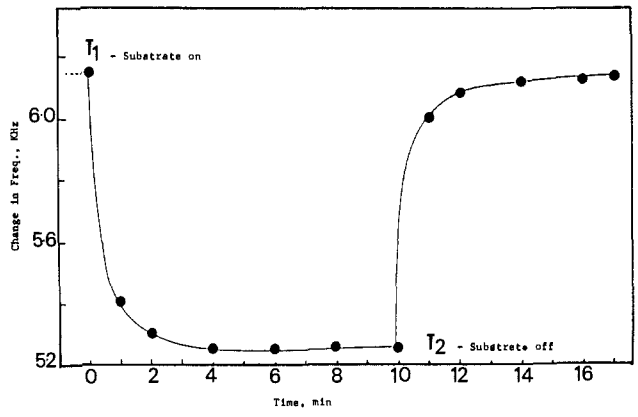

Figure 2. Typical piezoelectric crystal response to parathion-saturated carrier gas, 35 ppb, at 30 °C.

Enough antibody was applied to each crystal to produce different base frequency shifts corresponding to the amount of antibody on the crystal. The coated crystals were then allowed to completely dry in a desiccator for at least an hour before use.

Measurements. Before any measurements were made, the entire system was purged for at least 24 h with the pesticide of interest, to ensure vapor saturation of the carrier gas and to make sure that the system contained only the substrate of interest. The carrier gas was bubbled at a known rate through a trap filled with the liquid sample of interest. The vapor-saturated carrier gas was then diluted with pure carrier gas flowing at a known rate. The diluent flow rate, as well as the sample flow rate, could be changed to produce different vapor concentrations of the sample. The resulting gas mixture was then allowed to flow through the detector cell while making contact with both sides of the crystal. The schematic of the setup is shown in Figure 1.

Gas chromatography was used to confirm vapor-phase concentrations generated by the evaporation method. 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 transferred to a volumetric flask and aliquots were injected into a Perkin-Elmer Sigma 1B chromatographic system. A 50 m × 0.25 μm fused silica WCOT capillary column, coated with 5% methyl phenyl SE52 (Quadrex Corp., Albany), was used with a nitrogen phosphorus detector.

With the aid of a four-port valve, vapor-saturated gas or pure carrier gas was allowed to alternately flow past the detector at all times. As adsorption took place, ensuing frequency changes were measured, using a digital frequency counter. Response times were generally within 1–2 min. When the substrate-saturated gas was turned off, a return to the original equilibrium state required 2–5 min.

Results and Discussion

Choice of a Suitable Flow Rate. Initial flow rate studies revealed considerable differences in response between the faster and slower flow rates. Generally the highest responses were obtained with the very slow flow rates, but the corresponding recovery times were very long, about 15 min or more. The exact opposite response was obtained with higher flow rates: smaller responses and fast recovery times. A flow rate of 100 mL/min proved to be a suitable compromise between the two extremes.

Responses. The results in Figure 2 depict a typical crystal response. An increase in crystal response was observed with an increase in the amount of antibody on the crystal. With larger amounts of antibody coatings there were large fluctuations in frequency changes with slower recovery times.

In order to study the effect of response as a function of vapor-phase concentrations, the temperature of the sample container was increased by increasing the temperature of the water bath. This increases the vapor pressure and consequently the amount of pesticide in the gas phase. The vapor-phase concentration of various gases as a function of temperature is shown in Table I.

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

pesticide	VP, mmHg	concn, ppm
	30 °C	
parathion	2.7×10^{-5}	3.6×10^{-2}
malathion	4.0×10^{-5}	5.3×10^{-2}
p-nitrotoluene	2.7×10^{-1}	357.7
	20 °C	
parathion	5.7×10^{-6}	7.5×10^{-3}
methyl parathion	9.7×10^{-3}	1.3×10^{-1}
disulfoton	1.8×10^{-4}	2.4×10^{-1}
ethion	1.5×10^{-6}	2.1×10^{-2}

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

vol 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^a	0.88	0.98	1.00	0.99
b^b	3.64	3.95	4.98	4.04

[a] r = correlation coefficient. [b] b = slope.

A linear increase in ΔF was observed with an increase in temperature. The response of an uncoated piezoelectric crystal is, however, known to be independent of temperature. Thus a linear relationship of ΔF to parathion concentration is observed.

None of the crystals with silver coated metal electrodes responded upon exposure to parathion vapors, even with very small amounts of antibody coatings. There is a possibility that reaction of the SH groups in the antibody molecules with the silver might have resulted in the denaturation of the antibody. However, all of the gold coated crystals gave fairly good results.

Carrier Gas. Different carrier gases were used in this study, including nitrogen, helium, compressed air, and lab air. The responses with the various gases are summarized in Table II. The frequency changes obtained when helium was the carrier gas were generally higher than those with only nitrogen. The inertness of helium might be the possible reason for this. The fact that none of the commonly available gases appeared to inhibit antigen/antibody interactions was very encouraging.

All the carrier gases were passed through a drying tube, about 60 cm long filled with calcium sulfate (drierite), before being bubbled through the sample trap. The effluent gas has a relative humidity of about 15%. If the gas was totally saturated with water vapor, by bubbling it through a water trap, the crystal very quickly became saturated with water droplets and stopped vibrating. Heating of the entire system will get rid of the condensed water and also prevent any further water condensation on the crystal.

Interference. Antigen/antibody reactions are theoretically very selective and specific, for only one substance, even in the presence of larger amounts of similar molecules. To test the validity of this theory in the gas phase, the coated crystals were exposed to known concentrations of pesticides other than parathion. Most of the pesticides showed some response to the parathion antibody coatings, but at substantially higher concentrations than parathion. Table III contains a summary of these studies.

It is well-known that some antibodies show severe cross reactivity with some metabolic intermediates or byproducts structurally similar to their haptens. This varies from one antibody preparation to another and is usually accompanied by differences in binding rate constants.[2,5]

To investigate the nonspecific absorption of these pesticides on protein molecules, some crystals were coated with bovine serum albumin (BSA) and with human immunoglobulin G (IgG). Very small irreversible responses (<5 Hz) to parathion were observed with either compound as coating material. The antigen selectivity of the parathion antibodies in the gas phase observed in this study

Table III. Comparison of Antibody Specificity in the Gas and Liquid Phases

pesticide	amt (μg) in solution[a]	amt (ppb) in gas phase[b]
parathion	50	36
malathion	70000	106
methyl parathion	15000	158
p-nitrophenol	10000	680
disulfoton		560
ethion		102

[a] Amount of pesticidal material (μg) causing 50% inhibition of parathion binding to antiserum in solution (Ercegovich et al *J. Agric. Food Chem.* **1981**, *29*, 559-563). [b] Amount (ppb) needed to produce a 400-Hz change in the gas phase.

parallels that observed in solution by Ercegovich et al.[1] In their study they found, as indicated in Table III, that parathion required only 50 μg to produce a 50% inhibition of parathion binding to its antiserum, as compared to 70000 μg required for malathion to produce a similar effect. In this same study, 10000 μg of *p*-nitrophenol and 275 μg of reduced parathion were required to produce a similar effect. These studies were carried out in solution with radioimmunoassay which is a much more sensitive technique than the one reported here. It is also much more involved and needs more extensive preparation and instrumentation than the current detection method reported here.

In our study, as shown in Table III, 36 ppb of parathion were required to produce a frequency change of 400 Hz. To obtain the same magnitude of change (as shown in Table III) required 106 ppb of malathion and 158 ppb of methyl parathion. These amounts of interferents are not as large as those found in solution studies but are consistent with them,[1] demonstrating moderate avidity and specificity. It must be remembered, however, that the interferents used in gas-phase studies were not small amounts in the presence of the substrate of interest, as is usually the case in determining antibody specificity in solution. When interferents were studied, the carrier gas was totally saturated with the interferent of interest, with no amount of the specific substrate, even as a trace. Even under such drastic conditions, a substantially higher gas-phase concentration of the interferent was required to produce a response approximately equal to half the response from 36 ppm of parathion, as shown in Table III. 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 nonspecific adsorption of the pesticides by the antibody molecules.

Lifetime and Reversibility. The average lifetime of these crystals was about a week with nonimmobilized antibodies. This was surprising, since antigen/antibody interactions are sensitive to temperature, pH, and slight variations in the reaction medium and have short lifetimes. The few crystals that lasted longer than a week generally showed a very rapid decline in response and sensitivity after one week, as shown in Figure 3.

The response of these antibody coatings was shown to be completely reversible: the same frequency signal was observed before and after repeated exposure to antigen (within the experimental reproducibility of 6%).

Discussion

Antigen/antibody reactions, like enzyme–substrate reactions, are unique in many ways. They are specific, sensitive, and fairly rapid, but unlike enzyme substrate reactions they are known to be concentration dependent. Excess of either antibody or antigen generally prevents or diminishes antigen/antibody interaction. For best results, very stringent antibody/antigen ratios have to be used within a very narrow pH range, generally in a physiological medium. Because of these requirements, most antigen–antibody reaction studies have been made in aqueous media. These requirements, and stringent conditions, have made this area less attractive to some researchers.

By varying the flow rate of the carrier gas and the temperature of the liquid sample, the vapor pressure and the amount of sample in the gaseous state can be increased. The concentration of the

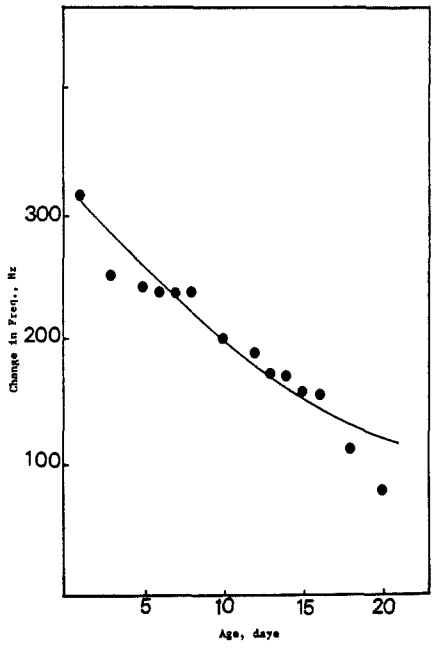

Figure 3. Long-term stability studies on the response of the coating with age.

substrate in the gas phase can be calculated by using the following equation[27]

$$C\ (\text{ppm}) = (1 \times 10^6)P_n/P$$

where P_n is the partial pressure and P the total pressure at the particular temperature. Such data are summarized in Table I. A calibration curve constructed with use of this approach shows a linearity between ΔF and parathion concentrations in the range of 2–35 ppb, with a relative standard deviation of 6%.

Antigen/antibody interactions are known to be a function of the antigenic site and involve noncovalent bonds that may or may not accompany conformational changes of either antigen or antibody. However, if conformational changes do take place, they are known not to lead to denaturation or loss of specificity. The ability of antibodies to recognize minute amounts of antigens or haptens has made their use very practical and essential in the detection of antigens and offers promise of being an outstanding analytical reagent.

Unfortunately, while a lot is known about antigen/antibody behavior in solution and physiological media, little or nothing is known about this behavior in the gas phase. The results obtained in this study demonstrate that a piezoelectric crystal coated with specific antibody could be used as an analytical tool in gas-phase analysis.

At the moment there is no one parameter that can be used as a measure of immunological specificity with absolute confidence. 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 selectivity. In this study the frequency response obtained with a known concentration of interferent, compared with the response from parathion, was used as a measure of how specific the parathion antibodies are to parathion and structurally related molecules. A summary of these data is presented in Table III. While all interferents showed some response on exposure to all antibody coated crystals, it should be noted that from 3 to 20 times more interferent than parathion was required to produce a comparable change in frequency. Because of the low vapor pressure of parathion and the need to conduct these experiments at low temperatures, it was not possible to generate higher concentrations of parathion than 35.5 ppb. This concentration was about one-third the amount of malathion required to produce a similar change in frequency. Under normal conditions, it is impossible to encounter a sample that is 100% in interferents. Methyl parathion, the close analogue of parathion tested which differs by only one CH_2 group, required almost five times (158 ppb) more substrate in the gas phase to produce a similar response. These observations of the reactivity in the gaseous phase confirm and further support what other workers found for parathion antibodies in solution using radioimmunoassay (see data from Ercegovich et al. in Table III).[1] Thus we predict the possible usefulness of some immunochemical reactions for analysis in the gaseous phase.

Antibodies produced from homologous antigens will usually have the ability to cross-react with other antigens, but to a much lesser extent than with their specific antigens. While this might be the case in this study, it also shows conclusively that the frequency change is not a result of protein–protein interaction (since IgG and Bovine Serum Albumin (BSA) show no reaction) but that of a specific antigen/antibody adsorption and desorption process, which parallels the reactivity in solution.

Conclusions

The results obtained in these studies are significant, in that up to the present time very little has been known about antigen/antibody activity in the gas phase. The fact that none of the common gases used seem to inhibit the antigen/antibody activity makes the future use of piezoelectric crystals as immunochemical sensors for direct measurement of antigens in the gas phase very promising. The absence of the need for an inert carrier gas is another reason for optimism.

Despite the very promising results, however, a number of unknown parameters must be determined before such devices can be used as routine immunochemical sensors. The degree of avidity and selectivity in solution as shown in Table III is a lot higher than we found in the gas phase. Because of this, the nature of antigen/antibody interactions in the gas phase has to be investigated more carefully. Binding affinities, association and dissociation rates and constants, and antigen/antibody ratios needed for detectable reactions have to be determined. The lifetime of coatings that are not chemically immobilized on the crystal has to be investigated further. The need for an independent analytical method to confirm antigen/antibody interaction might be the most serious problem, in that it defeats the purpose of such a device (a fast, cheap and sensitive immunochemical sensor). Such an independent confirmation, fortunately, needs to be done only once, for each antibody type.

On the basis of the results obtained in this study, this technique deserves further investigation. Work is currently underway directed at resolving some of these problems, through the use of reversible anchoring reagents for immobilization. If successful, this will provide answers to some of the problems mentioned above and allow for an easy replacement of inactive molecules on the crystal.

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Registry No. Parathion, 56-38-2; quartz, 14808-60-7.

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