similar for practical purposes.

The aluminum oxide column offers a simple and cheap way of preparing samples that can be placed in ampules of an automatic injector for continuous analysis during the day and night.

ACKNOWLEDGMENT

We thank Richardus A. Hilhorst for statistical calculations and J. Christopher Rigg (Centre for Agricultural Publishing and Documentation, Wageningen) for help in drafting the manuscript.

LITERATURE CITED

De Wit, J. C.; Notermans, S.; Gorin, N.; Kampelmacher, E. H. *J. Food Prot.* **1979**, *42*, 222.

Dunmire, D. L.; Otto, S. E. *J. Assoc. Off. Anal. Chem.* **1979**, *62*, 176.

Gorin, N. *J. Agric. Food Chem.* **1979**, *27*, 195.

Gutman, I. In "Methods of Enzymatic Analysis", 2nd Engl. ed.; Bergmeyer, H. U., Ed.; Academic Press: New York, 1974; Vol. 3, p 1186.

Trammell, K. W.; Peterson, C. E. *J. Am. Soc. Hortic. Sci.* **1976**, *101*, 205.

Natalio Gorin*
Frouwke T. Heidema

Sprenger Institute
6700 AA Wageningen, The Netherlands

Received for review April 28, 1980. Accepted August 12, 1980.

Automated Method for Determining in Vitro Cholinesterase Inhibition by Experimental Insecticide Candidates

An automated method for the determination of electric eel cholinesterase (ChE) inhibition by experimental insecticide candidates was developed. It was based on the manual procedure of Ellman et al. [Ellman, G. L.; Courtney, K. D.; Andres, V., Jr.; Featherstone, R. M. *Biochem. Pharmacol.* **1961**, *7*, 88–95] using acetylthiocholine iodide (ATChI) as the substrate and 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) as the color reagent. The substrate and color reagent concentrations were individually varied in order to determine optimum reaction conditions. The ChE assay conditions were found to be 1.5 mM ATChI and 0.38 mM DTNB. The reproducibility of the automated method was evaluated by the simultaneous analysis of 10 aliquots from each of three electric eel ChE solutions containing 247, 413, and 1054 units/L ChE. Coefficients of variation (CV) of 5.9–9.0 were obtained for each sample, respectively. Variation between runs was examined by comparing the three electric eel ChE solutions containing 270, 513, and 1247 units/L ChE for 10 different days. A CV of 3.7–7.9% was obtained for each sample, respectively. The test requires an initial 30 min of reagent preparation and thereafter as many as 400 samples/h can be analyzed. The sample volume requirement is as little as 20 mL of enzyme solution.

Screening for insecticidal activity of experimental carbamates and organophosphates may be done by determining the cholinesterase (ChE) inhibition produced by the insecticide candidates. As a ChE enzyme source, housefly head (Hellenbrand, 1967) and bovine erythrocyte (Hastings et al., 1970) are used. Many automated, as well as manual, methods are being used for the assay of ChE activity. However, a centrifugal analyzer, CentrifiChem (Union Carbide Corp.), which is extensively used in hospital laboratories for automated blood clinical chemistries, has not been applied for this purpose. This study reports the application of centrifugal analyzer methods for ChE inhibition studies using the electric eel enzyme preparation. The optimum reaction conditions for ChE assay for the centrifugal analyzer were examined and the reproducibility of the procedure for ChE assay was evaluated. On the basis of this methodology, the I_{50} value (defined as the amount of chemical required to inhibit 50% of the control ChE activity) for thiofanox, 3,3-dimethyl-1-(methylthio)-2-butanone *O*-[(methylamino)carbonyl]oxime, was established.

MATERIALS AND METHODS

Reagents. Electric eel (Sigma Chemical Co.; No. C-3389; 355 units of acetylcholinesterase/mg) was used as the enzyme source for the determination of optimum conditions. One milligram was dissolved in 100 mL of 0.05 M tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.4). This yielded a stock solution of 3.55 units/mL. This stock enzyme solution was stable for at least 2 weeks if kept refrigerated when not in use.

The 0.05 M Tris buffer (pH 7.4) was prepared by combining 6.64 g of NaCl, 6.05 g of tris, and 45 mL of 1 N HCl. This was diluted to 900 mL with distilled water. The pH was adjusted to 7.4 with 1 N HCl and the volume was adjusted to 1 L with distilled water. The substrate used was a 38 mM solution of acetylthiocholine iodide (ATChI), made by adding 109 mg of ATChI to 10 mL of distilled water. The color reagent, DTNB [(5,5-dithiobis(2-nitrobenzoic acid)], was prepared by dissolving 100 mg of DTNB in 500 mL of 0.05 M Tris buffer (pH 7.4). Both ATChI solution and color reagents were stable at least for 4 weeks at 4 °C.

Apparatus. The CentrifiChem 400 Analyzer was used for analyses. The CentrifiChem Pipettor was used for delivery of samples, reagents, and diluents.

Basic Reaction Conditions. All determinations were made by using 20 μL of enzyme samples, 50 μL of H_2O, and 350 μL of reagent. An initial absorbance reading was taken at 15 s after the start of the reaction, and the second reading was taken after an additional 15 s. The wavelength used was 405 nm.

In order to find optimal analytical conditions, the analyses were carried out while individually varying the

0021-8561/80/1428-1342$01.00/0

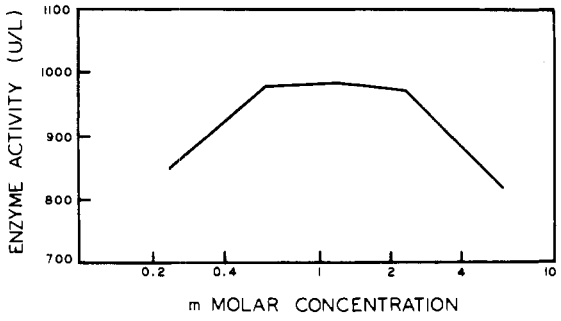

Figure 1. Effect of ATChI concentration in a final reaction mixture on ChE activity.

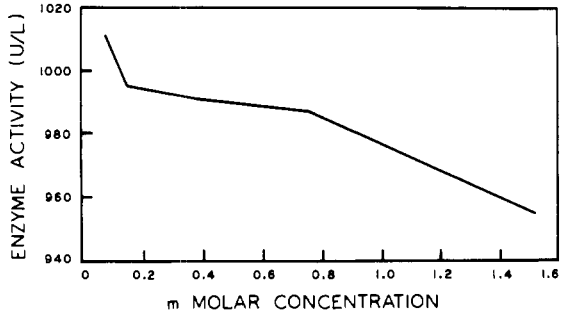

Figure 2. Effect of DTNB concentration in a final reaction mixture on ChE activity.

conditions of ATChI and DTNB concentration and buffer pH.

Effect of ATChI Concentration. The activity of electric eel ChE as a function of AtChI concentration was determined by running final concentrations of ATChI ranging from 0.24 to 5.9 mM. The pH and final DTNB concentration in reaction mixture were kept at 7.4 and 0.38 mM, respectively.

Effect of DTNB Concentration. The color reagent, DTNB, was prepared at final concentrations ranging from 0.076 to 1.52 mM in 0.05 M Tris buffer (pH 7.4). The ATChI concentration in the final reaction mixture was kept at 1.5 mM.

Effect of pH. Tris buffer solutions (0.05 M) were made to pH values ranging from 7.0 to 8.0 in 0.2 pH unit intervals by adjusting the pH of the buffer with concentrated HCl. By use of portions of these same buffer solutions, the final ATChI and DTNB concentrations were kept at 1.5 and 0.38 mM, respectively. Nonenzymatic hydrolysis of substrate under various pH values was examined without enzyme.

Inhibition of Electric Eel ChE by Thiofanox. Dimethyl sulfoxide (Me_2SO) was used as a solvent for making solutions of thiofanox (DS-15647) ranging in concentration from 0 to 1×10^{-3} M. The lowest concentration of thiofanox used was 1×10^{-8} M. The incubation at 37 °C was conducted by mixing 4.95 mL of electric eel ChE (1.8 units/mL) with 50 μL of various concentrations of inhibitors for 10 min, resulting in final molar concentrations of thiofanox ranging from 0 to 1×10^{-5}. The I_{50} value was determined from the plot of the percent inhibition vs. the log molar concentration of inhibitor.

Within-Run Precision. The reproducibility of the automated method was evaluated by the simultaneous analysis of 10 aliquots from each of the three electric eel ChE solutions containing 247, 413, and 1054 units/L ChE.

Day-to-Day Precision. The day-to-day reproducibility of the method was examined by comparing the three electric eel ChE solutions containing 270, 513, and 1247 units/L ChE for 10 working days.

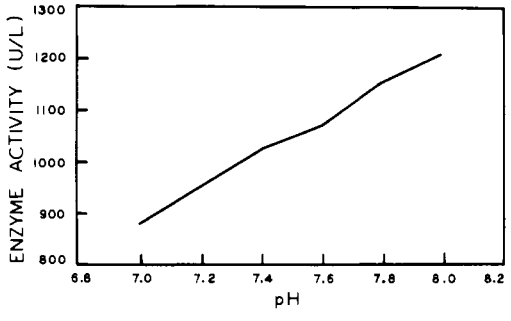

Figure 3. Effect of pH on ChE activity.

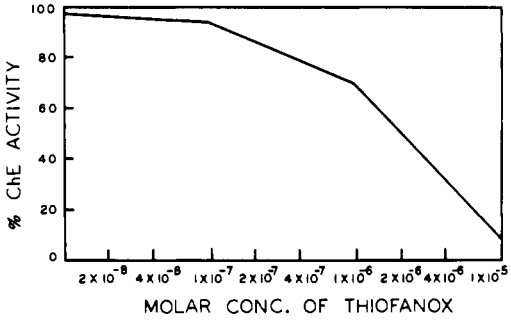

Figure 4. Inhibition of ChE by molar concentrations of thiofanox.

Table I. Precision of Automated Cholinesterase Assay

		cholinesterase act., units/L	
mean	n	SD	coeff of variation, %
	Within Run		
1054	10	70.8	6.7
413	10	24.4	5.9
247	10	22.2	9.0
	Between Run		
1247	10	46.1	3.7
513	10	40.5	7.9
270	10	17.9	6.6

RESULTS

Effect of ATChI Concentration. The effect of substrate concentration on ChE activity is shown in Figure 1. When the concentration of substrate in the final reaction mixture was kept from 0.5 to 2.5 mM, relatively constant ChE values were found. Therefore, 1.5 mM, the midpoint of plateau, was used throughout the experiment.

Effect of DTNB Concentration. When the enzyme and the substrate concentrations were held constant and the amount of DTNB in DTNB–buffer reagent was varied, a curve shown in Figure 2 was obtained. There were only small changes in ChE activity over a wide range of concentrations of DTNB, indicating that the concentration of color reagent was not critical. Therefore, the 0.5 mM solution of DTNB was used throughout the experiment. The concentration of this color reagent in the final reaction mixture is calculated to be 0.38 mM.

Effect of pH. Figure 3 shows that the ChE activity increases with the pH values. There was no pH optimum. The extent of nonenzymatic hydrolysis of substrate was equivalent to 2 and 13 units/L at pH 7.0 and 8.0, respectively. The magnitude of this nonenzymatic hydrolysis was equivalent to ~1% of the total ChE activity measured.

Inhibition of Electric Eel Cholinesterase by Thiofanox. Thiofanox was used as the inhibitory agent with Me_2SO used as a solvent. The results of the various

concentrations are shown in Figure 4, with the I_{50} value determined to be 2×10^{-6} M. This value agrees well with the I_{50} values determined manually by Findak (1979). This value also agrees well with I_{50} value determined by Magee and Limpel (1977) and Chin et al. (1974), who used bovine erythrocyte cholinesterase as the enzyme source.

Within-Run and Day-to-Day Precision. The results of the within-run and between-run (day-to-day) precision checks are shown in Table I. The within-run CV ranged between 5.9 and 9.0%, while the between-run CV ranged from 3.7 to 7.9%.

The preceding data demonstrate the establishment of a reliable and reproducible automated method for cholinesterase I_{50} values. In addition to these advantages, high rate of throughput (400 samples/h) and low reagent cost are typical advantages for the centrifugal analyzer method. Therefore, this method can be used to facilitate compound development and provide for more thorough structure–activity relationship studies.

LITERATURE CITED

Chin, B. H.; Tallant, M. J.; Sullivan, L. J. "Abstracts of Papers", 168th National Meeting of the American Chemical Society, Atlantic City, NJ, Sept 9–13, 1974; American Chemical Society: Washington, DC, 1974; PEST 58.

Findak, D. C., Diamond Shamrock Corp., personal communication, 1979.

Hastings, F. L.; Main, A. R.; Iverson, F. *J. Agric. Food Chem.* **1970**, *18*, 497–502.

Hellenbrand, K. *J. Agric. Food Chem.* **1967**, *15*, 825–829.

Magee, T. A.; Limpel, L. E. *J. Agric. Food Chem.* **1977**, *25*, 1376–1382.

Byong Han Chin*
Nancy Spangler

Diamond Shamrock Corporation
T. R. Evans Research Center
Painesville, Ohio 44077

Received for review March 3, 1980. Accepted August 29, 1980.

CORRESPONDENCE

On the Odor of 2-Methylisoborneol

Sir: In a recent paper in this journal, Tyler et al. (1978) described the odor of both enantiomers of 2-methylisoborneol (1,2,7,7-tetramethyl-*exo*-bicyclo[2.2.1]heptan-2-ol and 1,2,7,7-tetramethyl-*endo*-bicyclo[2.2.1]heptan-2-ol) as camphoraceous rather than earthy. A similar characterization was earlier reported by Gerber (1969). Since several studies mentioned by Tyler et al. (1978) indicate 2-methylisoborneol as an agent responsible for a musty or earthy odor in water or garden soil (Buttery and Garibaldi, 1976; Collins et al., 1970; Medsker et al., 1969; Wood and Snoeyink, 1977), an additional note on this seeming discrepancy is warranted.

A well-known phenomenon, although generally anecdotically reported, is that the odor character of a chemical compound may depend on the concentration of the compound (Polak et al., 1978). This is also the case for 2-methylisoborneol. Persson and York (1978), working with synthetic (−)-2-methylisoborneol, reported that the pure compound exhibited a camphoraceous odor, but that solutions of 0.01–10.0 μg of 2-methylisoborneol/L of water (twice distilled) exhibited a musty or muddy odor. Many judges considered aqueous solutions of 100 μg of 2-methylisoborneol/L as clearly camphoraceous, some considered solutions of 10.0 μg/L as camphorlike, and a few even characterized the odor of 1.0 μg of 2-methylisoborneol/L of water as camphoraceous (Persson and York, 1978; Persson, 1979). On consideration of these facts, the odor characterization delivered by Tyler et al. (1978) fits well into the picture, since they worked on the pure compounds and solutions of 1 ppt and 1 ppm in water.

It should be noted that Wood and Snoeyink (1977) worked on solutions containing 0.1–10.0 μg of 2-methylisoborneol/L and described these as earthy/musty. Medsker et al. (1969) described the odor of 2-methylisoborneol as camphoraceous, but indicated that the compound may be responsible for an earthy or musty taint in water. Zoeteman and Piet (1973), working with a large consumer panel (708 participants) in the Netherlands, described the odor of a solution of 0.12 μg of methylisoborneol/L of water as clearly earthy ("bosgrond" = woodland; "aarde" = earth, mold).

Thus, it appears that the discrepancy in the odor characterization of 2-methylisoborneol is to some extent due to work on different concentrations of the compound, although it is evident that experimental conditions have varied in other respects, too. Nevertheless, the conclusion implied by Tyler et al. (1978) that (−)-2-methylisoborneol does not exhibit a musty odor is premature; i.e., it is true for concentrated solutions of the compound. It may be concluded that odor characterization of compounds in the pure state may not be relevant for situations where these compounds appear as trace contaminants, exhibiting different odor characteristics, as is the case for 2-methylisoborneol in natural waters.

Literature Cited

Buttery, R. G., Garibaldi, J. A., *J. Agric. Food Chem.* **24**, 1246 (1976).
Collins, R. P., Knaak, L. E., Soboslai, J. W., *Lloydia* **33**, 199 (1970).
Gerber, N. N., *J. Antibiot.* **22**, 508 (1969).
Medsker, L. L., Jenkins, D., Thomas, J. F., Koch, C., *Environ. Sci. Technol.* **3**, 476 (1969).
Persson, P.-E., *Aqua Fennica* **9**, 48 (1979).
Persson, P.-E., York, R. K., *Aqua Fennica* **8**, 83 (1978).
Polak, E., Trotier, D., Baliguet, E., *Chem. Senses Flavour* **3**, 369 (1978).
Tyler, L. D., Acree, T. E., Butts, R. M., *J. Agric. Food Chem.* **26**, 1415 (1978).
Wood, N. F., Snoeyink, V. L., *J. Chromatogr.* **132**, 405 (1977).
Zoeteman, B. C. J., Piet, G. J., H_2O **6**, 174 (1973).

Per-Edvin Persson

University of Helsinki
Department of Limnology
Viikki
SF-00710 Helsinki 71
Finland

0021-8561/80/1428-1344$01.00/0