Enzyme-Linked Immunosorbent Assay for Paraquat and Its Application to Exposure Analysis

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An enzyme-linked immunosorbent assay (ELISA) was developed for the determination of paraquat in human exposure samples. Using an antibody dilution of 1/5000, concentrations of paraquat cation in the range 0.1–27 ng/mL could be measured. Limit of detection ranged from 0.1 to 1.0 ng/mL depending on the matrix analyzed. The method had good precision, with less than 5% between-run and less than 4% within-run variation, and was selective for paraquat showing minimal cross reactivity with ethylparaquat, diquat, and other compounds. In comparison with a gas chromatographic method, the ELISA gave higher recoveries, was less labor intensive, and was more sensitive. The ELISA was applied to paraquat in high volume glass fiber filters, personal air monitors, worker clothing patches, and hand washes collected during aerial spraying of cotton. When the same filters were analyzed by both ELISA and GC, the ELISA consistently resulted in higher values in keeping with the greater efficiency of the sample preparation steps of ELISA.

Due to their selectivity, sensitivity, and simplicity, immunoassays are useful for determining a wide variety of compounds (*1*). Previous reports on the development of pesticide-specific antibodies indicate such immunochemical procedures can offer advantages over gas chromatography (GC) and high-performance liquid chromatographic (HPLC) methods (*1, 2*). For instance, immunoassays for *S*-bioallethrin (*3, 4*), parathion (*5*), and other pesticides (*6–11*) demonstrate the applicability of immunochemical technology in environmental areas. For water-soluble compounds or compounds with low volatility, immunoassays can be faster, cheaper, and significantly more sensitive and reproducible than analytical procedures now employed.

Paraquat, an extensively used herbicide, was chosen in order to evaluate the advantages and disadvantages of immunoassays with field-generated samples. While the acute oral toxicity of paraquat is well documented (*12*), the hazard to humans through inhalation or other routes of low level exposure has not been fully investigated (*13–15*). For example, recent studies suggest that some varieties of Parkinsonian-type disorders may be linked to an environmental component such as exposure to paraquat or paraquat-like compounds (*16*). The sensitivity and selectivity of an ELISA could be useful in correlating environmental levels of the pesticide with incidences of these disorders. The pathogenesis of acute paraquat toxicity is not fully understood (*13*), but it is known to affect levels of glucose, insulin, catecholamine, and enzyme activity (*17*). Regardless of the route of exposure, paraquat toxicity is usually centered in the lungs (*18*). Animals exposed to paraquat aerosols developed pulmonary fibrosis (*19*). Although the field safety record of paraquat is good, there remain several areas of potential concern. Paraquat spray drift of over 200 m from the source can severely damage nontarget plants (*20*). An EPA advisory opinion regarding paraquat use on cotton recommends that agricultural workers and human residences not be situated within 400 m downwind of paraquat spray operations (*21*). In addition, airborne residues of dust generated during the harvesting of cotton 4 weeks after applications of paraquat contain significant levels of paraquat and 70% of the harvest dust is in the respirable range (*22*).

Availability of a rapid and inexpensive assay could facilitate collection of data on exposure levels in such situations, to assess potential health effects from occupational and incidental contact with the herbicide. Most of the analytical methods reported for paraquat lack the desired characteristics. For example, the radical cation formed by reduction of paraquat can be measured spectrophotometrically with a sensitivity of 0.1 ppm for a 50-g sample of crop, soil, or water (*23–26*); however, the reduction must be preceded by rather laborious acid extraction, cleanup, and concentration by cation-exchange chromatography. For analysis by GC, samples are prepared similarly to the spectrophotometric procedure and then either fully reduced (*27*) or reduced to the mono- and diunsaturated derivatives (*28*). Both ion pair (*29*) and cation-exchange (*30*) HPLC procedures have also been developed for prepared samples yielding sensitivities in the nanogram range. Procedures such as these are relatively cumbersome, which limits the scope of studies conducted to assess human exposure. Immunochemical methods reported for measuring low levels (5 ng/mL) of paraquat in plasma (*31–33*) offer ways to overcome the disadvantages in existing methods. Monoclonal antibodies for paraquat have also been reported for use in plasma (*34*).

This study was undertaken to develop a simple indirect, enzyme-linked immunosorbent assay (ELISA) for paraquat and assess its application to environmental samples such as those generated in worker exposure studies. We include details on the synthesis of paraquat haptens and their subsequent conjugation to proteins, and applications of antibodies as analytical reagents for determining paraquat on glass fiber filters, cotton clothing patches, and hand washes generated during an aerial application of paraquat to cotton. We also show the applicability of the assay to plasma, urine, lymph, and protein samples.

EXPERIMENTAL SECTION

Reagents. Alkaline phosphatase goat anti-rabbit IgG conjugate was purchased from Miles Laboratory (Elkhard, IN). Conalbumin (ConAl), ovalbumin (OA), bovine serum albumin (BSA), human serum albumin (HSA), keyhole limpet hemocyanin (KLH), and complete Freund's adjuvant (CFA) were obtained from Calbiochem (San Diego, CA). Paraquat dichloride, *p*-nitrophenyl phosphate, and additional KLH were from Sigma Chemical Co. (St. Louis, MO). Isobutyl chlorocarbonate, tri-*n*-butylamine, and 4,4′-bipyridine were purchased from Eastman Kodak Co. (Rochester, NY). Ethylparaquat dibromide and paraquathexanoic acid derivative were supplied by Imperial Chemical Co. (Jealott's Hill Research Station, U.K.). Methyl-^{14}C-paraquat and paraquat were supplied by R. Krieger (University of Idaho, Moscow, ID). Monomethylpyridone was supplied by P. C. Kearney (USDA,

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Beltsville, MD). The paraquatvaleric acid derivative used in initial experiments was supplied by Factori and Hunter (National Radiological Protection Board, Glasgow). Incomplete Freund's adjuvant (IFA) was from GIBCO (Grand Island, NY). Ethyl 5-bromovalerate, ethyl 4-bromobutyrate, ethyl 3-bromopropionate, and iodomethane were purchased from Aldrich Chemical Co. (Milwaukee, WI). Solvents were of analytical grade.

Instrumentation. An Hewlett-Packard (HP) 5710A gas chromatograph with a 30-m fused silica DB-1 capillary column equipped with a nitrogen/phosphorus detector was used throughout the analysis. Quantitative measurements based on peak height were performed by a HP 3390A integrator recorder. Temperatures were 180 °C (column) and 250 °C (injector port and detector). NMR spectra were obtained with a Varian EM360 90-MHz spectrometer from D_2O solutions referenced against sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS). A Cary 219 scanning spectrophotometer was used to obtain UV spectra of proteins and haptens. Radiolabeled paraquat was monitored by a Packard Model 2506 Tri-Carb liquid scintillation spectrometer. Decomposition points of synthesis products were obtained on a Fischer-Johns melting point apparatus. The ELISA was performed in polystyrene cuvettes (Gilford) and read in a Gilford manual EIA reader equipped with a 405-nm filter. Elemental analyses were done by Galbraith Laboratories, Knoxville, TN.

Synthesis of Monoquat. 4,4′-Bipyridine and iodomethane were reacted in 1.05:1.00 molar ratio in dry chloroform with stirring at 4 °C under a calcium chloride drying tube (*32*). After complete addition the reaction mixture was stirred overnight at room temperature. The product, *N*-methyl-4-(4-pyridyl)pyridinium iodide (monoquat), was collected by suction filtration, washed with dry chloroform, and dried in a vacuum desiccator. Recrystallization from 95% ethanol gave a yield of 80%. The compound was dried in an Abderhalden apparatus for 24 h and then stored in a vacuum desiccator. The product had a decomposition point of 244 °C. Structure confirmation was done by NMR showing ring proton resonance at 8.8 (4 H, m), 8.35 (2 H, d), and 7.85 ppm (2 H, m) and alkyl proton resonance at 4.35 ppm (3 H, s).

Synthesis of Paraquat Derivatives. Monoquat and ethyl 5-bromovalerate were reacted in a 1:2 molar ratio by stirring in freshly distilled dry dimethylformamide (DMF) at 120 °C for 4 h and then at room temperature overnight. The product was collected by suction filtration and washed with dry DMF. NMR of the intermediate confirmed the desired ester: ring proton resonances were at 9.2 (4 H, m) and 8.6 ppm (4 H, m); alkyl proton resonances at 4.75 (2 H, t), 4.5 (3 H, s), 4.2 (2 H, q), 2.5 (2 H, t), 2.2 (2 H, m), 1.7 (2 H, m), and 1.15 ppm (3 H, t). The signal from the CH_2– directly attached to N was partially obscured by the H_2O peak. The ester was hydrolyzed to the corresponding acid by refluxing with concentrated HCl for 60 min. The acid solution was evaporated to dryness with a rotary evaporator (60 °C bath temperature) leaving a solid residue of the desired product. The residue was crystallized from a minimum volume of acetone and recrystallized from 95% ethanol giving a 60% yield and a decomposition point of 220 °C after drying for several days in an Abderhalden apparatus. NMR showed loss of the ethyl ester indicating that the product was the free acid, *N*-(4-carboxybut-1-yl)-*N*′-methylbipyridilium, with traces of acetone impurity not removed by drying. Elemental analysis indicated that both bromide and chloride ions were present, which caused difficulty in calculating the theoretical elemental composition. Anal. Calcd for $C_{18}H_{20}N_2O_2Br_2$: C, 44.4; H, 4.6; N, 6.48; Br, 37.0. Found: C, 41.62; H, 4.52; N, 6.4; Br, 36.19; and Cl, 1.85. The same general synthesis was used to prepare the butyric and propionic acid derivatives of paraquat. Both the ester intermediates and final products of each synthesis were initially oils requiring several attempts before crystallization. NMR was used to confirm the final product as well as to identify the ester intermediate in each synthesis. NMR of the carboxylic acid final product was the same as that of the ester intermediate except for the loss of the alcohol. NMR analysis of the butyrate ester intermediate showed ring proton resonances at 9.2 (4 H, m) and 8.6 ppm (4 H, m) and alkyl proton resonances at 4.7 (2 H, t), 4.5 (3 H, s), 4.2 (2 H, q), 2.6 (2 H, t), 2.4 (2 H, m), and 1.2 ppm (3 H, t). The acid final product was obtained in a 50% yield and had a decomposition point of 211 °C; NMR confirmed complete hydrolysis of the ester. The propionic acid derivative, obtained in a 31% yield, had a decomposition point of 202 °C. The ester intermediate showed ring proton resonance at 9.20 (4 H, m) and 8.8 ppm (4 H, m) and alkyl proton resonance at 5.1 (2 H, t), 4.5 (3 H, s), 4.2 (2 H, q), 3.3 (2 H, t), and 1.2 ppm (3 H, t). Anal. Calcd for $C_{13}H_{18}N_2O_2Br_2$: C, 41.59; H, 4.0; N, 6.9; Br, 39.6. Found: C, 42.53; H, 4.90; N, 7.53; Br, 24.60; and Cl 8.91. Discrepencies were due to acetone entrapped in the crystals and partial exchange of Cl for Br during acid hydrolysis.

Antigen Preparation. Haptens were coupled to protein using the mixed anhydride method (*35*, *36*). The conjugation of hapten to protein was done in a molar ratio of either 30:1 or 15:1. The haptens were dissolved with stirring in a minimum amount of dry dioxane and formamide. To the stirred solution was added tri-*n*-butylamine (0.2 mmol excess) followed after 1 min by isobutyl chlorocarbonate (0.2 mmol excess). The resulting mixture was stirred at room temperature for 30 min and then added dropwise to a solution of protein (50 mg/10 mL) in a 0.2 M borate buffer (pH 8.7) followed by stirring at room temperature overnight. The conjugates were then dialyzed in the cold for 3 days in 7.0 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl (0.02% sodium azide—if antigen not used for injecting). Protein concentrations of the conjugates were determined spectrophotometrically using molar extinction coefficients of free haptens to calculate the amount present in the conjugates. The degree of hapten substitution was calculated at 4 mol/100 000 daltons of protein. Proteins used for conjugation included KLH, OA, HSA, BSA, and ConAl. Although two molar ratios were employed in the preparation of coating antigens, there appeared to be no significant difference between the two preparations in their ability as coating antigen and in the detection and quantitation of paraquat in the ELISA. The KLH conjugate (PQ-KLH) was found to be the superior coating antigen in spite of a somewhat high assay background as is commonly observed with KLH. The ConAl conjugate was found to be the best immunizing conjugate and was used throughout the study. Conjugates were stored at −20 °C.

Immunization. New Zealand white rabbits were immunized with paraquat antigen in 7.0 mM phosphate buffer (pH 7.4) containing 0.15 M NaCl (phosphate buffered saline, PBS) emulsified with an equal volume of complete Freund's adjuvant. A total volume of 1.2 mL of the emulsion was injected at multiple subcutaneous and intradermal sites. Booster injections of the same antigen emulsified in incomplete Freund's adjuvant were given at monthly intervals. Blood was obtained from the rabbit's ear artery a week to 10 days after the injection. Serum was separated by centrifugation and stored at −20 °C. With ELISA, anti-paraquat antibodies were seen 3 months after the initial injection. Repeated immunizations with the same antigen did not increase the titer of specific antibody, as rabbits having the highest antibody titer initially remained the high antibody producers during the entire course of the study.

ELISA. A solid phase indirect competitive ELISA was used for quantitation (ref 37, Figure 1). Cuvettes of polystyrene, contained in Gilford EIA plates, were sensitized by passive adsorption with 250 μL of a 1 μg/mL solution of the coating antigen PQ-KLH in a 0.1 M sodium carbonate–bicarbonate buffer (pH 9.6). The cuvettes were covered with Parafilm and kept overnight in a moist chamber at 4 °C. On the same day, rabbit antiserum containing paraquat-specific antibodies was diluted to 1/5000 in phosphate buffer saline solution (pH 7.4) containing 0.5% Tween 20 and 0.02% sodium azide (PBS–Tween), and added to dried acid (6 N HCl) extracts of samples containing paraquat. Several serial dilutions of these samples were made in polystyrene tubes with antibody in PBS–Tween to obtain at least one dilution within the linear region of the standard curve. The antibody was allowed to react with the samples overnight at 21 °C. The standard curve was made by taking an aliquot of the stock solution of paraquat (1.02 mg/mL based on cation) in 6 N HCl, evaporating the aliquot to dryness in polystyrene tubes, adding antibody, and making several serial dilutions. The standard curve could be generated in this way as the acid salts contained in the small aliquot were shown to not interfere with the assay. One or two standard curves were routinely run per assay. The ELISA was performed in polystyrene tubes on samples after extraction with 6 N HCl and

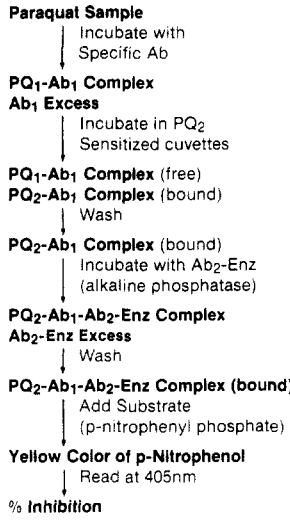


Figure 1. Schematic of ELISA procedure for paraquat.

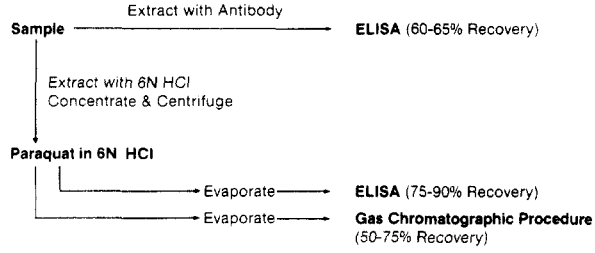


Figure 2. Analytical scheme for paraquat accommodating two variations for ELISA and the gas chromatographic procedure. For the GC procedure and ELISA with acid extraction, recoveries are based on three determinations of four spiking levels and checked using radiotracers. Variation in recovery for GC ranged from 50 ± 12% at the 50 ng level to 72 ± 9% at 1.0 μg. Recoveries for ELISA range from 77 ± 9% at the 50 ng level to 93 ± 5% at 1 μg.

evaporation (Figure 2). On the following day, the plates of cuvettes were washed with PBS–Tween to remove all unbound coating antigen. The incubation mixture of sample and antibody was added in 250-μL amounts to the cuvettes and incubated for 2 h at 21 °C. After the plates were washed again with PBS–Tween, a 1/2500 dilution of goat anti-rabbit IgG labeled with alkaline phosphatase was added to each cuvette. Following another 2 h incubation at 21 °C the plates were washed for a final time and 250 μL of the substrate *p*-nitrophenyl phosphate (1 mg/mL) in 10% diethanolamine buffer (pH 9.8) was added. The amount of degradation of substrate was indicated by the librated yellow color of *p*-nitrophenol. The color intensity was inversely proportional to the amount of paraquat in the samples. The phosphatase activity was stopped 30 min later by the addition of 50 μL of 3 N NaOH. Absorbance was read at 405 nm in a Gilford manual EIA reader. Maximum binding was assessed by adding no inhibitor to the antibody and background was assessed by adding no antibody to the system. Using normal rabbit serum rather than the paraquat antiserum showed virtually no binding to sensitized cuvettes, indicating nonspecific background binding in the assay was negligible. Even though biological samples were not obtained from workers, the utility of ELISA for such samples was assessed by spiking protein, human urine, and sheep lymph and plasma with paraquat followed by analysis in the same manner as for the aqueous standard curves.

Because paraquat is known to bind to many types of surfaces, nonspecific binding of paraquat to polystyrene and glass was evaluated as follows. Dilutions of methyl-^{14}C-paraquat, in PBS–Tween without antibody, were made following the same procedure as was used in the standard ELISA and allowed to stand overnight. Concentrations of radiolabeled compound corresponding to 22, 11, 5.5, 2.7, and 1.4 ng/mL were analyzed in quadruplet for both polystyrene and glass test tubes. For the polystyrene tubes 100% of the radioactivity was recovered for the entire concentration range. For the glass tubes the percentage decreased with each decreasing concentration. The amount of radioactivity obtained was 87%, 66%, 18%, 11%, and 6% for the dilutions used.

Exposure Study. Five workers were monitored to estimate their exposure levels to paraquat used as a cotton defoliant. The tank formulation consisted of 0.47 L of Ortho paraquat (29.1%, a.i.) dissolved in 38 L of water applied per acre of cotton. The tank mix also included magnesium chlorate, Agridex (mixture of paraffins and fatty acid esters), and Nalcol-Trol. The exposure group consisted of one pilot, one mixer/loader, two flaggers, and one control. Exposure was estimated by the use of clothing patches, air sampling in worker breathing zone, high-volume air sampling in the worker's vicinity, and hand washes. Each subject wore disposable overalls to which were pinned patches consisting of 65/35 dacron/cotton cloth, underlined with a 12-ply layer of 100% cotton gauze and aluminum foil. The central 7 × 7 cm section of the patch was removed for analysis. Each person was outfitted with nine patches positioned as follows: posterior neck (1), upper chest (2), upper arms (2), wrist (2), and anterior thighs (2). The only exception was the pilot, who did not wear disposable overalls, but instead wore a short sleeved shirt and trousers; the wrist patches were thus not placed on this subject. The posterior neck patch was used to estimate exposure to back of head and neck, chest patches for face and neck, shoulder patches for upper arms, chest and posterior neck for trunk, thigh patches for legs, and sleeve patches for hands (*38*). The corresponding left and right cloth patches for each individual were combined for analysis. The posterior neck patch was kept separate. Patches were stored in glass jars, put on dry ice in the field for transporting to the lab, and stored at −20 °C until analyzed. Airborne concentrations of total paraquat in the breathing zones of all workers and one control were determined with MSA portable pumps, Model S, personal air samples. Instruments were operated at a flow rate of 700 mL/min with a glass fiber filter (1.8 cm in diameter) situated in the sampling portion attached in the breathing zone of the workers. The hands were rinsed with 100 mL of 95% ethanol at the beginning and the completion of the application. High-volume air samples (Sierra-MISCO, Berkeley, CA) were fitted with 8 in. × 10 in. Gelman type A glass filters and operated at a flow rate of 1 m^3/min. For the samples taken before, during, and after application, the sampling durations were 1, 2.5, and 0.75 h, respectively.

Comparative Determination of Paraquat by Colorimetry and Gas Chromatography. Accuracy of the ELISA was determined by comparing the assay with a colorimetric procedure (*39*) on fortified samples of water and agricultural wastewater. The GC analysis was performed essentially as previously reported (ref 22, Figure 2). Correlation coefficients of the two methods were determined for fortified water and acid extracts of glass fiber filters. The high-volume air filters obtained in the field were cut in half, with half analyzed by ELISA and the other half by GC. For GC analysis, the half filters were again cut in half, each half placed in a 50-mL Nalgene test tube, covered with 30 mL of 6 N HCl, and sonicated for 60 min. The supernatant was decanted into a 300-mL round-bottom flask. The filter was rinsed 3 times with 5 mL 6 N HCl, with each rinsing added to the flask. The acid extract was next concentrated by rotary evaporation and transferred to a centrifuge tube. Samples were then centrifuged 3 times, and the supernatants were combined and evaporated. The dried residues were extracted 3 times with NH_4HCO_3, evaporated to dryness, and reduced with $NaBH_4$ (*22*). The reduced products were extracted 3 times with ethyl acetate, concentrated, and analyzed by GC using an NP detector. The extraction schemes for both methods were monitored with methyl-^{14}C-paraquat.

Antibody Used for Extraction. Glass fiber filters were cut in quarters and each quarter spiked with a 6 N HCl paraquat stock solution at 4, 0.25, or 0.062 μg. The spiked filters (triplicates of each spiking level) were kept at room temperature overnight to ensure evaporation of the acid. Each filter was placed in a 50-mL polyethylene test tube, covered with 20 mL of PBS–Tween (pH 7.4) and macerated with a Tekmar tissumizer (setting at 40) for 1 min. The pH was adjusted to 7.4 with NaOH. An antibody solution in PBS–Tween (pH 7.4) was added to each tube to give a final antibody dilution of 1/2500. Tubes were stoppered and secured on a shaker table in the cold (4 °C) for 1–2 days. After centrifugation, aliquots were removed and analyzed by ELISA. Using an antibody dilution of 1/2500 only slightly decreased assay

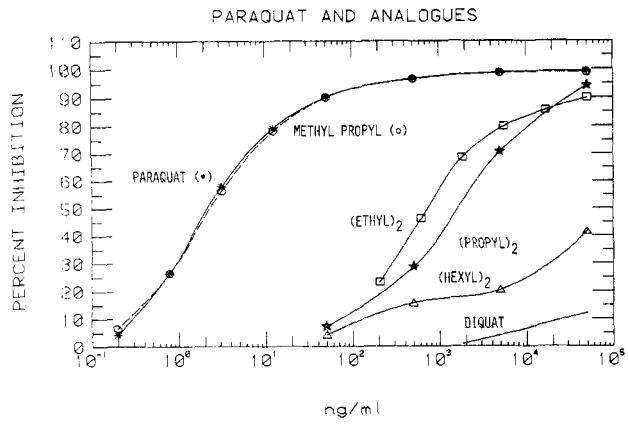


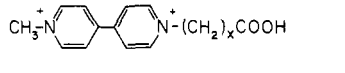
Figure 3. ELISA standard curves for paraquat and five related bipyridilium salts. Results based on triplicate determinations, within run variations 5% or less.

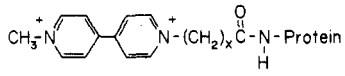
sensitivity, giving I_{50} values of 2.5 ng/mL and 5.0 ng/mL, respectively, for the 1- and 2-day incubations.

RESULTS AND DISCUSSION

Assay Development and Optimization. Inhibition studies were conducted to investigate the ability of several different compounds to prevent the binding of paraquat antiserum to sensitized cuvettes. Compounds tested included several structural analogues of paraquat, metabolites, and several commerical products including those used in conjunction with paraquat. The results (Table I) are expressed as the amount of compound in μg/mL giving the maximum percent inhibition of a standard amount of antibody. As expected, paraquat required the least amount to give the strongest inhibition among all the compounds tested, with the exception of the methylpropyl analogue, which closely paralleled the curve for paraquat (Figure 3). Based upon the immunizing and coating antigen used, one would expect the assay to be highly specific for bipyridinium compounds containing at least one *N*-methyl group. Some variation in the other substituent would not be detected, as was shown by the methylpropyl analogue; however no such compounds are used commercially nor are they metabolites of paraquat.

Development of a useful indirect ELISA requires the optimization of coating antigen structure and concentration, as the choice of coating antigen influences assay sensitivity and specificity. Sensitivity is often enhanced when the antibody population has a higher affinity for the compounds to be analyzed than for the coating antigen. Conversely, antibodies with too high an affinity for the coating antigen relative to the compound being analyzed usually lead to low assay sensitivity. Early studies indicated that KLH yielded the highest sensitivity and the lowest background of the four carrier proteins examined as coating antigens. A variety of antisera and coating antigen combinations were evaluated, but none appeared as useful as the valeric acid system. For example, when propionic and hexanoic acid handles were used on the paraquat coating antigen with the standard antiparaquat antiserum, the assay was less sensitive for paraquat (I_{50} 5 ng/mL and 12 ng/mL, respectively) and less capable of distinguishing between paraquat and propylparaquat.





x = 2–5

In subsequent studies the hapten with the 4 carbon spacer arm (valeric acid derivative) was used as both the injecting and coating antigen. The optimal coating antigen concentration and antibody dilution was determined by checkerboard titration (*37*). Positive readings were detected for sera with a concentration of coating antigen as low as 156 ng/mL, and the plateau was reached at concentrations greater than 1 μg/mL for the antiserum dilution used. A solution of 1 μg/mL for the coating antigen was selected for this study. When KLH or *S*-bioallethrin conjugated to KLH was used as the coating antigen, titers <100 were observed, compared to 5000 when PQ-KLH was used. These results indicate the high selectivity of the antisera to paraquat.

Each sample concentration was run in triplicate to calculate the within-run variation. The standard deviations for all points of the inhibition curves were usually 4% of the absorbance values. The coefficient of variation for the percent inhibition for any single concentration of paraquat was 4% of the values obtained from several runs performed on widely separate occasions. The ELISA correlated well with a colorimetric procedure when fortified water (r = 0.914) and agricultural wastewater (r = 0.995) were analyzed. The ELISA also compared well with a GC procedure when analyzing fortified water (r = 0.983) and acid extracts of glass fiber filters (r = 0.995).

Antibody Extraction. The antibody was used for extraction of paraquat as well as its detection. Average recoveries were 60% (I_{50} 40 ng/mL) for 2-day incubation and 45% (I_{50} 70 ng/mL) for 1-day incubation. Recoveries were lower if the filters were cut into small pieces rather than macerated. To determine if the antibody was actively extracting paraquat, spiked glass fiber filters were incubated for 1 to 2 days in buffer alone. Aliquots were removed, reacted with specific antibody, and analyzed by ELISA. With this extraction procedure no paraquat could be detected. Thus paraquat was not simply being removed by the buffer but was being actively extracted by the antibody. In our subsequent assays of filters and all other solid matrices, the acid extraction was used rather than the antibody extraction to conserve antisera. When antibodies were used for extraction, a large buffer volume was needed in macerating the samples, requiring a large amount of antisera. Concentration of buffer containing filter particles prior to addition of antisera could circumvent this problem, but was not pursued. Samples such as soil or ground plant material, which may not need macerating, would be better adapted to the antibody extraction. As larger amounts of antibody become available, possibly via hybridoma technology (*34*), antibody extraction of pesticides from difficult matrices could become the method of choice. The sample could simply be incubated with antibody, eliminating acid extraction, prior to ELISA determination.

Worker Exposure Samples. The concentrations in high-volume air samples taken during the application of paraquat as a cotton defoliant were almost 2 orders of magnitude higher than in samples taken immediately after spraying (Table II). The background sample taken before spraying contained a low level of paraquat indicative of ambient contamination with the herbicide in the cotton field environment. The ELISA gave higher values than GC in all samples due to the greater efficiency of the ELISA methodology (75–90%, Figure 1) compared with GC (60–65%). At low levels of paraquat, quantitation by GC was difficult due to interfering peaks in the retention region of the monoene and diene reduction products of paraquat.

From personal air samples taken in the worker's breathing zones, flaggers showed the highest respiratory exposure followed by the mixer/loader, pilot, and control (individual in the field not directly working with paraquat, Table III). These observations correlate well with results reported from other worker-exposure studies (*14*, *40*) but are lower than those

Table I. Maximal Inhibitions Caused by Several Compounds in the ELISA Specific for Paraquat

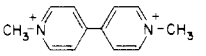
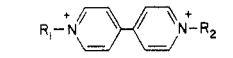
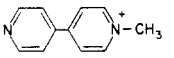
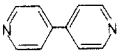
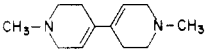
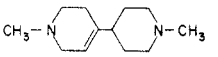
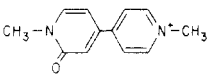
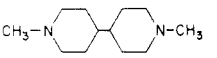
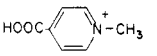
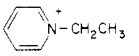
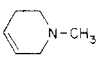
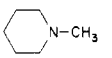
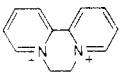
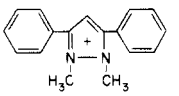
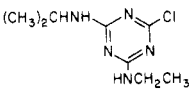
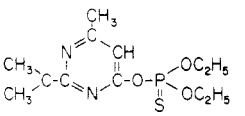
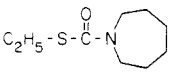
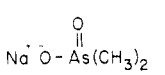
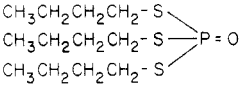
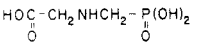
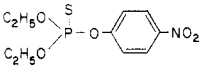
chemical name or category	structure	% maximum inhibition	amt giving max inhib, μg/mL
paraquat		100	0.2
paraquat analogues			
	$R_1 = C_2H_5$; $R_2 = C_2H_5$	89	50
	$R_1 = CH_3$; $R_2 = C_3H_7$	100	0.2
	$R_1 = C_3H_7$; $R_2 = C_3H_7$	94	50
	$R_1 = C_6H_{13}$; $R_2 = C_6H_{13}$	42	50
	$R_1 = C_8H_{17}$; $R_2 = C_8H_{17}$	32	100
	$R_1 = t\text{-}C_4H_9$; $R_2 = t\text{-}C_4H_9$	18	50
	R_1 = benzyl; R_2 = benzyl	45	100
paraquat metabolites and related compounds		31	200
		14	50
		0	100
		0	100
		32	50
		0	100
		8	100
		7	100
		5	100
		5	100
diquat		20	50
difenzoquat		14	50
atrazine		0	100
diazinon		20	100
molinate		31	100
sodium cacodylate		15	100
DEF		12	100
glyphosate		16	37
parathion		16	100

Table II. Comparison of GC and ELISA Results for High-Volume Air Samples

time	sample no.	GC amt, μg	GC concn, μg/m³	ELISA amt, μg	ELISA concn, μg/m³
before application (background)		0.20	0.10	0.35	0.18
during application	1	18.02	7.21	29.91	11.96
	2	31.12	12.45	55.82	22.33
1 h after application	1	–[a]	–[a]	0.52	0.26
	2	0.36	0.48	0.51	0.68

[a] Interferences precluded quantitation.

Table III. Paraquat Found in Breathing Zone Air Samples of Workers by ELISA

subject	vol of air sampled,[a] L	paraquat found amt, ng	paraquat found concn, ng/m³
pilot	82.5	16.9 ± 0.37[b]	205 ± 5[b]
mixer loader	114.0	48.4 ± 2.22	422 ± 15
flagger 1	79.5	87.7 ± 0.66	1103 ± 7
flagger 2	87.0	215 ± 12.56	2471 ± 134
control	138.0	12.3 ± 0.73	89.4 ± 5.3

[a] Sampling duration 120 min. [b] Standard deviation for three determinations.

Table IV. Paraquat Found by ELISA in Hand Rinse Samples of Workers before and after 1-Day Shift[a]

	before application,[b] ng	after application,[c] ng
pilot	117.6	5799 ± 234
mixer loader	35.9	180 ± 18
flagger 1	50.0	2807 ± 170
flagger 2	125.7	3161 ± 268
control	*d*	6.6 ± 0.1

[a] Handwash bottles rinsed 3 times with 6 N HCl. [b] Average of two determinations. [c] Average and standard deviation of three determinations. [d] Not available.

reported for workers using boom applications of paraquat on tomatoes and citrus (*41*). Results were consistent with the amount of exposure each of these workers would be expected to encounter based upon our observations of their tasks. All respiratory values were much lower than the threshold limit value of 0.5 mg/m³ (*42*). For analysis of hand exposure, workers hands were rinsed with ethanol before and after their occupational activity with the chemical. It appeared that the paraquat became adsorbed on the walls of the glass sample bottles during storage. Thus, the emptied sample bottles were washed 3 times with 6 N HCl, to recover adsorbed paraquat. Each subject's hands had very low levels of paraquat before the field application of the compound (Table IV). After application there was an increase in paraquat in all hand rinses, particularly for the pilot and flaggers. The increase observed for the pilot were due to his adjusting the spray nozzles on the plane between applications bare-handed. The mixer/loader had relatively low levels for hand-rinse samples taken both before and after exposure, which could be attributed to rubber gloves worn by this worker. The efficiency of ethanol to remove paraquat from the hands is unknown so that the values determined in this study may underestimate the total hand residues on the workers.

The results from the cotton clothing patches were used to estimate dermal exposure (Table V). The total body surface area (*43*), and the surface area proportions of various anatomic regions were calculated for each individual (*44*). The two flaggers had the highest level of exposure as would be expected due to their close proximity to direct fall out. Upper body surface of each showed the highest levels, consistent with the route of exposure. Upper arms showed high levels due to raising of arms for flagging. For all the workers, the dermal exposure (Table V) far exceeded the respiratory exposure (Table III). Dermal and respiratory exposures from a study of workers operating conventional low-pressure spray equipment were comparable to our data (*40*). From 20 different exposure situations studied, the highest dermal exposure was 3.4 mg/h with respiratory exposures ranging from nondetectable to 2.0 μg/h (*40*). Because the efficiency of absorption of dermal doses of paraquat is not known, it may well be that the respiratory exposure is a biologically significant route even though it is much smaller in quantity than the amount deposited on the skin.

This exposure study was viewed as a preliminary test of the ELISA's applicability to various environmental samples. As the ELISA performed well here, a full scale study with larger numbers of workers and work intervals can be conducted.

Biological Samples. A full-scale worker-exposure study could include samples of plasma to assess the extent to which various exposure levels lead to absorption of the chemical. Current GC methods using ethylparaquat as internal standard can detect plasma concentrations of 0.1 μg/mL with FID or 0.025 μg/mL with NP-TSD detector (*45*); however the method has been reported to be quite complex (*46*). By use of the ELISA, sheep plasma diluted 1:1 with PBS was analyzed with no cleanup, with a limit of detection of 2 ng/mL for whole plasma (Figure 4). With such sensitivity plasma concentrations could be monitored several days after exposure to

Table V. Summary of Worker Exposures to Paraquat

body regions	% of total body exposure	exposure per body region,[a,b] μg pilot	mixer loader	flagger 1	flagger 2	control
face & neck	6.9	7.04 ± 0.36	97.0 ± 5.9	153 ± 4	305 ± 11	7.04 ± 0.25
back of head & neck		2.22 ± 0.08	6.15 ± 0.37	778 ± 24	397 ± 19	1.82 ± 0.05
upper arms	10.0	574 ± 28.8	24.7 ± 0.3	2.36 ± 0.07	2.45 ± 0.08	1.30 ± 0.16
forearms	7.2	NA[c]	187 ± 6	434 ± 12	751 ± 16	5.42 ± 0.34
hands	4.2	NA[c]	110 ± 3.27	255 ± 7	441 ± 9	3.49 ± 0.22
trunk	27.0	34.4 ± 0.98	305 ± 16	6.08 ± 0.05	3.89 ± 0.03	31.8 ± 0.68
legs	12.5	28.0 ± 0.80	865 ± 39	241 ± 23	600 ± 42	26.6 ± 1.44
respiratory exposure,[d] μg		0.007	0.015	0.040	0.039	0.003
est total body exposure, mg/h			0.80	0.94	1.25	0.04

[a] Based on clothing patch samples. [b] Based on three determinations. [c] NA = not analyzed. [d] Based on personal air monitors.

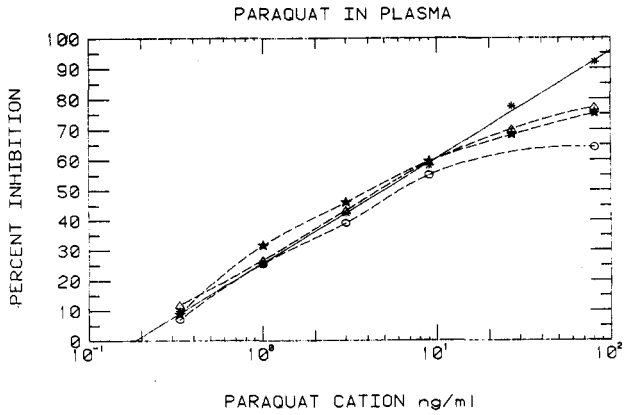


Figure 4. ELISA standard curves for paraquat in sheep plasma diluted with phosphate-buffered saline solution. Results based on triplicate determinations, within-run variations 8%. The symbols and corresponding correlation coefficients (r^2, for the linear portion of the curve only) are as follows: *, aqueous solutions, 0.999; O, 50% plasma, 0.998; ☆, 25% plasma, 1.00; Δ, 10% plasma, 1.00.

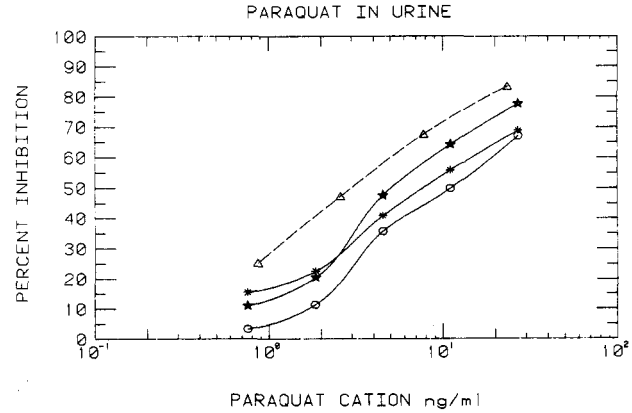


Figure 5. ELISA standard curves for paraquat in human urine diluted with phosphate-buffered saline solution, with pH adjusted to 7.4. Results based on duplicate determinations, within-run variations 6%. The symbols and corresponding correlation coefficients (r^2, for the linear portion of the curve only) are as follows: Δ, aqueous solutions, 0.999; *, 50% urine, 0.999; O, 25% urine, 0.996; ☆, 10% urine, 0.997.

assess the progress of poisoning victims, as plasma concentrations of paraquat are indicative of the extent of poisoning (*47*).

For urine samples, the alkali/dithionite test (*48*) is useful for confirming severe paraquat poisoning, but with its limited sensitivity (1 μg/mL) and susceptibility to false positives, it is of doubtful clinical value (*47*). By use of urine diluted 10:1, 4:1, or 1:1 with buffer a paraquat concentration of 9 ng/mL was determined by ELISA without cleanup or preconcentration (Figure 5).

Lymph had a positive effect on ELISA results and increased assay sensitivity. With lymph diluted 1:1 without any cleanup, the limit of detection was 700 pg/mL for whole lymph (Figure 6). The reason for the enhanced sensitivity is not known, but it was more pronounced with increasing amounts of lymph. The assay was more sensitive in lymph diluted 1:1 than for 4:1 or 10:1 dilutions in buffer.

As paraquat is known to interact with protein (*49*) the ELISA was tested for analysis of paraquat in the presence of various proteins. Various amounts of paraquat were incubated with KLH, HSA, or BSA, at protein concentrations of 1.0 mg/mL, 0.5 mg/mL, and 0.1 mg/mL. Subsequent determination of paraquat with ELISA showed slight protein interference at 1 mg/mL and no interference at 0.1 mg/mL even at a paraquat concentration of 0.333 ng/mL. One explanation for this phenomenon could be the strong affinity of the antibody for paraquat. At a high protein level, protein could

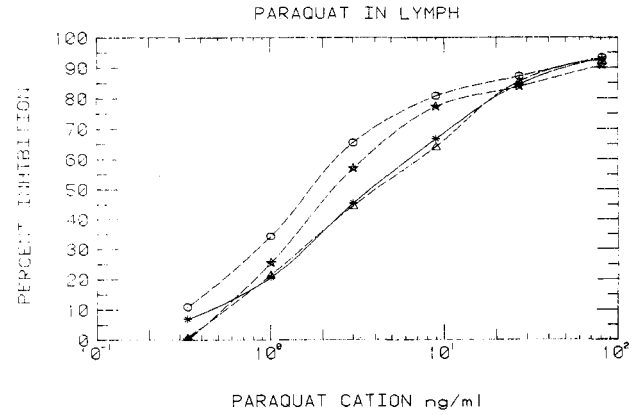


Figure 6. ELISA standard curves for paraquat in sheep lymph diluted with phosphate-buffered saline solution, with pH adjusted to 7.4. The symbols and corresponding correlation coefficients (r^2, for the linear portion of the curve only) are as follows: *, aqueous solutions, 0.998; O, 50% lymph, 0.993; ☆, 25% lymph, 0.996; Δ, 10% lymph, 1.00.

be precipitated before performing the ELISA, or the standard curve could simple be run in the presence of protein.

CONCLUSIONS

The ELISA described in this study is promising for analyzing the presence and fate of paraquat in a variety of physical and biological samples. For worker-exposure studies, ELISA provided lower detection limits and higher recoveries and was considerably faster than a published GC procedure. Such worker-exposure or epidemiological studies generate a large number of samples for which ELISAs are ideally suited. For screening samples, ELISA could also be applied directly to the sample without acid extraction and concentration of the extract, but with some sacrifice in recovery. The use of antibodies to extract or concentrate sorbed compounds from biological or physical matrices could be of general utility in analytical chemistry.

Our findings demonstrate that a simple indirect ELISA can be used in determining the level of paraquat in a variety of sample matrices and could be applied to field use due to its speed and simplicity. Although the assay as described here had sufficient sensitivity, assay sensitivity could be increased by sample cleanup and concentration or by changing the substrate from *p*-nitrophenyl phosphate to a fluorescent substrate such as methylumbelliferyl phosphate. The ELISA's ability to quantitate paraquat in the presence of proteins, plasma, lymph, and urine could also prove useful in pharmacokinetic studies. The inhibition values demonstrate that the selectivity of the paraquat antiserum is sufficient for use in studying the metabolism and pharmacokinetics of paraquat.

ACKNOWLEDGMENT

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Registry No. $Br(CH_2)_4CO_2H$, 14660-52-7; paraquat, 4685-14-7; monoquat, 35022-69-6; *N*-(4-ethylcarboxybut-1-yl)-*N'*-methylbipyridilium bromide, 101697-52-3; *N*-(4-carboxybut-1-yl)-*N'*-methylbipyridilium chloride, 73088-18-3; *N*-(4-carboxybut-1-yl)-*N*-methylbipyridilium bromide, 101697-53-4.

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Quantitative Study of Acoustic Emission from a Model Chemical Process

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The potential of acoustic emission (AE) as a method for monitoring chemical processes has been demonstrated. For this study, a model process, the hydration of silica gel, has been investigated in some detail. As silica gel hydrates, gas is evolved which causes the granules to fracture and produce sound. Integrated AE signals have been used to give quantitative information as to the amount of material present, the average particle size, and the percentage water content of the partially hydrated silica gel. Pattern recognition techniques have classified four types of AE signals from the reaction. Kinetic information has also been obtained about the reaction mechanism involved by analysis of the time intervals between events. This analysis suggests that the hydration of silica gel is a two-step process with a parallel, non-AE-producing pathway. These results indicate that AE monitoring will prove to be an effective method of gaining often unique information about many difficult-to-study chemical systems.

The purpose of this paper is to investigate the usefulness of acoustic emission (AE) monitoring as a method for studying certain physical and chemical processes. Betteridge et al. (*1*) have demonstrated that many chemical reactions are accompanied by the emission of acoustic energy. These range from the obvious such as the "fizz" that accompanies effervescent reactions to the low-level emissions that are produced by certain crystalline-phase changes. Other workers have observed AE from the precipitation of dichloro(pyrazine)zinc(II) (*2*), the gelation reaction of sodium carbonate and calcium chloride (*3*), phase transitions of *p*-cresol and MBBA liquid crystals (*4*), and the polymorphic transformations of a number of DTA temperature standards (*5*). AE has found more widespread use in areas other than the strictly chemical; it is used for corrosion monitoring (*6*) and is routinely used in the plastics industry for studying fracture mechanisms and in nondestructive testing. Applications in these areas have been comprehensively reviewed by Drouillard and Hamstad (*7*). However, in our opinion, it has not been established whether AE is a useful quantitative tool for studying chemical reactions, or what the appropriate analytical techniques are for extracting useful chemical information from AE signals.

For this study, the hydration of silica gel was selected as a model system. This reaction is both acoustically active and not readily amenable to other monitoring techniques. It was

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