ANALYTICAL BIOCHEMISTRY 166, 353–360 (1987)

Use of a 96-Well Microplate Reader for Measuring Routine Enzyme Activities

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Received February 12, 1987

A method is described for the routine determination of the rate of colorimetric enzyme reactions using a 96-well microtiter plate reader commonly used in immunoassay. This approach is illustrated by monitoring esterase activity using three common products: release of thiol, release of ethanol, and release of *p*-nitrophenylate ion. Examples include monitorings of the rate of hydrolysis of acetylthiocholine iodide by eel acetylcholinesterase and the rate of hydrolysis of malathion and nonconventional esters such as *O*-methyl, *O*-ethyl, and *O*-isobutyl carbonates of *p*-nitrophenol by commercial porcine liver carboxylesterase. Data obtained from the plate reader were compared to those obtained, under similar conditions, in a conventional spectrophotometer. Absorbance measurements made in both machines on the same solution, as well as absorbance changes measured over time, were similar. The use of the 96-well plate format tremendously increased the number of enzyme assays carried out per person and the interface with a personal computer allowed rapid manipulation of the absorbance values to calculate the desired rate data. This approach should be generally applicable to many routine colorimetric assays in the research laboratory.

KEY WORDS: semiautomated, enzyme assay; carboxylesterases; 96-well plate reader, enzyme rates; nitrophenyl carbonates; cholinesterase; microtiter plates, enzyme rates.

Enzyme assays are used extensively in clinical settings. For example, serum enzyme levels are routinely measured for diagnostic purposes. In these settings the instrumentation for assessing this activity is designed to handle many samples simultaneously and automatically. However, this instrumentation is expensive and is not easy to adapt to a research environment involving many users and many different assay formats.

In the research laboratory setting, it is often desirable to run a battery of enzyme assays. Using several substrates with the same enzyme source and/or several enzyme sources with one substrate is common. Many enzyme assays are carried out as point assays, that is, stopping the reaction at a given time and measuring the resulting product. Alternatively, one can measure the rate of an enzyme reaction, which reduces interference from background and differences due to optical purity of cuvettes while providing more valuable kinetic data.

The enzyme-linked immunosorbent assay (ELISA)[2] is a common immunoassay format based on a colorimetric rate or endpoint (1,2). Typically in these assays release of a colored product is related to the concentration of an analyte with the assays run in plastic plates consisting of 96 small cuvettes. We have applied the ELISA format to many routine colorimetric endpoint and rate

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[2] Abbreviations used: ELISA, enzyme-linked immunosorbent assay; INT, *p*-iodonitrophenyltetrazolium violet; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); IEF, isoelectric focusing; SGOT, serum glutamic-oxaloacetic transaminase.

0003-2697/87 $3.00

assays performed in this laboratory, and Schuman *et al.* (3) have demonstrated an endpoint assay for butyrylcholinesterase (EC 3.1.1.8) activity based upon the change in absorbance of phenol red, caused by the release of butyric acid from the substrate, using a microtiter plate. Since automated readers for 96-well ELISA plates are inexpensive instruments that are now commonly available in biochemistry laboratories, this technology could be generally applicable. As examples of this technology, we report a comparison of enzyme rates determined on both a 96-well plate reader and a spectrophotometer using two commercial esterase preparations.

Influence of hydrolytic enzymes on the action of xenobiotics and the role of xenobiotics in inducing or inhibiting hydrolase action on endogenous substrates have been the subjects of intense research efforts. Carboxylesterases (EC 3.1.1.1) are a number of distinct enzymes which hydrolyze xenobiotics containing an ester, thioester, and amide group (4–7). Acetylcholinesterase (EC 3.1.1.7) is found in nervous tissue of animals. The regulation of the enzyme activity, its localization, and its inhibition have been studied (8–12), and its utility in diagnosis and prognosis has been reported (13,14). In the present study we demonstrate the use of a 96-well microplate format for monitoring enzymatic hydrolysis involving release of thiol, ethanol, and *p*-nitrophenol. The assessment of acetylcholinesterase and carboxylesterase activities was conducted simply, rapidly, and quantitatively.

MATERIALS AND METHODS

Chemicals. Malathion (*O,O*-dimethyl-*S*-[1,2-bis(ethoxycarbonyl)ethyl]phosphorodithioate, 99.2%) was provided by Dr. M. Mallipudi (American Cyanamid Corp., Princeton, NJ). *p*-Nitrophenol, methyl chloroformate, ethyl chloroformate, and isobutyl chloroformate were purchased from Aldrich Chemical Co. (Milwaukee, WI). *p*-Iodonitrophenyltetrazolium violet (INT), alcohol dehydrogenase, NAD, NADH diaphorase, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and acetylthiocholine iodide were obtained from Sigma Chemical Co. (St. Louis, MO). *p*-Nitrophenyl carbonates were synthesized from the appropriate chloroformate and *p*-nitrophenol and purified by repeated recrystallization and/or flash chromatography to yield one spot in several TLC systems and no absorbance in the 400-nm region. NMR, ir, and MS were consistent with the assigned structures.

Enzyme sources. Acetylcholinesterase (EC 3.1.1.7) from electric eel (Type VI-S, 190 U/mg solid, 218 U/mg protein) and partially purified carboxylesterase (EC 3.1.1.1) from porcine liver (Type I, 10.9 mg protein/ml, 160 U/mg protein) were obtained from Sigma Chemical Co. and diluted with buffer before use.

Standard curve. A series of dilutions of *p*-nitrophenol in 0.1 M Tris–HCl buffer, pH 7.5, was made. Each solution was read in triplicate in a Titertek Multiskan 96-well microtiter plate reader (Flow Laboratories, McLean, VA) and a Varian–Cary 219 uv/visible spectrophotometer (Varian, Palo Alto, CA) at 405 nm.

Enzyme assays. The rates of hydrolysis of a thioester by acetylcholinesterase and *p*-nitrophenyl and diethyl esters by porcine liver carboxylesterase were monitored spectrophotometrically using a 96-well microtiter plate format. A total volume of 300 μl incubation mixture was used for each well and enzyme assays were initiated by the addition of substrate. Typically 72 wells could have substrate added within 2 min. The plate was placed in the reader chamber (31 ± 0.5°C) and absorbances were measured at 1.5-min intervals for up to 20 min. It took about 55 s to read 96 wells. The Titertek was interfaced (RS232C) with an IBM personal computer. After each reading the data were stored using a program called PC-EIA (Dorian Software, Wheaton, MA). Later the stored data were transferred into a user-specified format for import into a Lotus 1-2-3 (Lotus Develop-

ment Corp., Cambridge, MA) spreadsheet using an in-house program. The "transferred" data were then imported into a Lotus 1-2-3 spreadsheet for which labels and simple formulas for calculations had already been entered to correct for spontaneous hydrolysis and express multiple point assays as rate data. The graphed data were viewed and the linear portion was selected for the determination of rate. (Usually this was the first five time points, although it varied depending on the linearity of the assay under the conditions used.) The rate was determined by performance of a simple linear regression as provided in Lotus 1-2-3, Release 2. Since the rate data were already in a spreadsheet, a few keystrokes converted the data to specific activity. The use of a spreadsheet for calculation allowed the assay format to be adapted readily to the assay format used by different investigators.

The Varian–Cary, interfaced with an Apple II computer (Apple Computer, Cupertino, CA), was used for rate measurements in 1-ml plastic cuvettes for comparison with data generated on the Titertek. The instrument was equipped with a five-position sample turret, time drive, and a temperature-controlled compartment. Assays were started by the addition of substrate. After a quick mixing, cuvettes were placed in the sample chamber and the data collection program was begun. Once data collection and storage were complete, the program could be manipulated to output the slope values for the rate measurement.

Acetylcholinesterase activity was assayed after Ellman *et al.* (15) with modifications (16). For the Titertek, 290 μl of the reagent buffer containing 0.01% DTNB in 0.05 M phosphate buffer, pH 7.4, and 6 μl of enzyme solution (20 ng solid/ml final concentration) were added to the well. The reaction was started by the addition of 6 μl of acetylthiocholine iodide to give a final substrate concentration of 5×10^{-4} M. Absorbance change was measured at 1.5-min intervals for about 10 min at 405 nm. A reagent blank was run in the same manner. For the Varian–Cary, 0.96 ml of reagent buffer (as prepared above) was added to a 1-ml cuvette. The enzyme was added in 20 μl, giving a final concentration of 20 ng solid/ml. The reaction was started by the addition of 20 μl of acetylthiocholine iodide solution to give a final concentration of 5×10^{-4} M. A reagent blank was used. Activity was monitored for 5 min at 405 nm. Using a non-enzyme blank against a buffer blank, no spontaneous hydrolysis could be detected under these conditions.

The rates of hydrolysis of malathion, *O*-methyl, *O*-ethyl, and *O*-isobutyl carbonates of *p*-nitrophenol by porcine liver carboxylesterase also were monitored. The spectrophotometric method of Talcott (17) was used to assay carboxylesterase activity on malathion. In brief, the release of ethanol from hydrolysis of the substrate was coupled to the reduction of INT with alcohol dehydrogenase and NADH diaphorase. For the Titertek the incubation mixture contained 150 μl of enzyme solution (16 μg protein/ml) and 150 μl of reagent mixture containing 1.8 mM NAD, 25 IU/ml alcohol dehydrogenase, 0.1 IU/ml NADPH diaphorase, and 437 μg/ml INT in 0.1 M Tris–HCl buffer, pH 7.5. The reaction was started by injecting 3 μl of an acetone solution of 3×10^{-2} M malathion into the well using a Hamilton repeating dispenser, yielding a final substrate concentration of 3×10^{-4} M. The well contents were mixed using the needle of the syringe. Reagent blank containing no enzyme was used. Absorbance readings were taken at 1.5-min intervals and monitored for a total of 20 min at 492 nm. The rate of malathion hydrolysis was also monitored at 500 nm for 5 min at 31°C in cuvettes containing 1.0 ml of incubation mixture using the Varian–Cary 219. A 0.5-ml aliquot of reagent mixture prepared as above was added to a 1-ml plastic cuvette with 0.5 ml of enzyme solution. A reagent blank was used as a reference. The reaction was started by injecting 1.0 μl of substrate in acetone into the cuvette and shaking. The

final malathion concentration was 3×10^{-4} M in this case as well.

Porcine carboxylesterase activities an *O*-methyl, *O*-ethyl, and *O*-isobutyl carbonates of *p*-nitrophenol were assayed according to the method of Ljungquist and Augustinsson (18) for detecting released *p*-nitrophenylate ion. For the Titertek, wells contained enzyme solution in 300 μl of 0.1 M Tris–HCl buffer, pH 7.5, and 3 μl of an acetone solution of substrate was injected into the well yielding a 0.15-mM final substrate concentration. The reagent blanks, containing no enzyme, were used. The production of *p*-nitrophenol was monitored for 20 min at 405 nm. For the Varian–Cary, the sample cuvette contained enzyme solution in 1.0 ml of the buffer. The assay was started by the addition of 1.0 μl of substrate in acetone, giving a final substrate concentration of 0.15 mM. The reference cuvette contained no enzyme. The rate of hydrolysis was monitored at 31°C for 5 min at 400 nm.

RESULTS

Standard curves for *p*-nitrophenol generated on both Titertek and Varian–Cary are shown in Fig. 1. Data obtained from each instrument were similar. However, at the higher concentration range (1.25×10^{-4}–1×10^{-3} M) the linear range was greater for the Titertek (Fig. 1A). The two curves were identical in the concentration range 3.91×10^{-6}–1.25×10^{-4} M (Fig. 1B), while at the lower range (1.22×10^{-7}–3.91×10^{-6} M) the Varian–Cary appeared slightly more sensitive (Fig. 1C). The average coefficients of variation were less than 4 and 5% for the Titertek and Varian–Cary, respectively. In the case of each substrate, enzyme reactions were carried out under conditions which gave values on the linear region of standard curves.

According to Beer's law, the equivalent absorbances at all concentrations in the standard curve observed with both Varian–Cary and Titertek indicate that the path length in the cuvette and the well should be 1 cm. For the Titertek, a volume of 0.3 ml per well (ca. 1 cm) was used, but since the path length in the Titertek is measured from the bottom of the plate through the solution rather than across a cuvette (Fig. 2), the accuracy of the volume in the well is extremely important. Additionally, the accuracy of the reader in reading through the same part of the well each time due to possible surface tension meniscus effects is critical. This problem was avoided, however, by the large number of replicates that were run. In fact, we have compared replicates along columns

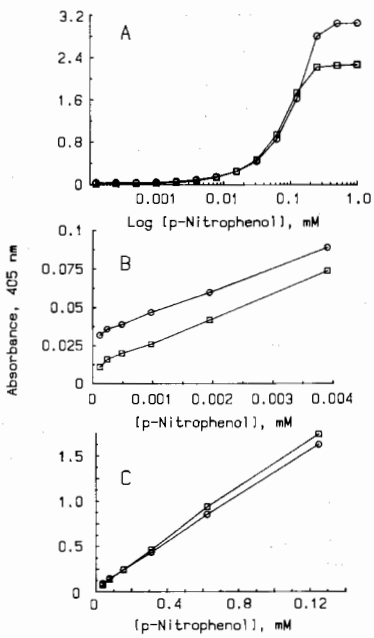

FIG. 1. Standard curve for *p*-nitrophenol as measured in the Varian–Cary 219 (□) and Titertek Multiskan (○) at 405 nm. *p*-Nitrophenol was dissolved in 0.1 M Tris–HCl buffer, pH 7.5, to give a final concentration of 1 mM and diluted in buffer to give the concentrations shown. At any one concentration the same preparation was read in three wells of a 96-well plate or in three 1-ml cuvettes and absorbances were recorded. Each point represents the mean of three different determinations. The average coefficients of variation were 3.6 and 4.8% for the Titertek and Varian–Cary, respectively. (A) Full concentration range tested, 1.22×10^{-7} to 1×10^{-3} M. (B) Concentrations from 3.91×10^{-6} to 1.25×10^{-5} M. (C) The low concentration range (1.22×10^{-7} to 3.91×10^{-6} M).

or rows of the 96-well plate and have seen very little difference among replicates. Evaporation from the wells during incubations would also affect path length and thus absorbency readings. Our experience with ELISA technology indicates no significant evaporation over a 60-min incubation. Additionally, if significant evaporation were occurring, the linearity of the time curves would be compromised, and this does not seem to be the case here. However, it is possible to read the plates through transparent plate covers.

The activities of eel acetylcholinesterase on acetylthiocholine iodide and of porcine liver carboxylesterase on malathion, *O*-methyl, *O*-ethyl, and *O*-isobutyl carbonates of *p*-nitrophenol were assayed using the Titertek. The enzyme activities obtained were compared with those determined on the Varian–Cary using the same molar concentrations of protein and substrate in each case.

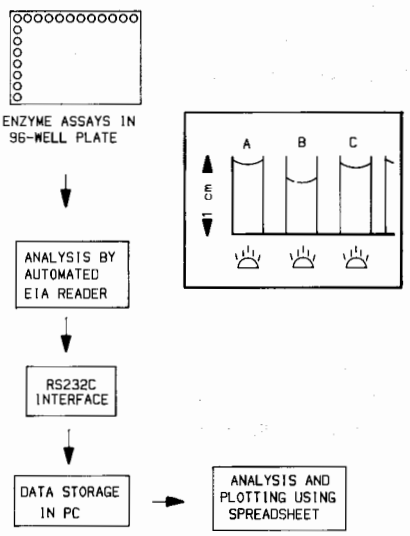

FIG. 2. Schematic of the Titertek plate reader and data collection system. Inset shows the path of light for each well of a 96-well microtiter plate in the Titertek. This drawing illustrates the importance of having the correct volume in the 96-well plates for accurate absorbance readings. It is also important to test the plate reader periodically for alignment, because small changes in the fiber optic alignment with the plate could cause readings through the edges of the meniscus instead of the center.

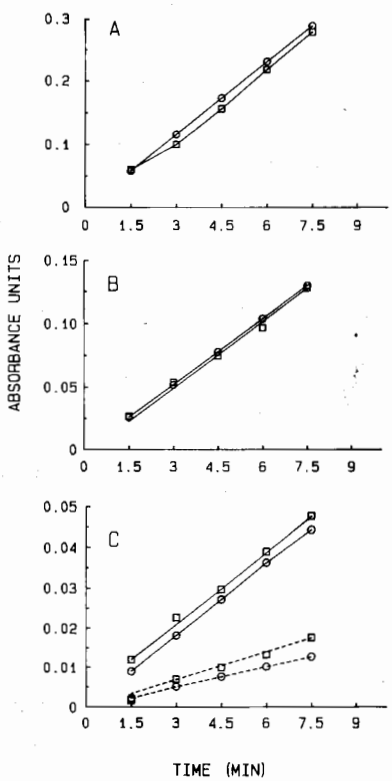

FIG. 3. (A) Absorbance for acetylthiocholine iodide (5×10^{-4} M) using eel cholinesterase (20 ng solid/ml, 0.0038 U, or 0.017 μg protein/ml) measured on the Titertek (○) and the Varian–Cary (□) at 405 nm. (B) Absorbances for malathion (3×10^{-4} M) were measured at 492 nm on the Titertek (○) and 500 nm on the Varian–Cary (□) using porcine carboxylesterase (1.6 μg/ml). (C) Absorbance for *p*-nitrophenyl ethylcarbonate (1.5×10^{-4} M) (—) and *p*-nitrophenyl isobutylcarbonate (1.5×10^{-4} M) (---) measured at 405 nm on the Titertek (○) and 400 nm on the Varian–Cary (□) using porcine carboxylesterase (81.75 ng protein/ml). Each point represents the mean of three different determinations. The average coefficients of variation for the three substrates used were 6.9% (range 2.6–11.1%) for the Varian–Cary and 2.7% (range 0.3–9.2%) for the Titertek.

Figure 3 shows that for the two enzymes, the rates of hydrolysis as measured by the absorbance changed over time were similar in both instruments; however, Titertek gave slightly higher rates for the hydrolysis of malathion and *O*-ethyl carbonate of *p*-nitrophenol. Table 1 presents specific activities of acetylcholinesterase and carboxylesterase on

TABLE 1

ACETYLCHOLINESTERASE AND PORCINE CARBOXYLESTERASE ACTIVITIES AS MONITORED ON VARIAN–CARY AND TITERTEK

Enzyme source	Substrate	Specific activity (μmol/mg protein/min) Varian–Cary	Titertek
Eel acetylcholinesterase	Acetylthiocholine iodide	141 ± 18.3[a]	136 ± 20.8[a]
Porcine liver carboxylesterase	Malathion	0.784 ± 0.06	0.806 ± 0.03
	p-Nitrophenylmethyl carbonate	4.32 ± 0.26	4.22 ± 0.56
	p-Nitrophenylethyl carbonate	4.44 ± 1.40	6.64 ± 1.93
	p-Nitrophenylisobutyl carbonate	1.25 ± 0.25	1.77 ± 0.37

[a] Each value represents the mean ± SD of three separate experiments. Assay conditions were as given under Materials and Methods. Rates were determined from regression of at least eight points on the linear region of an absorbance vs time plot.

the substrates used. Interestingly, it was found that the *p*-nitrophenol carbonate esters were hydrolyzed by porcine liver carboxylesterase at a much faster rate than malathion. At the same time, *O*-methyl and *O*-ethyl carbonates of *p*-nitrophenol were hydrolyzed more rapidly than the isobutyl ester. The rates of the hydrolysis of the thioester (acetylthiocholine iodide) by eel acetylcholinesterase and of diethyl ester (malathion) and *p*-nitrophenyl ester (*O*-methyl, *O*-ethyl, *O*-isobutyl carbonate of *p*-nitrophenol) by porcine carboxylesterase as monitored on Titertek were correlated to those obtained on Varian–Cary (r^2 = 0.99, with a slope of 1.0).

DISCUSSION

This study demonstrates the development and evaluation of a 96-well microplate format for the routine monitoring of esterase activities on substrates of toxicological and pharmacological interest. The use of this approach for the performance of such assays as well as the associated data analysis gives results that are sensitive, accurate, reproducible, and generally applicable.

The Varian–Cary 219 is a highly sophisticated spectrophotometer, and it is invaluable for performing difficult spectrophotometric assays. However, the routine measurement of multiple enzyme rates is very tedious with such complex instrumentation. Under the best conditions an investigator can run five simultaneous assays which can be completed in about 10 min including preparation and incubation time. A routine sampler attachment can increase sample throughput for point assays, but we have found it to reduce throughput for rate determination. Using the Titertek, an investigator can easily complete 72 assays in about 30 min with no additional automation. If multichannel pipets and diluters are available, the number of assays per hour can be greatly increased. The 96-well format is amenable to integration with simple "pick and poke" robotic systems allowing complete automation of routine assays.

A current disadvantage to the 96-well plate format is that these assays cannot be done using substrates with absorbance maxima below 340 nm with most commercially available instruments and plates. It is likely that the spectral range available will be expanded in the near future. Also the Multiskan does not have a temperature-controlled chamber. Although we found the temperature of the plate chamber to remain around 31°C over the course of a day, this temperature may be limiting for some applications

and shorter incubation times would lessen any temperature effects. Several manufacturers are considering the introduction of thermostated plate chambers. Finally, current instrumentation does not allow us to run assays in which the incubation time is brief. However, an interactive interface for the 96-well format in which the computer controls the collection of data could speed the number of readings that may be taken in a given unit of time.

In mammalian liver microsomes there are multiple forms of carboxylesterase with different substrate specificities (18–21). To further characterize these important hydrolytic enzymes, one needs tools for distinguishing groups of these enzymes by using diagnostic substrates and inhibitors. Also, simple high-yield methods of purification are needed. For quickly purifying and characterizing specific enzymes, it is critical at each step of purification to relate the enzymes assayed to previously described esterases in the literature. Thus large numbers of assays involving a battery of substrates must be run on each fraction, which requires a great effort involving very expensive technician time or else a very expensive automated device for monitoring enzyme activities. The present study indicates that it is possible to automate the performance of such assays as well as the associated data analysis rapidly using inexpensive equipment. Moreover, this technique using carbonate and thioesters will have great utility in analysis of esterase activities for genetic or clinical reasons, as they provide a wide range of possible structures for detecting different enzymes.

This approach can be used to evaluate the influence of hydrolytic enzymes on the action of xenobiotics and the role of xenobiotics in inducing or inhibiting hydrolase action on endogenous substrates. Also, it can be used for the evaluation of hydrolytic enzymes as markers of disease or toxin-induced physiological changes. Talcott *et al.* (22) noted that esterases acting on malathion were at low levels in the sera or apparently normal patients. These levels, however, were elevated in patients with a history of liver damage, and malathion carboxylesterase activity and serum glutamic-oxaloacetic transaminase (SGOT) activity were correlated positively.

The use of ELISA plate readers and especially the 96-well format has been applied to all colorimetric procedures commonly used in this laboratory. This application not only results in a dramatic increase in productivity, but it frees our spectrophotometers for more detailed studies of enzyme kinetics. For instance, all protein assays in the laboratory employ an ELISA reader and the techniques presented above have been used for the analysis of carboxylesterase activities on numerous substrates, enzyme inhibition in mouse and rat, as well as several insect systems. This approach was particularly useful for applications which result in many repetitive samples such as monitoring carboxylesterase activity from *Drosophila melanogaster* on malathion in slices of isoelectric focusing (IEF) gels. The 96-well plate reader will not replace a more advanced spectrophotometer such as Varian–Cary 219 for detailed enzyme kinetics or a variety of other procedures nor will it compete with automated analyzers in a large diagnostic laboratory. However, for routine colorimetric assays it offers a cost-effective way to reduce the expense of repetitive assays while dramatically increasing productivity and maintaining flexibility.

Since the work for this manuscript was conducted, a commercial plate reader has been marketed specifically for kinetics in 96-well plates and many plate reader companies are developing or marketing kinetic software programs to interface with existing 96-well plate readers. These more sophisticated systems will make the technology presented in this manuscript even more attractive to biochemistry laboratories. An evaluation of several plate readers indicates that the software system discussed in this manuscript is a very valuable tool for adapting the second-generation plate reader technology to the needs of

multiple users by allowing the output of the reader to be placed into a spreadsheet in a user-specified format.

ACKNOWLEDGMENTS

This work was supported by NIH Grant ES02710-07. B.D.H. is a recipient of the Burroughs Wellcome Toxicology Award.

REFERENCES

1. Voller, A., Bidwell, D. E., and Bartlett, A. (1976) *Bull. W. H. O.* **53,** 55–65.
2. Ngo, T. T., and Lenhoff, H. M. (1985) Enzyme Mediated Immunoassay, p. 489, Plenum, New York.
3. Schuman, R. F., Brimfield, A. A., and Hunter, K. W. (1984) *Biosci. Rep.* **4,** 149–154.
4. Krisch, K. (1971) *in* The Enzymes (Boyer, P. D., Ed.), pp. 43–69, Academic Press, New York.
5. Heymann, E. (1980) *in* Enzymatic Basis of Detoxication (Jakoby, W. B., Ed.), Vol. 2, pp. 291–323, Academic Press, New York.
6. Heymann, E. (1982) *in* Metabolic Basis of Detoxication: Metabolism of Functional Groups (Jakoby, W. B., Bend, J. R., and Caldwell, J., Eds.), pp. 229–245, Academic Press, New York.
7. Fonnum, F., Sterri, S. H., Aas, P., and Johnsen, H. (1985) *Fundam. Appl. Pharmacol.* **5,** S29–S38.
8. Froede, F. C., and Wilson, I. B. (1971) *in* The Enzymes (Boyer, P. D., Ed.), Vol. 5, pp. 87–114, Academic Press, New York.
9. Hagopian, M., and Tennyson, V. M. (1971) *J. Histochem. Cytochem.* **19,** 376–381.
10. Lee, P. W., Allahyari, R., and Fukuto, T. R. (1978) *Pestic. Biochem. Physiol.* **8,** 146–157.
11. Massoulie, J., and Bon, S. (1982) *Annu. Rev. Neurosci.* **5,** 57–106.
12. Gelb, M. H., Svaren, J. P., and Abeles, R. H. (1985) *Biochemistry* **24,** 1813–1817.
13. Changeux, J. P., Podleski, T., and Meunier, J. C. (1969) *J. Gen. Physiol.* **54,** 225S–244S.
14. Festoff, B. W., Popiela, H., and Beach, R. L. (1984) *in* Proceedings, Second International Meeting on Cholinesterases, Bled, Yugoslavia, Sept. 17–21, 1983 (Brzin, M., Barnard, E. A., and Sket, D., Eds.), pp. 317–330, de Gruyter, Berlin.
15. Ellman, G. L., Courtney, K. D., Andres, V., Jr., and Featherstone, R. M. (1961) *Biochem. Pharmacol.* **7,** 88–95.
16. Hammock, B. D., Abdel-Aal, Y. A. I., Mullin, C. A., Hanzlik, T. N., and Roe, R. M. (1984) *Pestic. Biochem. Physiol.* **22,** 209–223.
17. Talcott, R. E. (1979) *Toxicol. Appl. Pharmacol.* **47,** 145–150.
18. Ljungquist, A., and Augustinsson, K. B. (1971) *Eur. J. Biochem.* **23,** 303–313.
19. Mallipudi, N. M., Talcott, R. E., Ketterman, A., and Fukuto, T. R. (1980) *J. Toxicol. Environ. Health* **6,** 585–596.
20. Mentlein, R., Heiland, S., and Heymann, E. (1980) *Arch. Biochem. Biophys.* **200,** 547–559.
21. Kao, L. R., Motoyama, N., and Dauterman, W. C. (1985) *Pestic. Biochem. Physiol.* **23,** 66–73.
22. Talcott, R. E., Pond, S. M., Ketterman. A., and Becker, C. E. (1982) *Toxicol. Appl. Pharmacol.* **65,** 69–74.