Inhibition of Beef Liver Hydrolytic Enzymes by Organophosphorus Pesticides

Quantal Inhibition Characteristics of Several Organophosphorus Compounds, Using *o*-Nitrophenyl Butyrate as Substrate

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Inhibitory properties of several organophosphorus compounds have been studied using a beef liver homogenate as enzyme source and *o*-nitrophenyl-butyrate as substrate. The development of the procedure for this purpose included a preliminary study of the effects of pH, incubation time, enzyme concentration, and alcohol on absorbance, as well as the effect of preincubation time and concentration of alcohol on inhibition. The final inhibition data were subjected to a probit transformation to obtain a linear relationship between inhibition and quantity of inhibitor. From the resulting curves, an inhibition characteristic (I_c^s) peculiar to each compound was determined.

It has been demonstrated that organophosphorus pesticides and some of their metabolites inhibit a large number of tissue esterases in vitro (Heath, 1961; Lawrence *et al.*, 1960; Ooms and Brebart-Hansen, 1965; Read and McKinley, 1963; van Aspern and Oppenoorth, 1960).

Enzymes of this type are classified on the basis of substrate specificity in the following manner: (a) the arylesterases or A-esterases, which are not inhibited by organophosphates, (b) the aliesterases or B-esterases which are inhibited by organophosphates, (c) the C-esterases and (d) the cholinesterases (Aldridge, 1953a; Aldridge, 1953b; Augustinsson, 1958; Bergman *et al.*, 1957; Bergman and Rimon, 1958). Recent evidence has shown, however, that certain proteolytic enzymes such as trypsin and chymotrypsin, have the capacity to hydrolyze carboxylic acid esters and are inhibited by substances known to be potent esterase inhibitors (Myers, 1960).

Carboxylesterases (previously referred to as aliesterases) have been identified in many different tissues from numerous species including blood serum and plasma (Aldridge, 1962; Bernsohn *et al.*, 1961; Lawrence *et al.*, 1960; Main and Braid, 1962; Seume *et al.*, 1960), human and rat brain (Barron *et al.*, 1961), human liver (Ecobichon and Kalow, 1961), and housefly homogenates (van Aspern and Oppenoorth, 1960). Much of the work dealing with the inhibition of carboxylesterases by organophosphorus pesticides has been done on crude tissue homogenates (Bergman and Rimon, 1960; Myers *et al.*, 1957; Read and McKinley, 1963), mainly because of the difficulty involved in the isolation and purification of these enzymes (Bergman and Rimon, 1960; Franz and Krisch, 1966).

The fact that these enzymes are inhibited by organophosphorus compounds is used in the authors' laboratories as the basis for the detection of organophosphorus pesticide residues (McKinley and Read, 1962; Mendoza *et al.*, 1967). This procedure, applicable to both paper and thin layer chromatography, involves the oxidation of the thiophosphate to an active esterase inhibitor with bromine, the inhibition of the esterases from the liver homogenate spray in the areas on the chromatogram occupied by the pesticide, and finally the development of a background color using a suitable substrate, usually α-naphthyl acetate or 5-bromoindoxyl acetate.

This study was undertaken to determine the inhibitory effects of several organophosphorus compounds on the carboxylesterases of beef liver that hydrolyze *o*-nitrophenyl butyrate. The work reported was designed to investigate the quantal characteristics of their inhibitory properties in order to obtain some criteria that could be used in assessing and estimating organophosphorus pesticide residues.

MATERIALS

Solution A. Clark and Lubs buffer, pH 8.0 (Clark, 1928), 46.80 ml. of 0.1*N* sodium hydroxide was added to 50 ml. of 0.1*M* potassium dihydrogen phosphate solution and diluted to 100 ml.

Solution B. Monosodium phosphate, 69.0 grams, was dissolved in distilled water and made up to a volume of 1 liter to give a 0.5*M* solution.

Solution C. Disodium phosphate, 134.04 grams, was dissolved in distilled water and made up to a volume of 1 liter to give a 0.5*M* solution.

Solution D. Solution B was added to 50 ml. of solution C to give a buffer solution of 0.5*M* with respect to phosphate and a pH of 6.60. This solution was diluted 1 to 10 with distilled water for use in the preparation of substrate.

Substrate (ONPB). *Ortho*-nitrophenyl butyrate (1.046 grams) was dissolved in 10 ml. of methanol containing a drop of glacial acetic acid; 0.3 ml. of this solution was pipetted into a 100-ml. volumetric flask, made up to 100 ml. with diluted buffer D, and the resulting solution was filtered through a fine sintered-glass funnel.

Inhibitor (DFP). One gram of diisopropyl phosphofluoridate (Aldrich Chemical Co.) was dissolved in 22 ml

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of isopropyl alcohol to give approximately 0.25*M* solution. DFP is highly toxic and should be handled with extreme caution.

Working Enzyme Solution. The crude tissue homogenate was prepared according to the method used by Read and McKinley (1963). One milliliter of this crude material was introduced onto a column (1.5 × 30 cm.) containing sephadex G-75 (medium) and eluted with buffer A. The crude fraction appeared as a brown band and its movement was followed easily as it was being excluded from the column. This procedure was adopted because of the unstable nature of the crude enzyme preparation. The resulting enzyme mixture after dilution with water showed very little loss of activity after several hours at room temperature.

The brownish material was collected in a 25-ml. volumetric flask and stored in the freezer. One milliliter of this was diluted to 400 ml. with distilled water to give the working enzyme solution. Fresh working enzyme solutions were prepared daily.

Organophosphorus Compounds. The pesticides and related compounds used in this study along with their source of supply are listed below. These compounds were obtained in the purest form available (research grade) and stock solutions were made up in absolute ethanol and stored at 8° C. Appropriate working solutions in 10% ethyl alcohol were prepared fresh from the stock solutions prior to use.

Trithion. *O,O*-Diethyl *S*-(*p*-chlorophenylthio) methyl phosphorodithioate, Stauffer Chemical Co.

R-1776. *O,O*-Diethyl *S*-(*p*-chlorophenylsulfonyl) methyl phosphorodithioate, Stauffer Chemical Co.

R-1777. *O,O*-Diethyl *S*-(*p*-chlorophenylsulfinyl) methyl phosphorothioate, Stauffer Chemical Co.

R-1990. *O,O*-Diethyl *S*-(*p*-chlorophenylsulfonyl) methyl phosphorothioate, Stauffer Chemical Co.

Systox. Mixture of *O,O*-diethyl *S*-(and *O*-)-2-(ethylthio) ethyl phosphorothioates, Chemagro Corp.

Dimethoate. *O,O*-Dimethyl-*S*-(methyl carbamoyl methyl) phosphorodithioate, American Cyanamid.

Dimethoate Carboxylic Acid. *O,O*-Dimethyl *S*-(methyl carboxylic) phosphorodithioate, American Cyanamid.

Dimethoate Oxygen Analog. *O,O*-Dimethyl-*S*-(methyl carbamoyl methyl) phosphorothioate, American Cyanamid.

Thimet. *O,O*-Diethyl *S*-(ethylthio) methyl phosphorodithioate, American Cyanamid.

Guthion. *O,O*-Dimethyl S-4-OXO-1,2,3-benzotriazin-3(4H)-ylmethyl phosphorodithioate, Chemagro Corp.

Guthion Oxygen Analog. *O,O*-Dimethyl *S*-4-OXO-1,2,3-benzotriazin-3(4H)-ylmethyl phosphorothioate, Chemagro Corp.

PROCEDURE

One milliliter of the pesticide working solution was pipetted into a 15-ml. glass-stoppered tube. Three milliliters of the working enzyme solution was added, and the mixture preincubated at 25° C., for 15 minutes exactly. Then 3 ml. of the ONPB solution was added, mixed thoroughly, and incubated for 15 minutes at the same temperature. The reaction was stopped after the elapsed time with the addition of one drop of DFP solution. A reagent blank was prepared by adding a drop of the DFP to the enzyme solution. The control was obtained by first incubating 1.0 ml. of 10% ethyl alcohol with the enzyme working solution and then carrying out the substrate hydrolysis as outlined previously. After the reaction was stopped, the solutions were transferred to 1-cm. silica cuvettes and the absorbance of each tube read at 370 mμ, against the reagent blank. The per cent inhibition was calculated from the following formula:

$$\% \text{ Inhibition} = \frac{\text{absorbance (control)} - \text{absorbance (pesticide)}}{\text{absorbance (control)}} \times 100$$

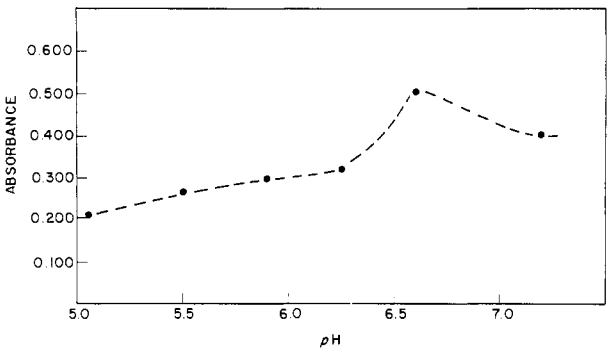

Figure 1. Effect of pH on absorbance

RESULTS

Study of the Variables. The following experiments were carried out using the same procedure as described previously except where specified. The conditions studied include: the effects of pH, incubation time, enzyme concentration, and alcohol on absorbance, as well as the effects of preincubation time and concentration of alcohol on inhibition.

Effect of pH on Absorbance. The effect of pH on absorbance is shown in Figure 1. Different buffer pH's were obtained by varying the amount of solution B relative to solution C while keeping the total volume constant. The graph shows that there is a gradual rise in the absorbance until a maximum was attained at a pH of 6.60 (the pH of solution D prior to dilution). Upon dilution and addition of the substrate, the resulting pH of the mixture was 6.30. Alkaline pH values are not desirable because the substrate undergoes spontaneous hydrolysis in this range.

Effect of Incubation Time on Absorbance. The effect of incubation time on absorbance is shown in Figure 2. Two milliliters of enzyme solution was incubated with 2 ml. of ONPB solution for time intervals of 5, 10, 15, and

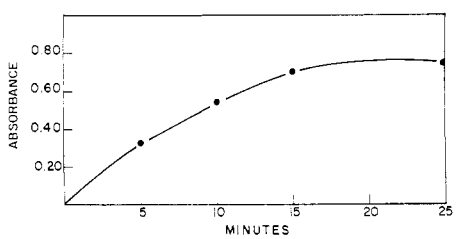

Figure 2. Effect of incubation time on absorbance

25 minutes. The reaction was stopped by the addition of 1 drop of DFP reagent and the color was read at 370 mμ on a Beckman B spectrophotometer against a reagent blank. The results revealed a marked increase in absorbance up to 15 minutes with little change over the next 10 minutes. An incubation time of 15 minutes was adopted for use in all subsequent studies.

Effect of Enzyme Concentration on Absorbance. The enzymatic activity expressed as absorbance at 370 mμ is plotted against enzyme concentration, liver (grams $\times 10^{-6}$) per 8 ml. of final solution, in Figure 3. In this case, 4 ml. of the ONPB was added, the solutions incubated at 25° C. for 15 minutes, and the reaction stopped by DFP. A gradual rise in absorbance with increasing enzyme concentrations was noted. Suitable absorbance was obtained with 500 to 1000 $\times 10^{-6}$ gram of liver. It was decided to use 3 ml. of enzyme solution with 3 ml. of ONPB solution in future experiments to ensure a good absorbance reading and suitable volume for spectrophotometric analysis. This mixture actually represented a concentration of 857 $\times 10^{-6}$ gram of liver per 8 ml. final solution. This concentration was selected after the activating effects of alcohol on enzyme activity were studied.

The Effect of Alcohol on Absorbance. Ethanol was chosen as the solvent in this study because most organophosphorus compounds are readily soluble in it, and at low levels its denaturant effect on enzymes is very slight. The following procedure was used: Three milliliters of the enzyme working solution was added to varying amounts of ethanol and made up to a total volume of 4 ml. with distilled water. After 15 minutes of preincubation at 25° C., 3 ml. of the ONPB solution was added to the mixture and the reaction allowed to proceed for 15 minutes at 25° C., followed by the addition of DFP and measurement of the absorbance at 370 mμ. The results as expressed in Figure 4 are in terms of ethanol concentration in the final solution. The least effect was observed at the 2.5% level. Lower concentrations were not tested because of the possibility of pesticide insolubility. In all subsequent experiments the compounds to be tested were added as 1-ml. samples of a 10% ethanolic solution so as to give a final concentration of 2.5% ethyl alcohol in the preincubation mixture.

Effect of Alcohol on Pesticide Inhibition. Three milliliters of enzyme working solution was incubated with the trithion in ethanol solutions of different concentration. The concentrations of ethyl alcohol in the final solutions were as follows: 2.5, 7.5, 12.5, and 25.0%. The results are expressed graphically in Figure 5. Increasing the ethyl alcohol levels caused a decrease in the inhibitory capacity of the pesticide. Because of these results and the ones from the previous experiment, the 2.5% concentration was used in subsequent experiments.

The Effect of Preincubation Time on Inhibition. Identical amounts of Trithion in 10% ethanol were added to several tubes containing the enzyme working solution. The preincubation times studied were 5, 10, 15, 20, 25, and 30 minutes. The results expressed graphically are shown in Figure 6. Maximum effect was achieved with the 20-minute preincubation period with no significant difference compared with the 15-minute interval. This latter period was chosen as the most suitable and was used in all further inhibition studies. Preincubation time was optimized only with trithion but for the sake of comparison of relative potencies the same preincubation period was used for all the pesticides.

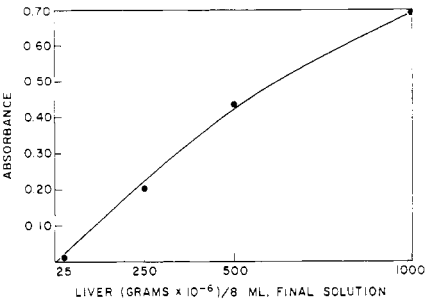

Figure 3. Effect of enzyme concentration on absorbance

Total volume in each case kept constant

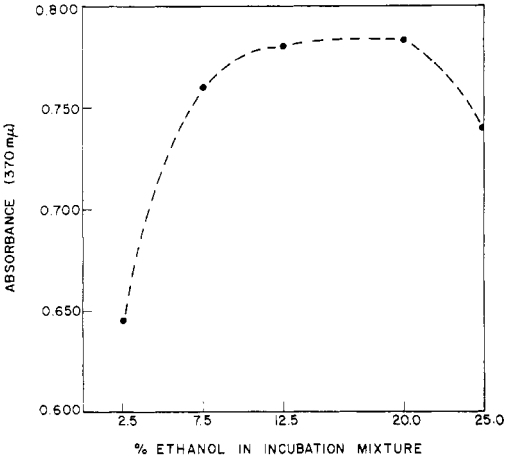

Figure 4. Effect of alcohol on absorbance

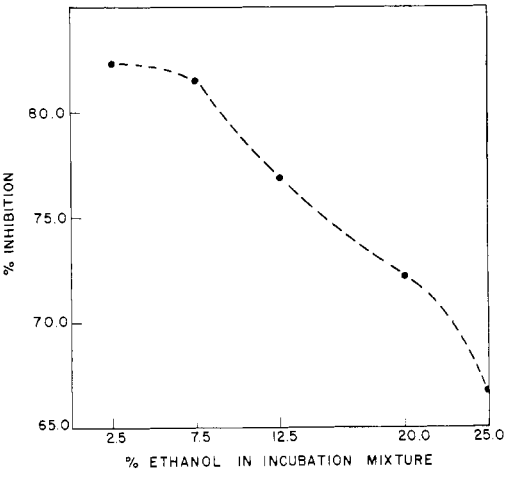

Figure 5. Effect of alcohol on enzyme inhibition

DISCUSSION OF RESULTS

Trithion was used as the inhibiting agent in preliminary experiments and the results were based on the average of two trials (with duplicates run at each trial). Several

methods of plotting these data were attempted before a linear relationship was obtained. Briefly, these were: quantity of inhibiting pesticide *vs.* per cent inhibition (Figure 7); logarithm of pesticide quantity *vs.* absorbance (Figure 8); logarithm of pesticide quantity *vs.* per cent inhibition (Figure 9).

When the first method was applied, the response (inhibition) was not linear and the graph had a sigmoidal appearance. The second and third methods also yielded a sigmoidal curve.

Because the third curve resembled the plot of in vivo toxicological response,—i.e., per cent killed *vs.* log pesticide concentration—the probit transformation was applied to the authors' data. The name "probit" was first proposed by Bliss (1934), but the procedure is actually a modification of Gaddum's normal equivalent deviate method. Finney (1952), defines probit in this manner: "The probit of the proportion P is defined as the abscissa which corresponds to a probability P in a normal distribution

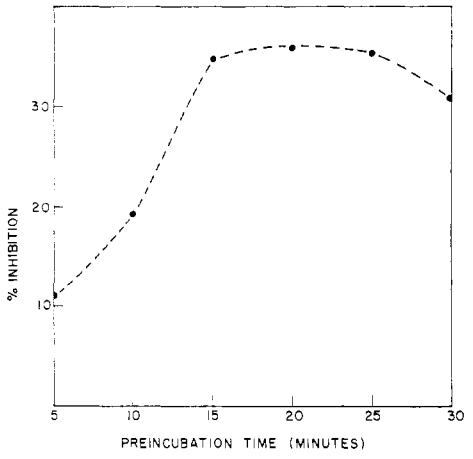

Figure 6. Effect of preincubation time on enzyme inhibition

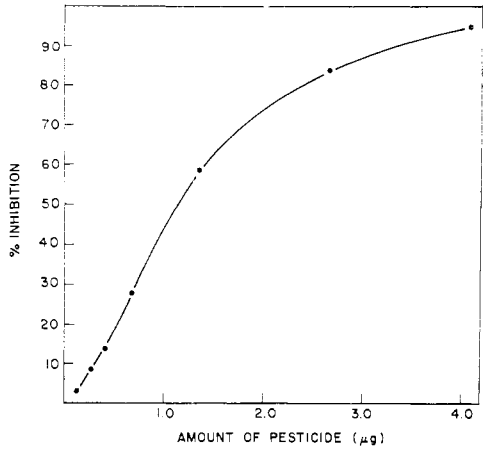

Figure 7. Inhibition results for trithion

% inhibition plotted against amount of pesticide

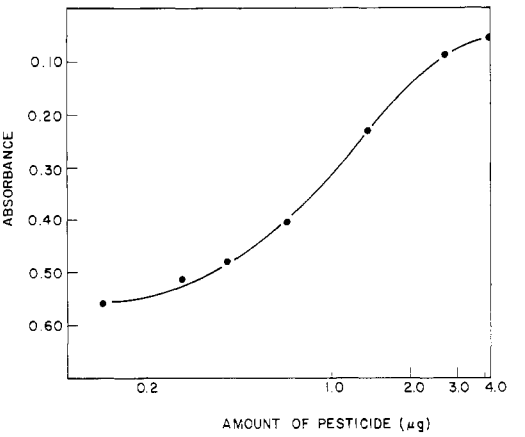

Figure 8. Inhibition results for trithion

Absorbance plotted against amount of pesticide

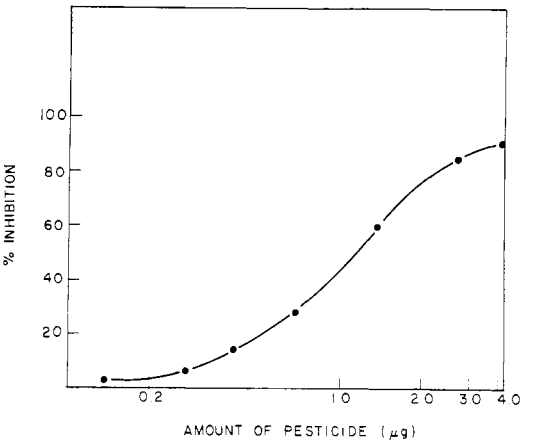

Figure 9. Inhibition results for trithion

% inhibition plotted against logarithm of pesticide quantity

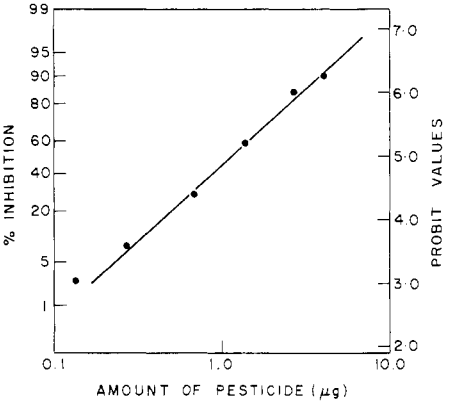

Figure 10. Effect of the probit transformation as applied to trithion inhibition data

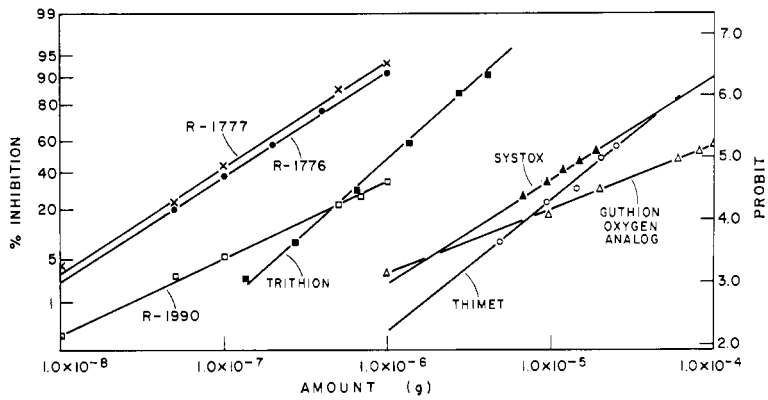

Figure 11. Inhibition results of some other compounds used in this study

with mean 5 and variance 1." When this transformation was applied to the authors' data a linear result was obtained (Figure 10). The results as shown here were plotted on a special type of graph paper called "probit paper" on which the per cent mortality (inhibition in this case) is converted directly into probits.

This transformation was applied subsequently to all the inhibition data obtained from this study, and the resulting curves are shown in Figure 11. There was a considerable range of response for the compounds tested with Trithion having an I_{50} (50% inhibition value) of 1.13×10^{-6} gram, while Guthion gave no inhibition with 1.0×10^{-4} gram. Moreover, the slopes of each of the representative lines varied, with the one for trithion being the steepest.

Table I gives the I_{50} for each compound as well as the slope of the line expressed as units of probit rise over a concentration differential represented by one logarithmic cycle. These two values are combined to give the inhibition characteristic, I_c^s, where s represents the slope of the probit curve, and c the quantity of material in grams that gives 50% inhibition. The inhibition values quoted are expressed in terms of weight of material incubated with 3 ml. of working enzyme solution. The indices reported are true only under the specific conditions used. These inhibition characteristics are presented in the last column of Table I.

Table I. Inhibition Characteristics (I_c^s) of Several Organophosphorus Compounds[a]

Compound	C[b]	S[c]	
Trithion	1.05×10^{-6}	2.35	$I_{1.05}^{2.35} \times 10^{-6}$
R-1777	1.30×10^{-7}	1.77	$I_{1.30}^{1.77} \times 10^{-7}$
R-1776	1.55×10^{-7}	1.67	$I_{1.55}^{1.67} \times 10^{-7}$
R-1990	2.10×10^{-6}	1.23	$I_{2.10}^{1.23} \times 10^{-6}$
Guthion oxygen analog	5.68×10^{-5}	1.02	$I_{5.68}^{1.02} \times 10^{-5}$
Thimet	2.18×10^{-5}	2.04	$I_{2.18}^{2.04} \times 10^{-5}$
Systox	1.70×10^{-5}	1.67	$I_{1.70}^{1.67} \times 10^{-5}$

[a] Other compounds used in this study gave no inhibition with 10^{-4} gram.
[b] Quantity of material in grams that gives 50% inhibition.
[c] The slope of the probit curve expressed as units of probit rise over a concentration differential represented by 1 logarithmic cycle.

The results indicate that the enzyme inhibition response could provide a basis for the qualitative and quantitative evaluation of the compounds concerned, and hence a method for their determination in pesticide residues. In addition, this enzyme inhibition method could provide a valuable confirmation of the results obtained by purely chemical methods based on gas-liquid and thin-layer chromatography. Further studies in this area are now underway.

LITERATURE CITED

Aldridge, W. N., *Biochem. J.* **53,** 110 (1953a).
Aldridge, W. N., *Biochem. J.* **53,** 117 (1953b).
Aldridge, W. N., *Biochem. J.* **84,** 255 (1962).
Augustinsson, K. B., *Nature* **181,** 1786 (1958).
Barron, K. D., Bernsohn, J. I., Hess, A., *J. Histochem. Cytochem.* **9,** 656 (1961).
Bergman, F., Rimon, S., *Biochem. J.* **70,** 339 (1958).
Bergman, F., Rimon, S., *Biochem. J.* **77,** 209 (1960).
Bergman, F., Segal, R., Rimon, S., *Biochem. J.* **67,** 481 (1957).
Bernsohn, J., Barron, K. D., Hess, A., *Proc. Soc. Exptl. Biol. Med.* **108,** 71 (1961).
Bliss, C. I., *Science* **79,** 38 (1934).
Clark, W. M., "Determination of Hydrogen Ions," p. 200, Williams and Wilkins, Baltimore, 1928.
Ecobichon, D. J., Kalow, W., *Can. J. Biochem. Physiol.* **39,** 1329 (1961).
Finney, D. J., "Probit Analysis," p. 20, Cambridge University Press, 1952.
Franz, W., Krisch, K., *Biochem. Biophys. Res. Com.* **23,** 816 (1966).
Heath, D. F., "Organophosphorus Poisons," p. 116, Pergamon Press, London, 1961.
Lawrence, S. H., Weimer, H. E., Salkin, D., *Proc. Soc. Exptl. Biol. Med.* **105,** 572 (1960).
MacRae, H. F., Randall, C. J., *Can. J. Biochem.* **14,** 1779 (1965).
Main, A. R., Braid, P. E., *Biochem. J.* **84,** 255 (1962).
McKinley, W. P., Read, S. I., *J. Assoc. Offic. Agr. Chemists* **45,** 467 (1962).
Mendoza, C. E., Wales, P. J., McLeod, H. A., McKinley, W. P., "Food and Drug Directorate," Canada, in press.
Myers, D. K., "The Enzymes," p. 475, Academic Press, New York, 1960.
Myers, D. K., Tol, J. W., de Jonge, M., *Biochem. J.* **65,** 223 (1957).
Ooms, A. J. J., Brebart-Hansen, J. C. A. E., *Biochem. Pharm.* **14,** 1727–38 (1965).
Read, Sheila I., McKinley, W. P., *J. Assoc. Offic. Agr. Chemists* **46,** 863 (1963).
Seume, F. W., Casida, J. E., O'Brien, R. D., J. AGR. FOOD CHEM. **8,** 43 (1960).
Van Aspern, K., Oppenoorth, F. J., *Entomol. Exptl. Appl.* **3,** 68 (1960).

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