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# Bioassays for Detection of Aldicarb in Watermelon

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During the summer of 1985, more than 1200 illnesses reported in California were attributed to the consumption of watermelons. Some clearly involved cholinergic symptoms. Similar illnesses in Oregon were associated with the presence in melons of aldicarb sulfoxide, the primary toxic metabolite of aldicarb. Ten of 250 tests of melons in California were positive for the sulfoxide. (Green et al. 1987). Anticipating that similar problems could occur in the future, a project was undertaken to examine biological methods to demonstrate the presence of carbamates and/or organophosphate esters in watermelons as adjuncts to current analytical methods.

## MATERIALS AND METHODS

Analytical standards (at least 98% pure) of aldicarb, aldicarb sulfoxide, aldicarb sulfone, acephate, and methamidophos were prepared at 1 mg/mL in ethyl acetate. The edible fruit (3 kg) of a ripe commercial watermelon (6.4 kg) was excised; samples of fruit (with no seeds) weighing 300 g were homogenized in a Polytron homogenizer-sonicator on high setting for 2-3 min and filtered under reduced pressure through a Buchner funnel containing Sharkskin filter paper. Residues retained on the filter were used for the pulp samples. Filtrates were refiltered through Whatman No. 934-AH glass microfiber filters under a slight vacuum to yield a colorless, translucent liquid used for the juice and concentrated juice samples. GC analysis of spiked watermelon filtrates was done in triplicate with an H-P 5710A gas chromatograph equipped with a N/P detector and a Durabond-1 capillary column. Recoveries of aldicarb, aldicarb sulfoxide and aldicarb sulfone averaged 106 $\pm$ 10% (SD).

Concentrated juice was prepared by rotary evaporation of the filtrate (60 mL) to 0.1 of its original volume, at 60 C. All samples were stored at 4 C. Sample preparation is summarized in Figure 1. Samples were prepared in triplicate using three concentrations of the organocarbamates or organophosphates to be

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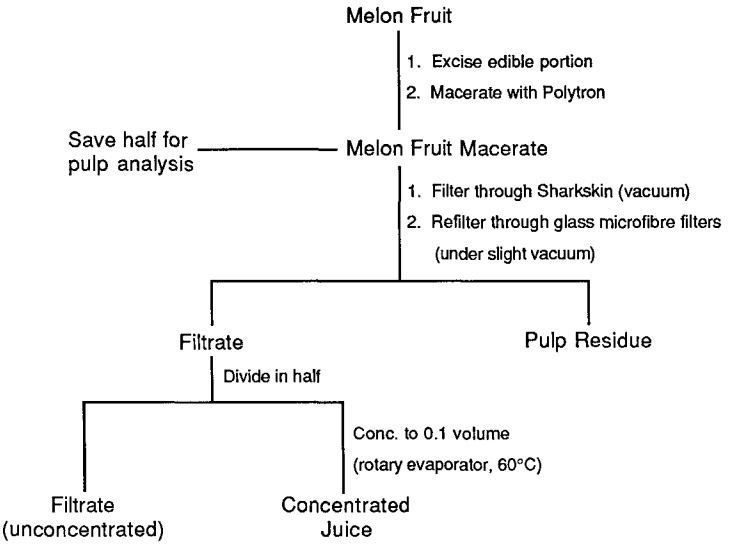


Figure 1. Sample preparation scheme.

tested. Each sample (either 30 g or 3 g) was divided into three aliquots (either 10 g or 1 g). All acetylcholinesterase (AChE) analyses were performed blind; bee bioassays were not. In addition to a control melon, four suspect melons were prepared exactly as described above and kept frozen until used.

The activity of eel AChE (E.C. 3.1.1.7, Type III, Sigma Chemical Co.) was assayed in the presence of the watermelon samples by a spectrophotometric method (Ellman et al. 1961) modified for use with an automated microplate reader. The enzyme was incubated for 12 min at room temperature with pH 8.0 phosphate buffer, dithionitrobenzoate, and watermelon extract. Total volume was 220 uL, AChE was 0.068 units/mL and watermelon extracts were 9.1% (v/v). Acetylthiocholine substrate was added to bring the volume to 250 uL; pathlength was 0.7 cm; wavelength was 410 nm. Three min later (elapsed time: 15 min) the microplate reader automatically began 6 readings at 1 min intervals. All assays were performed in triplicate. Controls included blanks where the juice was replaced with buffer and blanks where the AChE was replaced with buffer. Data were transmitted to a portable computer (Radio Shack TRS 80, Model 100), and later to a Zenith Z-100 computer for processing. Time points were analyzed by linear regression (using a program written by B.W. Wilson) to obtain slopes of absorbance per min for each well on the micro plate. Correlation coefficients for the triplicate samples were above 0.98 and often 1.00; standard deviations of the slope of replicates were usually less than 3% of the mean. The mean slope of the triplicate assays was an estimate of the relative AChE activity for each watermelon sample. Percent inhibition was determined by comparing AChE activity in the spiked samples to the activity in the unspiked controls. I50 values (concentrations

of pesticides needed to give a 50% inhibition of AChE activity) were derived by linear regression from log-linear plots.

The concentrations of pesticides used in the calculations were those during the 15 min incubation of the enzyme with the watermelon samples. Since the activity of carbamate-inhibited AChE did not recover during the short period of the assay itself, we assumed that most of the inhibition occurred during the incubation period and that the addition of substrate, at a concentration much greater than that of the pesticides, prevented further inhibition during the course of the assay itself.

Two-story hives were maintained at the Bee Biology Facility with the queen confined with an excluder. Hives were supplied with 40% sugar syrup (v/v) through an opening in the top cover. Adult worker bees were removed from the hive and placed inside a pyramid shaped metal wire cage with a tube (290 mm diam.) affixed to the top opening. The cages were made from strips of metal screen (9 mesh/$cm^2$) 20 cm long and 9 cm wide, stapled to form a circular insert of 5.7 cm in diameter. Five concentrations of chemical with four replicates usually were used for each log-dosage mortality test. Replicates consisted of 25 $\pm$ 5 bees. Each concentration was repeated three times.

Each sample of approximately 200 g watermelon pulp was blended at high speed for one min; an equal weight of 80% sugar syrup (v/v) was added. The mixture was blended for two min to produce a watermelon-sugar mixture to feed the bees. One mL of insecticide stock solution was added to 39 mL of watermelon mixture or 50% sugar syrup and the mixtures vibrated for one minute more to ensure homogeneity. Approximately 10 mL of each replicate was placed in 55 x 20 mm disposable petri dishes, a wire screen (9 mesh/$cm^2$) placed on the surface of the mixture and the cylindrical cage fitted over the petri dish.

Bees were confined for two hours before being given access to the insecticide bioassay solution. Temperature was maintained at 27 $\pm$ 1 C and relative humidity at 50-60%.

## RESULTS AND DISCUSSION

The first filtrate was dark pink and contained bits of fine pulp that plugged the glass microfiber filters during the second filtration. Consequently it was necessary to change filters after passage of every 20 mL of filtrate. The second filtrate was clear of pulp and murky yellow in color. Rotary evaporation was chosen over lyophilization to concentrate the filtrate; we thought it less likely to cause loss of pesticide through volatilization, from an aqueou solution due to aldicarbs low Henry's law constant (Suntio et al. 1987) and 50 mL of juice could be concentrated to 5 mL in less than one hour by using a strong vacuum and a temperature of 60 C compared to 18 hours to process 25 ml of juice with lyophilization. The final concentrate was as thick as honey and had a similar color. Some

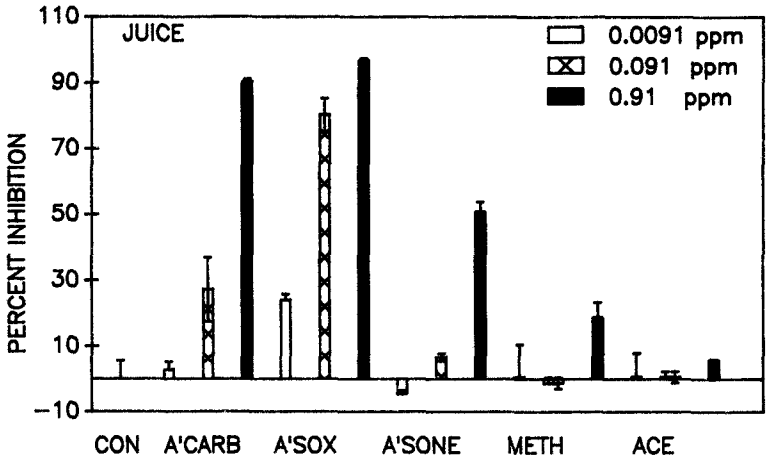


Figure 2. Inhibition of electric eel ACHE by watermelon juice samples spiked with pesticides (unspiked control, aldicarb, aldicarb sulfoxide, aldicarb sulfone, methamidophos, acephate) at the concentrations shown. Error bar is SEM.

of the syrup stuck to the round-bottom flask; consequently the 10:1 concentration factor given in the flow sheet is an approximation.

A radiometric AChE assay method (Johnson and Russell 1976) was incompatible with the watermelon samples; it increased the apparent hydrolysis of acetylcholine as if the watermelon extracts interfered with the organic/aqueous distribution of label in the counting vials. All the data and results presented here were obtained with the modified spectrophotometric method of Ellman et al. (1961).

The inhibitions of AChE activity caused by the various pesticides for juice, pulp and juice concentrate are summarized in Figures 2,3 and 4, respectively. The I50 values calculated from them are shown in Table 1. Aldicarb sulfoxide was a relatively potent AChE inhibitor while methamidophos and particularily acephate were poor AChE inhibitors at the concentrations tested. Relative inhibitory ability of the pesticides (in decreasing order) was: aldicarb sulfoxide, aldicarb, aldicarb sulfone, methamidophos, and acephate.

There was an apparent inhibition of AChE activity in the control watermelon samples compared to blanks which contained no watermelon. Unspiked pulp inhibited 8.7 $\pm$ 4.4%, juice inhibited 15.3 $\pm$ 4.7% and concentrated juice inhibited AChE activity 43.2 $\pm$ 9.6% compared to melonless blanks. One possibility was that natural substances in watermelon interfered with the assay. The watermelon juice did not inhibit AChE activity in several

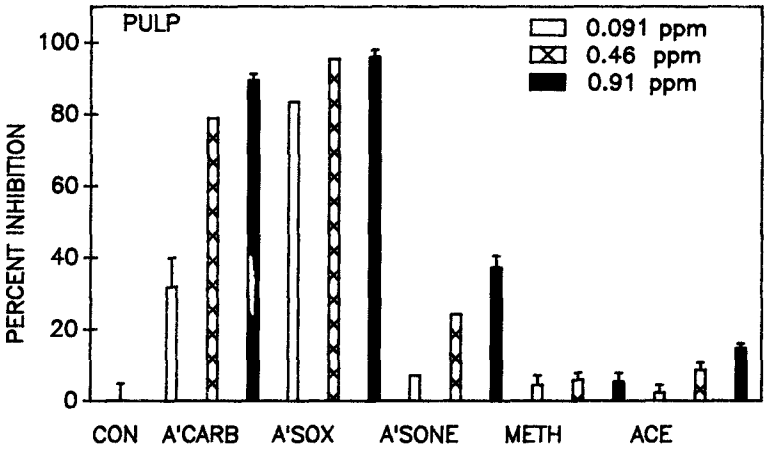


Figure 3. Inhibition of electric eel ACHE by edible watermelon pulp samples spiked with pesticides (unspiked control, aldicarb, aldicarb sulfoxide, aldicarb sulfone, methamidophos, acephate) at the concentrations shown. Error bar is SEM.

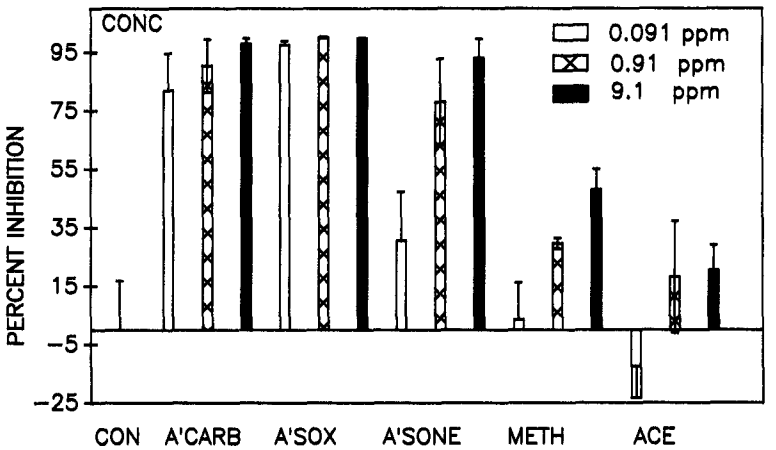


Figure 4. Inhibition of electric eel ACHE by concentrated watermelon juice samples spiked with pesticides (unspiked control, aldicarb, aldicarb sulfoxide, aldicarb sulfone, methamidophos, acephate) at the concentrations shown. Error bar is SEM.

experiments using a pH Stat method of determining AChE activity (Worthington 1972). The apparent inhibitions of AChE caused by juice and pulp samples from suspect melons are summarized in Table 2; they were not significantly different from that caused by the unspiked control samples.

Table 1. I50 Estimates of Pesticides

| Compound | ppm | mole/l | |
|---|---|---|---|
| Aldicarb | 0.18 | $9.3 \times 10^{-7}$ | (a) |
| A. Sulfoxide | 0.031 | $1.5 \times 10^{-7}$ | (b) |
| A. Sulfone | 1.3 | $6.0 \times 10^{-6}$ | (b) |
| Methamidophos | 9.7 | $6.9 \times 10^{-5}$ | (c) |
| Acephate | --- | --- | |

Derived from (a) pulp, (b) juice or (c) concentrated juice data.

Table 2. Percent Inhibition of AChE by Suspect Melons

| Sample | Pulp | Juice |
|---|---|---|
| Control | 8.7 ± 4.4 | 15.3 ± 4.7 |
| B 25634 | 11.7 ± 4.7 | 7.1 ± 1.2 |
| B 23423 | 7.2 ± 2.6 | 8.4 ± 3.1 |
| F7-H | 5.1 ± 1.8 | 9.6 ± 0.7 |
| F7-F | 3.9 ± 1.2 | 4.2 ± 0.6 |

(a) Mean ± s.d.

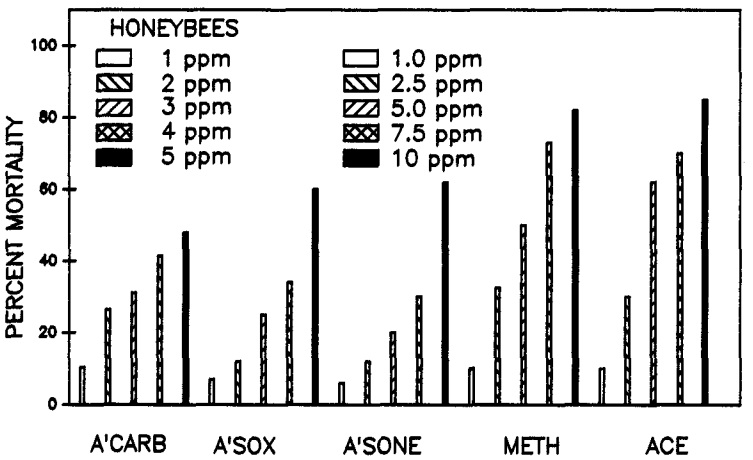


Figure 5. Mortality of honeybees fed sugar syrup containing pesticides (aldicarb, aldicarb sulfoxide, aldicarb sulfone, methamidophos, acephate) at the concentrations shown. Aldicarb was used at the lower set of concentrations and the remaining 4 chemicals at the higher set of concentrations.

Mortalities of bees fed spiked syrup are shown in Figure 5. LD50s were above 1 ppm, and were similar for aldicarb and its metabolites. The four watermelons suspected of contamination with aldicarb did not cause increased mortality of the honeybees unless the samples were spiked with aldicarb.

The methodology developed in this study demonstrates the feasibility of preparing watermelon extracts without organic solvents and using in vitro enzyme assays and honeybee bioassays to study their potential toxicity. The Ellman assay for AChE with the eel enzyme was faster and more sensitive than the honeybee mortality test in revealing toxicity of aldicarb and its metabolites. The most potent inhibitor was aldicarb sulfoxide. I50's were below 1 ppm, in a region expected for contaminated watermelons.

There is evidence aldicarb sulfoxide is the major residue of aldicarb in watermelons. In a separate study conducted a year before the one reported here, watermelon vines were grown in soil treated with aldicarb granules (Temik) at rates of 1, 2 and 4 kg/ha (Seiber and McChesney 1986). Gas chromatographic analysis indicated that the highest residues were in the leaves. Approximately 10 days after treatment of the soil, average leaf residue values ranged from a maximum of 0.82 ppm for aldicarb sulfoxide to 0.28 ppm for aldicarb sulfone. Very little of the parent aldicarb was found in the leaves. A melon harvested 17 days after treatment had the highest residue observed (0.13 ppm of the sulfoxide). Most harvested melons showed sulfoxide residues below 0.13 ppm; the amount of sulfone residues were insignificant in comparison with the level of sulfoxide and, again, parent aldicarb was not observed. Even though these treatments were made in September, relatively late in the growing season, and may not have led to optimal uptake of the pesticide, nevertheless, we conclude that aldicarb is transferred primarily as the sulfoxide when systemically applied to melons, and that contamination of the melons can occur.

Although the enzyme assay was more sensitive than the honeybee test, both appear suitable for studying possible contaminated melons. J.M. Witt and S.L. Wagner reported in a letter (July 24, 1985, personal communication) to Oregon Extension agents that, "The laboratory analyses in Oregon showed aldicarb sulfoxide residues of 1.1 to 3.1 ppm (with one value at 6.3 ppm) in melons implicated in illnesses and in California they found residues of 1 -2.5 ppm." However, a later report (Green et al. 1987) noted levels as low as 0.01 ppm were associated with symptoms. Data from 4 human volunteers in an unpublished study of Union Carbide also discussed in the review of Risher et al. (1987) led Witt and Wagner (1985, personal communication) to estimate a No-Observed-Effect-Level for man for aldicarb of 0.050 mg/kg, corresponding to 1.85 ppm in watermelons (0.050 mg/kg x 50 kg body weight/1350 gram portion of watermelon x 1000).

Spontaneous hydrolysis of carbamylated AChE tends to occur more readily than spontaneous hydrolysis of phosphorylated enzyme. This makes it difficult to find decreases in the blood cholinesterases of people exposed to organocarbamates beyond the first few hours (Risher et al. 1987). The results of this study suggest that it would be possible to develop a protocol for cholinesterase assays of the melons themselves that would be rapid and sensitive enough to be useful in establishing whether they contained anticholinesterase agents. This would be particularily important when analytical methods appropriate to a potential toxicant are inapplicable because its chemical nature is not known with some degree of certainty. The eel AChE enzyme is available from commercial sources and the specrophotometric assay can be adapted to the variety of colorimeters and spectrophotometers found in small hospitals and laboratories. Contamination of foodstuffs with anticholinergic compounds is likely to be of continuing concern. For example, as this article is being finalized, there are reports in the press of illnesses alleged to be due to the presence of aldicarb in cucumbers (Sacramento Bee, April 11, 1988).

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