EFFECTS OF DDT AND 2,4-D (SODIUM SALT) ON VARIOUS ENZYMES OF *MICROPTERUS SALMOIDES*

Presented to the Graduate Council of
Southwest Texas State College
In Partial Fulfillment of
The Requirements

For the Degree of

MASTER OF ARTS

By

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May, 1969

ACKNOWLEDGMENTS

The author wishes to express his appreciation to Dr. C. R. Willms, Professor and Chairman of the Department of Chemistry, for his assistance, guidance, and patience during the experimentation and preparation of this thesis.

Acknowledgments are also extended to Dr. John Fitch, Assistant Professor of Chemistry, and to Dr. Francis Horne for their constructive criticisms of this manuscript.

The author is especially indebted to the Aquatic Station, Southwest Texas State College, and to The Texas State Fish Hatchery, San Marcos, Texas, for providing the fish used in this study, to Dr. W. E. Norris, Jr., Dean of the School of Science, and Dr. M. H. Whittenberg, Associate Professor of Biology, for the use of special equipment, and most of all to Miss Alverta Hollie for moral support without which this work would not have been accomplished.

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CHAPTER I

INTRODUCTION

DDT [1,1,1-Trichloro 2,2-Bis (chlorophenyl) ethane] was synthesized in 1874, but its effectiveness as an insecticide was not realized until 1939 by Paul Muller of the Geigy Company in Switzerland. Since its patent in 1942, it has been one of the most persistent and most universally used insecticides available.

Derivatives of 2,4-D (2,4-Dichlorophenoxyacetic acid) are highly toxic to plants and therefore are used extensively as herbicides. However, this economic use of the compound is threatened by an observed toxic effect on fish and wildlife.

In spite of extensive research, the mode of toxicity of DDT and 2,4-D remains unknown. DDT has been shown to accumulate in the viscera and gill; thus many investigators feel that it somehow affects respiration. Some classical studies have been made on the respiratory enzymes with isolated mitochondria.

This study is an attempt to determine the enzyme system(s) attacked by the two compounds mentioned above and to establish the basis for their toxicity. Five

enzymes, some of which have been shown to have enzymatic activity in semipurified preparations of fish, were assayed in homogenate preparations and the effect of DDT, 2,4-D, and several solvent systems determined.

Literature Review

Despite the widespread use of DDT and the ensuing research designed to determine the mechanism of its toxicity to animals, no real advance has been made to this end. In addition to displaying toxic effects on insects and other pests, DDT has been shown to affect wildlife in very low concentration. This toxic effect on insects as well as vertebrates has prompted intensive revaluation of DDT as an insecticide and has stimulated more research into the nature of its toxicity.

Much research has been done on DDT's effect on insects and as a result a new enzyme was discovered by J. Sternburg, C. W. Kearns, and H. Moorefield[1] which was found to be responsible for the detoxification of DDT. This enzyme, DDT-dehydrochlorinase,converts DDT to DDE, [1,1-dichloro-2,2 bis (chlorophenyl) ethane], a non-toxic analog, by dehydrochlorination.

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[1] J. Sternburg, C. W. Kearns, and H. Moorefield, "DDT Dehydrochlorinase, an Enzyme Found in DDT-Resistant Flies," Journal of Agricultural and Food Chemistry, II (1954), 1125-1130.

The effect of DDT on certain insects is a characteristic deterioration of the central nervous system as described below by O'Brien.

> The symptoms of poisoning in insects suggest nervous impairment. In the American cockroach, for example, there is tremor throughout the body and appendages along with hyperexcitability followed by slow ataxia and apparent paralysis which may be total at 24 hours. Similarly, in mammals there is hyperexcitability and tremor, which is particularly evident in the fact, and there are convulsions, which may be both tonic (i.e. the animal is rigid) and clonic (frenzied and uncoordinated movements). Finally there is weakness and prostration.[2]

Such response aroused speculation that the site of attack was in the nervous system, specifically on cholinesterase, and subsequent investigation yielded varying results. A. Richards and L. Cutkomp[3] showed that DDT did not inhibit cholinesterase of nerve cords of Periplaneta americana when added in vitro. Also, cholinesterase measurements on nerve cords from this insect in various stages of DDT poisoning showed no inactivation of this enzyme.[4]

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[2]R. D. O'Brien, Insecticides; Action and Metabolism, 1967, p. 169.

[3]A. Richards and L. Cutkomp, "The Cholinesterase of Insect Nerves" Journal of Cellular and Comparative Physiology, XXVI (1945), 57-61.

[4]J. Tobias, J. Kollros, and J. Savit, "Acetylcholine and Related Substances in the Cockroach, Fly and Crayfish and the Effect of DDT," Journal of Cellular and Comparative Physiology, XXVIII (1946), 159-182.

similar negative results were obtained with cholinesterase in Musca domestica, Leptinotarsa decemlineata, sheep brain, and horse erythrocytes.[5] Yet, J. Tobias, J. Kollros, and J. Savit[6] found that the amount of acetylcholine in the ventral nerve cord of the American cockroach increased considerably during the prostrate stage of DDT poisoning but not in the hyperactive stage.

In support of the hypothesis that the increased acetylcholine content resulted from the conversion of a bound form such as acetylcholine-lipoprotein complex to the free ester, Tobias et al.[7] found that in the normal roach cord about 2 percent of the acetylcholine was in the bound form. No similar increase in acetylcholine content could be demonstrated in frogs and rats poisoned by DDT.

A. Anderson, R. March, and R. Metcalf[8] showed DDT and DDE at 3.3 X 10^{-4}M to inhibit succinoxidase, cytochrome oxidase, and succinic dehydrogenase in housefly thorax. The

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[5]D. Vincent, R. Truhaut, and A. Abadie, "Mechanism of the Toxic Action of DDT," Comptes Rendus des Seances de la Societe de Biologie et de ses Filiales, CXLII (1948), 1500.

[6]Tobias, Kollros, and Savit, loc. cit.

[7]Ibid.

[8]A. Anderson, R. March, and R. Metcalf, "Inhibition of the Succinoxidase System of Susceptible and Resistant Houseflies by DDT and Related Compounds" Annals of the Entomological Society of America, XLVII (1954), 595-602.

same effect was observed by C. Johnson[9] in rat heart, while J. Judah[10] found no evidence that DDT affected aldolase, ATPase, glutamic dehydrogenase, choline oxidase, hexokinase, or succinoxidase. However, Torda and Wolff[11] found that DDT apparently increased cytochrome oxidase activity in rat muscle.

F. H. Premdas[12] exposed salmon underyearlings to 1 ppm C^{14}DDT and determined the amount absorbed internally compared with the amount absorbed externally in fish killed by the exposure. At all times, up to and including the time of death, high concentrations of DDT were found in the gills, liver, spleen, heart, kidneys, gonads, and swim-bladder. Much smaller concentrations occurred in the stomach, intestines, brain, and spinal cord; the smallest amounts were found in muscles, bones, and integument. He concluded that DDT enters mainly through the gills, from which it is transported throughout the body via the circulatory system.

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[9]C. Johnson, "The In Vitro Effect of DDT and Related Compounds on the Succinoxidase System of Rat Heart," Archives of Biochemistry and Biophysics, XXXI (1951), 375-382.

[10]J. Judah, "Metabolism and Mode of Action of DDT," British Journal of Pharmacology, IV (1949), 120-131.

[11]C. Torda and H. Wolff in R. L. Metcalf, Organic Insecticides, p. 169.

[12]F. H. Premdas, Bureau of Sport Fisheries and Wildlife Circular 224 (May 1965), p.28.

While 2,4-D has not been subjected to as intense a study as DDT, it has, nonetheless, been given substantial review. 2,4-D and many of its derivatives are used extensively as herbicides. At recommended concentrations no animal mortality above normal has been observed.[13] However, at levels of 0.05 to 1.00 ppm a toxic effect on energy production in bluegills is evident. In his studies to date, Robert Hiltibran[14] found that concentrations as high as 10^{-4} g/ml. of the sodium salt were required to inhibit succinoxidase and alpha-ketoglutarate activities.

The major works on fish metabolism include M. Gumbman and A. L. Tappel's study of the tricarboxylic acid cycle[15] and of fatty acid oxidation in carp liver,[16] W. D. Brown's study of glucose metabolism in carp,[17] and R. C. Hiltibran's

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[13]C. Rawls and F. Beaven, Bureau of Sport Fisheries and Wildlife Circular 224 (May 1965), p. 10.

[14]R. C. Hiltibran, Abstracts of 7th International Congress of Biochemistry, Tokyo, Japan, 1967.

[15]M. Gumbman and A. L. Tappel, "The Tricarboxylic Acid Cycle in Fish," Archives of Biochemistry and Biophysics, XCVIII (1962), 262-270.

[16]W. D. Brown and A. L. Tappel, "Fatty Acid Oxidation by Carp Liver Mitochondria," Archives of Biochemistry and Biophysics, LXXXV (1959), 149-158.

[17]W. D. Brown, "Glucose Metabolism in Carp," Journal of Cellular and Comparative Physiology, LV (1960), 81.

studies on oxidative metabolism in bluegills.[18, 19, 20]

One might surmise that metabolism in all fish is similar or that a specific enzyme reacts in all organisms in the same manner. This is more often not the case, and not until such hypotheses are confirmed by experimentation, do they become law, and only for the species examined.[21] Most of the work on fish metabolism has been performed on carp and bluegill, ostensibly because of their ready accessibility, but no reported data are available on the important game fish Micropterus salmoides (largemouth black bass).

In this study, the effect of DDT and 2,4-D sodium salt is examined with respect to five enzyme systems of Micropterus salmoides:

1. Succinoxidase (a $FADH_2$ generating system) and malic dehydrogenase (a NADH generating system) are examined to determine if oxidative metabolism is inhibited by the pesticides. Since these two enzymes represent the only

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[18] R. C. Hiltibran, "Oxidation of Succinate by Bluegill Liver Mitochondria," Transactions-Illinois Academy of Science, LVIII (1965), 176-182.

[19] R. C. Hiltibran, "Oxidation of Alpha-Ketoglutarate by Bluegill Liver Mitochondria," Transactions-Illinois Academy of Science, LX (1967), 244-249.

[20] R. C. Hiltibran, "Oxidative Phosphorylation by Bluegill Liver Mitochondria," Abstracts 6th International Congress of Biochemistry, (1964), p. 780.

[21] Many reports exist of varying toxicities of pollutants to different species of fish.

known entry paths to the electron-transport system through which oxidative metabolism occurs, enzymatic inhibition of either would infer inhibition of oxidative metabolism and suggest the path through which inhibition occurs.

2. Cytochrome oxidase is examined in order to estimate more precisely the point of attack on the electron-transport chain. Since cytochrome oxidase is a terminal enzyme in the respiratory chain, inhibition of its activity by the pesticides would suggest that attack on this enzyme is the basis for toxicity in fish.

3. Catalase, the enzyme catalyzing the reduction of H_2O_2 to H_2O, was selected as another respiratory enzyme for study.

4. Acetylcholinesterase catalyzes the hydrolysis of the acetylcholine ester which results in relaxation of neural impulses. Inhibition of this enzyme would cause the classical symptoms of DDT poisoning previously noted. Because DDT is believed to affect acetylcholinesterase activity, this enzyme was selected for study. Its selection as a possibility is also supported by the extreme insolubility of DDT in aqueous medium. Since DDT has been shown to accumulate in fatty tissue, it is conceivable that the toxic effect of DDT may be due to action on a lipase rather than a respiratory enzyme.

CHAPTER II

METHODS AND MATERIALS

The fish, Micropterus salmoides, used in these studies were obtained from the San Marcos River in the vicinity of the City Park, Aquatic Station of Southwest Texas State University, and the Texas State Fish Hatchery, San Marcos, Texas. The animals were fasted at least two days before being used.

The DDT was acquired as a commercial impure preparation of 50 percent p,p'-DDT. It was recrystallized three times from 95 percent ethanol, and, after final purification, the melting point was determined to be 107°C.

The 2,4-D was acquired as the commercially available impure lithium salt. It was acidified and recrystallized until it had a constant melting point of 137°C and then made alkaline and precipitated with dilute NaOH.

Preparation of Homogenates

The animals were sacrificed by decapitation, and the desired tissue excised and rinsed in cold homogenizing medium. The composition of the homogenizing media which were used for the various enzyme assays are described later in this chapter. The weighed tissue was then placed in a graduated beaker or test tube which was then filled to the desired volume with the homogenizing medium, and homogenized in the cold for two minutes in a Servall Omni-Mix. Centrifugation where applicable was at 600 X g for 5 minutes unless otherwise stated.

Succinoxidase and Cytochrome Oxidase

Four g. of gill filament or 2 g. of liver were placed in 0.25M sucrose and the final volume adjusted to 20 ml. prior to homogenization. The gill tissue used in all experiments was gill filament excised (with scissors) from the cartilagenous bronchial section.

Malic Dehydrogenase

Six g. of gill filament were homogenized in a final volume of 20 ml. with 0.25M sucrose.

Catalase

Three hundred g. of muscle homogenate was suspended

in cold water and the final volume adjusted to 600 ml. with water, homogenized, squeezed through two thicknesses of cheesecloth, and centrifuged for 30 minutes.

Acetylcholinesterase

Thirty-five g. of muscle tissue were frozen, then placed in a container with a solution of 0.1M NaCL-0.04M $MgCl_2$. The final volume was made to 200 ml. After homogenization the pH was adjusted to 7.4 with about 1 or 2 ml. of 0.1M NaOH. The preparation was then kept cold and used as such (without centrifugation) for all subsequent assays.

Assays

Succinoxidase, malic dehydrogenase, and cytochrome-oxidase activity were estimated by a modification of the manometric method described by Potter[1] at 37.5°C. In this method oxygen uptake was estimated by measuring the pressure change in a Warburg reaction flask attached to a Warburg manometer suspended in a constant temperature bath. The total reaction volume was 3.0 ml. The center well of the flask contained 0.2 ml of 20 percent KOH and a 1 X 2 cm strip of accordionized Whatman #1 filter paper to absorb CO_2. The technique entailed the following:

1. Calibration of manometers and flasks.
2. Equilibration of constant-temperature bath at 37.5°C.
3. Insertion of assay components into the flasks (flask contents detailed in Tables I, II, and III).
4. Attachment of reaction flasks and manometers to the bath.
5. Equilibration while shaking for 15 minutes.
6. Closing of stopcock and taking initial readings of flasks at 30-second intervals.
7. Three additional readings at 30-second intervals every 15 minutes.

In the succinoxidase experiments the assay medium consisted of 0.016M potassium phosphate (pH 7.4), 1.6 X 10^{-5}M cytochrome C (molecular weight 13000), 0.05M sodium

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[1]V. R. Potter, "Homogenate Technique" in Manometric Techniques, ed. W. W. Umbreit, R. H. Burris, and J. E. Stauffer, 1964, pp. 174-175.

succinate and 3 X 10^{-3}M $CaCl_2$, 3 X 10^{-3}M $AlCl_3$ and 0.3 ml. homogenate in a total volume of 3.0 ml. Table I shows the individual succinoxidase assay systems in detail.

In the malic dehydrogenase assay system the reaction medium contained 0.27M potassium phosphate (pH 7.4) 0.01M nicotinamide, 0.05M sodium malate, 0.05M sodium glutamate, 0.033 percent nicotinamide adenine dinucleotide (NAD), 4 X 10^{-5}M cytochrome C, 0.3 ml. homogenate, and varying concentrations of solvent and solvent pesticide solution. Table II shows the experimental details of the various assay systems.

The reaction vessels in the cytochrome oxidase experiments contained 0.8 X 10^{-4}M cytochrome C, 1.14 X 10^{-3}M sodium ascorbate, and varying aliquots of solvent or solvent pesticide solution and homogenate. The various assay systems are illustrated in detail in Table III.

In order to determine the reproducibility of the method, eleven flasks were prepared for two series of experiments. Six flasks contained water as solvent and five contained 2.7 percent ethanol as solvent (see assay 4 of Table I); the succinoxidase experiment was conducted as before, and the probable error was determined from the maximum difference between oxygen uptakes of any two flasks of the same series. This difference was expressed as a percentage of the maximum oxygen uptake.

Catalase was assayed by the method of von Euler and

Josephson.[2] Fifty ml. of cold 0.015N H_2O_2 -0.115M potassium phosphate buffer (pH 7.4) was placed in a 250 ml. flask. Ten ml. of solvent (35% dioxane), 35% dioxane-0.015M 2,4-D-Na,[+] or 35% dioxane-1.5 X 10^{-4}M DDT was added and the reaction flask and homogenate were allowed to equilibrate at 0°C for 10 minutes. Three ml. of the homogenate were then added and, at timed intervals, 5 ml. aliquots were transferred to 10 ml. of 2N H_2SO_4 to stop the reaction. The aliquots were titrated with 0.0491N $KMnO_4$ to estimate the amount of H_2O_2 consumed.

Acetylcholinesterase was assayed by the method of D. Nachmansohn and I. B. Wilson[3] in a solution of 0.1M NaCl-0.04M $MgCl_2$. Fifteen minutes prior to an assay, 10 ml. of cold homogenate was added to an incubation mixture which consisted of 10 ml. of solvent (10% ethanol) and/or pesticide (10^{-5}M DDT or 10^{-2}M 2,4-D) and 10 ml. of 0.2M NaCl-0.04M $MgCl_2$. The mixture was allowed to incubate at room temperature (25-26°C) for 15 minutes, after which 1 ml. of freshly prepared 2.2M acetylcholinchloride was added with stirring. The stirring was continued until the assay

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[2]H. von Euler and K. Josephson, "Uber Katalase I," Annalen Der Chemie, LDII (1927), 158-181.

[3]D. Nachmansohn and I. B. Wilson, "Acetylcholinesterase," (Vol. 1 of Methods in Enzymology, ed. S. P. Colowick and N. O. Kaplan. 50 vols.), pp. 647-648.

was complete. After 1 minute the pH was carefully adjusted to 7.4 with 0.02N NaOH. The volume of 0.02N NaOH required to maintain pH at 7.4 was plotted as a function of time in order to evaluate acetylcholinesterase activity.

Solubilization of DDT and DDE

Preweighed aliquots of DDT or DDE were dissolved in a minimum amount of pure solvent. Immediately, prior to use, the solvent pesticide solution was diluted with water to obtain a 10 percent acetone, alcohol, or 2M dimethyl sulfoxide (DMSO) solution, and an aliquot was then added to the reaction flask. A fresh preparation was used for each assay to safeguard against precipitation of the pesticide.

CHAPTER III

RESULTS

Succinoxidase

Solvent Effects

A series of preliminary experiments was performed to find a suitable solvent for DDT. Solvent controls were used with all experiments which are described later in this chapter.

Digitonin.--A concentration of 0.1 percent digitonin formed a seemingly homogeneous emulsion of concentrations of DDT as high as 10^{-4}M and did not affect oxygen uptake. However, the supply of digitonin was limited; so a search for a solvent was continued. The DDT appeared to form a homogeneous suspension in hot solution but settled out in a matter of hours. The oxygen uptake data with digitonin are shown in Table IV.

Ethanol.--By the use of various ethanol concentrations and by measuring succinoxidase activity, a concentration of 2.7 percent ethanol (0.8 ml. of 10 percent ethanol in 3 ml.

total volume) was found to be optimum.[1] The oxygen uptake in a medium containing 2.7 percent ethanol in the absence of succinate is compared with oxygen uptake for the same substrate in the aqueous solution in Table V. This series of assays was used as a check for accuracy. The greatest difference between any two oxygen uptake rates is 0.26 and 0.38µl/mg/hr for aqueous control and ethanol respectively. Expressed as a percent of the highest uptake rate in each series, the maximum error is 9.8 percent and 13.7 percent. These data show that, within the range of experimental error, ethanol at a final concentration of 2.7 percent has no effect on oxygen uptake of bass liver or gill homogenate when succinate is the substrate.

Dimethyl sulfoxide (DMSO).--Reaction media which contained different concentrations of DMSO, when assayed for succinoxidase activity, showed no pronounced differences in oxygen uptake as shown in Table VI.

Effect of DDT on Succinoxidase

Table VII is a summary of the effect of DDT on succinoxidase activity of bass gill tissue. These data show that at concentrations as high as 3 X 10^{-5}M DDT no

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[1]Two and seven-tenths percent ethanol does not inhibit enzymatic activity appreciably; yet it solubilizes DDT up to 10^{-4}M.

effect on succinoxidase is observed. However, at 6.67×10^{-4}M DDT in 0.53M DMSO, oxygen uptake in the presence of sodium succinate was inhibited by 72 to 83 percent with the use of liver homogenate as shown in Table VIII.

Effect of 2,4-D (Sodium Salt) on Succinoxidase

Tables IX and X illustrate the effect of 2,4-D on succinoxidase activity (µl oxygen consumed/mg/hr) in bass bill and liver homogenates. In concentrations up to and including 3×10^{-3}M 2,4-D (sodium salt) no effect on succinoxidase activity was observed, while at 8×10^{-2}M 2,4-D, inhibition was almost total.

Malic Dehydrogenase

The Effect of 2,4-D (sodium salt) on Malic Dehydrogenase

The effects of 2,4-D on malic dehydrogenase are summarized in Table XI. These data infer no inhibition of succinoxidase activity in bass gill homogenates when 2,4-D concentration is as high as 2×10^{-3}M. Additional experiments using liver homogenates showed almost complete inhibition when the 2,4-D concentration was increased to 5×10^{-2}M as shown in Table XII.

Effect of DDT on Malic Dehydrogenase

For the assay of malic dehydrogenase in the presence of DDT, acetone was employed as a solvent. A thorough investigation of solvent effect was not performed; however, precursory experiments inferred 1.6 percent acetone (0.5 ml. of 10 percent acetone in 3 ml. reaction media) to be a better solvent for this system. Table XIII shows that 1.6 percent acetone has very little effect (if any) on malic dehydrogenase activity of fresh gill tissue.

Cytochrome oxidase

Effect of 2,4-D (sodium salt)

Table XIV shows the effect of 2,4-D on cytochrome oxidase activity of fresh bass liver homogenate. A comparison of control with activity in the presence of 6.67×10^{-2}M 2,4-D shows approximately 71 percent inhibition. This change in activity is much too large to be attributed to experimental error. Thus cytochrome oxidase activity is definitely inhibited by 6.67×10^{-2}M 2,4-D.

Effect of DDT

Table XV shows the control (0.53M DMSO) activity to be 27.0 and 29.7 µl/mg/hr compared with 11.1 and 10.2 µl/mg/hr in the presence of 6.67×10^{-4}M DDT. Such comparison infers 40-65 percent inhibition of cytochrome oxidase activity by 6.67×10^{-4}M DDT. This decrease in activity must be attributed to inhibition by 6.67×10^{-4}M DDT.

Effects of DDE

The effect of 6.67×10^{-4}M DDE in 0.53M DMSO on cytochrome oxidase activity of bass liver homogenate is shown in Table XVI. From the table, 11.4 and 10.5 µl/mg/hr activities in the presence of 6.67×10^{-4}M DDE compared with 29.7 and 27.0 µl/mg/hr activity in the control

represents 35-40 percent inhibition by these concentrations of DDE.

Catalase

A plot of log a/a-x, initial $[H_2O_2]$ / final $[H_2O_2]$, vs. time for catalase assay in 5.5 percent dioxane, 5.5 percent dioxane-2 X 10^{-5}M DDT, and 5.5 percent dioxane-2.4 X 10^{-3}M 2,4-D Na^+ is shown in Figure 1. The slopes of the plots per g. tissue, specific rate constants, are shown to be 0.0204 $min^{-1}g^{-1}$, 0.018 $min^{-1}g^{-1}$ and 0.019 $min^{-1}g^{-1}$ respectively. These data indicate, within the limits of experimental error, that DDT and 2,4-D Na^+ do not inhibit catalase activity at this concentration (2.4 X 10^{-5}M DDT and 2.4 X 10^{-3}M 2,4-D Na^+).

Acetycholinesterase

Bass liver homogenate was found to exhibit acetylcholinesterase activity. These data are shown graphically in Figures 2-5. The specific activities of the various assays are summarized in Table XVII, specific activity being defined as micromoles of acetic acid liberated per gram of tissue per hour.

The acetylcholinesterase activity was found to increase slightly with time; the specific activity at the beginning of the assays was 570 $g^{-1}min^{-1}$ while at the end of the assay, a period of approximately three hours, the activity was 660 $g^{-1}min^{-1}$. Thus a control was run immediately after each assay, and the close correlation in date shows that the change during an experiment was negligible. Each experiment had a duration of about 10 minutes.

Accumulation of DDT Residues in Organs

With the use of the Schecter-Haller method for determination of DDT a reproducible standard curve was obtained. After consideration of the sensitivity of the method, it was concluded that due to the great number of specimens required (approximately 50 g. of each organ or tissue for each determination), the study would have to be discontinued. An analysis of the pesticide residues in the tissues required a clean-up procedure as outlined in the Official Methods of Analysis of Agriculturist Chemists.[2]

When the extract was passed through acid-treated Celite 545, the eluate was repeatedly dark brown and black; actually darker eluates were obtained when the clean-up was attempted. The celite was sold as "Filter-aid" by a local chemical firm. The tradename "filter aid" apparently does not represent the same product as the John Mansfield product, Celite 545. This faulty chemical was presumed to be the source of complications that were found with this assay procedure.

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[2]Association of Official Agricultural Chemists, Official Methods of Analysis, 1965, pp. 391-394.

CHAPTER IV

DISCUSSION

Throughout these studies, solubility of the pesticide was a dominating factor. These studies were started with the use of ethanol at a final concentration of 2.7 percent. In order to obtain a homogeneous suspension of DDT (concentrations up to 3 X 10^{-5}M), weighed quantities of pesticide were dissolved in hot ethanol and diluted to the desired volume. However, at 6.67 X 10^{-4}M, the DDT would dissolve in the hot alcohol and recrystallize when cooled or diluted.

Digitonin appeared to hold the DDT in suspension unless allowed to set for over an hour, but the solution had to be heated before the digitonin itself would dissolve. One could never be certain if the crystals in solution were digitonin or DDT.

Acetone proved to be a very good solvent. It readily dissolved the DDT, and, when diluted to volume, the pesticide did not recrystallize immediately.

Earlier work by K. J. Wilson[1] showed that succinoxidase

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[1]K. J. Wilson, "The Effect of Dimethyl Sulfoxide on the Oxidative Metabolism of Saccharomyces ceremisiae and Escherichia coli," Unpublished Master's Thesis, Southwest Texas State College, 1966.

activity is only slightly affected by DMSO. Preliminary studies (Table III) indicated no appreciable effect of DMSO on oxygen uptake when succinate is substrate. Since the pesticides were readily soluble in DMSO, it was used as solvent in all the assays that required 6.67×10^{-4}M DDT.

Care had to be taken also in 2,4-D (sodium salt) solutions of the higher concentration. The salt dissolved in warm solution but readily recrystallized on cooling.

DDT was shown to affect succinoxidase and cytochrome oxidase activity at 6.67×10^{-4}M. The inferred point of attack is the fourth complex of the multi-enzyme system proposed by Green and Tzagoloff[2] which contains cytochrome oxidase. Proof of this point would require demonstration that DDT also inhibits NADH-generating systems and any enzyme occurring in the electron transport system between substrate and cytochrome oxidase.

This study suggests DDT has no effect on malic dehydrogenase activity at 1.6×10^{-5}M DDT. But, similarly, at that concentration no effect was observed with DDT on succinoxidase activity. Determination of the concentration of DDT required to produce a toxic effect was outside the

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[2]D. E. Green and A. Tzagoloff, "The Mitochondrial Electron Transfer Chain," Archives of Biochemistry and Biophysics, CXVI (1966), 293-304.

scope of this study, but it is obvious from the data that the concentration required was between 1.6 X 10^{-5}M and 6.67 X 10^{-4}M since the former concentration produced no effects and the latter produced almost complete inhibition. Thus, malic dehydrogenase must be examined at a higher DDT concentration before it can be concluded that it is unaffected by DDT.

Doubt is also cast on the fourth complex as the point of attack because, although cytochrome oxidase is affected, its inhibition is perhaps the sum effect(s) of inhibition of two or more enzyme systems between substrate and actual oxygen consumption. The direct consequence of this line of reasoning is that cytochrome C reductase should also be affected since it resides at complex III.

Another consideration is the low concentration of DDT normally required to demonstrate toxic effects. DDT in concentrations of 11.5-44 ppb will kill 50 percent of various fish in 96 hours[3]; the solubility of DDT in water at 25° is 1.2 ppb.[4] One would certainly expect inhibition at concentrations of 100 ppb or 284mμm/liter. Yet these data show that at 3 X 10^{-5}M DDT, one thousand times the lethal concentration, no effect is observed *in vitro* on the systems assayed.

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[3]O'Brien, *op. cit.*, p. 303.

[4]*Ibid.*

Furthermore, if the inhibition of respiration observed is the source of DDT toxicity, markedly less inhibition should be observed with the use of the relatively non-toxic analog, DDE. But Table V shows that the inhibition at 6.67×10^{-4}M DDE is almost identical to DDT.

Apparently this investigator encountered the same blocks as others in their attempts to elucidate the effects of DDT on respiration. Although DDT affects respiration, it does not follow, nor is it strongly suggested that this effect is the basis for DDT toxicity to fish. More investigation is required to determine the effect of higher concentrations of DDT on malic dehydrogenase. The effects then of DDT (if any) should be compared to the effects of an analog of DDT that exhibits no toxicity to fish. Only in the absence of appreciable activity of the enzyme in the presence of the non-toxic DDT analog can one relate the toxicity of DDT to its effect on respiration.

Hiltibran has inferred that 2,4-D sodium salt is toxic to bluegills at levels of 1.0 to 40 ppm.[5] This suggests that the salt might have a toxic effect on one or more enzyme systems at a level around 2×10^{-4}M. The data presented here show no inhibition at such levels of 2,4-D sodium salt and still none at 3×10^{-3}M of the salt. But

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[5]R. C. Hiltibran, Abstract, Presented at the Weed Society of America Meeting in St. Louis, Missouri, February 1966.

at 8 X 10^{-2}M, succinoxidase, malic dehydrogenase, and cytochrome oxidase activity is almost completely inhibited. The inhibition of cytochrome oxidase activity might suggest inhibition of all enzymes that transfer electrons through the electron-transport chain since cytochrome oxidase is near the terminus of that system through which electrons must pass in vivo to affect oxidation of substrate and reduction of oxygen.

These data are in consonance with those obtained by Hiltibran.[6] He reports that the sodium salt of 2,4-D inhibits oxygen uptake in the presence of succinate and alpha-ketoglutarate; however, there is a lack of available data that shows the levels of 2,4-D that were used or the exact levels of 2,4-D sodium salt that causes the toxic effect to bass.

The studies of catalase and acetylcholinesterase should be repeated at the pesticide concentrations shown to cause inhibition. However, at the level tested, neither DDT nor 2,4-D had any effect on enzymatic activity.

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[6]Ibid.

CHAPTER V

SUMMARY

In summary, the effect of DDT and 2,4-D on five enzyme systems has been examined. At physiological-inhibition concentrations around 10^{-5}M, no effects on the rates of enzymatic catalyzed reactions were observed. However, at 6.67×10^{-4}M, DDT inhibited activity of succinoxidase and cytochrome oxidase, and at 2,4-D concentration of 8×10^{-2}M inhibition of these enzymes and malic dehydrogenase was virtually complete. Because the concentrations of DDT and 2,4-D required to cause inhibition were very high one cannot conclude that attack on oxidative metabolism is the basis for toxicity to bass. However, these data suggest that a possible point of attack of 2,4-D might be cytochrome oxidase.

(1) H_2O Control.
(2) Digitonin control.
(3) Succinoxidase assays in presence of 3 X 10^{-6}M DDT in 0.016% digitonin.
(4) Ethanol control - total ethanol concentration in reaction flask is 2.7%.
(5) Succinoxidase assays in presence of 3 X 10^{-13} to 3 X 10^{-5}M DDT in 2.7% ethanol.
(6), (7) DMSO controls.
(8), (9) Succinoxidase assays in presence of 3 X 10^{-5}M and 6.67 X 10^{-4}M DDT in 0.53M DMSO.
(10) Succinoxidase assays in presence of 3 X 10^{-13} to 8 X 10^{-2}M 2,4-D Na^+.

TABLE I. SUMMARY OF SUCCINOXIDASE ASSAY SYSTEMS

Warburg Flask's Contents	Individual Assay Systems									
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)
0.5M Na Succinate (ml) (pH 7.4)	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
1 X 10^{-4}M Cytochrome C (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
0.1M Phosphate pH7.4 (ml)	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
3 X 10^{-3}M $AlCl_3$ (ml)	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
3 X 10^{-3}M CaCl (ml)	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Homogenate (30% Gill) or 10% Liver (ml)	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
20% KOH (ml)	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2
H_2O (ml)	0.8	-	-	-	-	-	-	-	-	0.8
0.1% Digitonin (ml)	-	0.8	0.8	-	-	-	-	-	-	-
DDT (M)	-	-	$3X10^{-6}$	-	$3X10^{-13}$	-	-	$3X10^{-5}$	$6.67X10^{-4}$	-
10% Ethanol (ml)	-	-	-	0.8	0.8	-	-	-	-	-
1M DMSO (ml)	-	-	-	-	-	0.8	-	-	-	-
2M DMSO (ml)	-	-	-	-	-	-	0.8	0.8	0.8	-
2,4-D (sodium salt) (M)	-	-	-	-	-	-	-	-	-	$3X10^{-13}$ - $8X10^{-2}$

TABLE II. SUMMARY OF MALIC DEHYDROGENASE SYSTEMS

Warburg Flask Contents	Individual Assay Systems (1)	(2)	(3)	(4)
0.1 M (ml) pH 7.4	0.8	0.8	0.8	0.8
0.1M Nicotinamide (ml)	0.3	0.3	0.3	0.3
0.5M Na-Malate (ml)	0.3	0.3	0.3	0.3
0.5M Glutamate (ml)	0.3	0.3	0.3	0.3
0.5% NAD (ml)	0.2	0.2	0.2	0.2
4 X 10^{-4}M Cytochrome C (ml)	0.3	0.3	0.3	0.3
Homogenate (30% gill) (ml)	0.3	0.3	0.3	0.3
20% KOH (center well) (ml)	0.2	0.2	0.2	0.2
H_2O_2 (ml)	0.5	-	-	0.5
10% Acetone (ml)	-	0.5	0.5	-
DMSO (M)	-	-	-	-
DDT (M)	-	-	$1.6X10^{-5}$	-
2,4-D (M) (sodium salt)	-	-	-	2 X 10^{-7} to 5 X 10^{-2}

(1) Water control.
(2) Acetone control. Total acetone concentration is 1.6%.
(3) Malic dehydrogenase assays in presence of $1.6X10^{-5}$M DDT in 1.6% acetone.
(4) Malic dehydrogenase assays in presence of aqueous solution of $2X10^{-7}$M to $5X10^{-2}$M 2,4-D Na^{+}.

TABLE III. SUMMARY OF CYTOCHROME OXIDASE ASSAYS

Warburg Flasks Contents	Individual Assay Systems			
	(1)	(2)	(3)	(4)
0.1M PO_4 Buffer pH 7.4 (ml)	1.0	1.0	1.0	1.0
4.8 X 10^{-4}M Cytochrome C (ml)	0.5	0.5	0.5	0.5
4 X 10^{-3}M $AlCl_3$ (ml)	0.3	0.3	0.3	0.3
0.0114M Na-ascorbate (ml)	0.3	0.3	0.3	0.3
Homogenate (30% gill or 10% liver) (ml)	0.1	0.1	0.1	0.1
20% KOH (ml)	0.2	0.2	0.2	0.2
H_2O (ml)	-	-	-	0.8
2M DMSO (ml)	0.8	0.8	0.8	
DDT (M)	-	6.67×10^{-4}	-	-
DDE	-	-	6.67×10^{-4}	-
2,4-D Na+ (M)	-	-	-	6×10^{-2}

(1) DMSO control. Total DMSO CONCENTRATION IS 0.53M
(2) Cytochrome oxidase assay in presence of 6.67×10^{-4}M DDT in 0.53M DMSO
(3) Cytochrome oxidase assay in presence of 6.67×10^{-4}M DDE in 0.53M DMSO
(4) Cytochrome oxidase assay in presence of aqueous 6×10^{-2}M 2,4-D Na+

TABLE IV

MICROLITERS OF OXYGEN CONSUMED PER MILLIGRAM OF FRESH GILL TISSUE PER HOUR IN 0.027 PERCENT DIGITONIN IN THE PRESENCE OF 0.05M SODIUM SUCCINATE

Digitonin Concentration (%)	0	0.027
Duplicate Q_{O_2}	0.444	0.486
	0.456	0.486

TABLE V

MICROLITERS OF OXYGEN CONSUMED PER MILLIGRAM FRESH LIVER PER HOUR IN THE PRESENCE OF 2.7 PERCENT ETHANOL AND 0.05M SODIUM SUCCINATE

ETHANOL Concentration (%)	0	2.7
	2.6	2.44
	2.44	2.60
	2.66	2.44
	2.40	2.68
	2.40	2.32
	2.40	
Q_{O2} Average	2.48	2.49

TABLE VI

MICROLITERS OF OXYGEN CONSUMED PER MILLIGRAM FRESH WEIGHT OF GILL TISSUE PER HOUR IN 0.05M SODIUM SUCCINATE WITH VARIOUS CONCENTRATIONS OF DIMETHYL SULFOXIDE

DMSO Concentration (M)	0	0.027	0.27	0.53
Duplicate Q_{O_2}	0.980	0.880	0.890	0.800
	0.880	0.790	0.890	0.800

TABLE VII

MICROLITERS OF OXYGEN CONSUMED PER MILLIGRAM FRESH WEIGHT OF GILL TISSUE PER HOUR IN 0.05M SODIUM SUCCINATE WITH VARYING SOLVENTS AND VARIOUS CONCENTRATIONS OF DDT

Duplicate Q_{O_2} in DDT Conc. (M)	0	$3X10^{-13}$	$3X10^{-11}$	$3X10^{-9}$	$3X10^{-7}$	$3X10^{-6}$	$3X10^{-5}$
2.7% ETOH	.525	.465	.420	.480	.426	.492	-
	.525	.465	-	-	.475	-	-
.027% Digitonin	.486	-	-	-	-	-	-
	.486	-	-	-	-	.454	-
.027% Digitonin	1.02	-	-	-	-	-	1.05
	1.02	-	-	-	-	-	1.11
0.53M DMSO	1.05	-	-	-	-	-	0.984
	0.810	-	-	-	-	-	0.890

TABLE VIII

MICROLITERS OF OXYGEN CONSUMED PER MILLIGRAM FRESH LIVER PER HOUR IN THE PRESENCE OF 0.05M SODIUM SUCCINATE AND 0.53M DMSO WITH VARYING CONCENTRATIONS OF DDT AND DDE

Pesticide Concentration (M)	0	DDT $6.67X10^{-4}$	DDE $6.67X10^{-4}$
Duplicate Q_{O_2}	4.50 5.25	1.27 1.05	1.20 1.20
	3.84 4.44	0.740 0.840	1.02 1.02

TABLE IX

MICROLITERS OF OXYGEN CONSUMED PER MILLIGRAM FRESH WEIGHT OF GILL TISSUE PER HOUR IN THE PRESENCE OF 0.05M SODIUM SUCCINATE AND VARYING CONCENTRATIONS OF 2,4-D

2,4-D Conc. (M)	0	$3X10^{-13}$	$3X10^{-11}$	$3X10^{-9}$	$3X10^{-7}$	$3X10^{-5}$	$3X10^{-3}$	$8X10^{-2}$
	1.05	1.08	1.02	1.00	1.00	0.900	1.00	-
	1.05	-	-	-	-	0.900	1.08	-
	2.48	-	-	-	-	-	-	0.35
	7.80	-	-	-	-	-	-	0.900
	5.64	-	-	-	-	-	-	0.78

TABLE X

MICROLITERS OF OXYGEN CONSUMED PER MILLIGRAM FRESH WEIGHT OF LIVER TISSUE PER HOUR IN THE PRESENCE OF 0.05M SODIUM SUCCINATE AND VARYING CONCENTRATIONS OF 2,4-D

2,4-D Concentration (M)	0	8×10^{-2}
Q_{O_2} (Duplicates)	2.48 7.80 5.64	0.350 0.900 0.780

TABLE XI

MICROLITERS OF OXYGEN CONSUMED PER MILLIGRAM FRESH WEIGHT OF GILL TISSUE PER HOUR IN THE PRESENCE OF 0.05M SODIUM MALATE AND VARYING CONCENTRATIONS OF 2,4-D

2,4-D Concentration (M)	0	$2X10^{-7}$	$2X10^{-5}$	$2X10^{-3}$
Q_{O_2}	2.16	2.19	2.04	2.19

TABLE XII

MICROLITERS OF OXYGEN CONSUMED PER MILLIGRAM FRESH WEIGHT OF LIVER TISSUE IN THE PRESENCE OF 0.05M SODIUM MALATE AND VARYING CONCENTRATIONS OF 2,4-D

2,4-D CONCENTRATION (M)	0	5×10^{-2}
Q_{O_2}	6.48	0.300
(Duplicates)	8.40	0.210

TABLE XIII

MICROLITERS OF OXYGEN CONSUMED PER MILLIGRAM FRESH GILL TISSUE PER HOUR IN THE PRESENCE OF 0.05M SODIUM MALATE WITH VARYING CONCENTRATIONS OF ACETONE AND DDT

Acetone Concentration (%)	0	1.6	1.6
DDT Concentration (M)	0	0	$1.6X10^{-5}$
Q_{O_2} (Duplicates)	1.41	1.35	1.38
	1.41	1.35	1.22
	1.41	1.35	1.11

TABLE XIV

MICROLITERS OF OXYGEN CONSUMED PER MILLIGRAM FRESH LIVER PER HOUR IN THE PRESENCE OF 0.05M SODIUM ASCORBATE, 8 X 10^{-5}M CYTOCHROME C AND VARYING CONCENTRATIONS OF 2,4-D

2.4-D Concentration (M)	0	6.0×10^{-2}
Q_{O_2} (Duplicates)	22.8	6.3
	21.3	6.0
	20.1	

TABLE XV

MICROLITERS OF OXYGEN CONSUMED PER MILLIGRAM FRESH LIVER PER HOUR IN THE PRESENCE OF 0.05M SODIUM ASCORBATE, 8 X 10^{-5}M CYTOCHROME C AND 0.53M DMSO WITH VARYING CONCENTRATIONS OF DDT

DDT Concentration (M)	0	6.67×10^{-4}
Q_{O_2} (Duplicates)	27.0	11.4
	29.7	10.5
	18.6	11.1
	21.0	10.2

TABLE XVI

MICROLITERS OF OXYGEN CONSUMED PER MILLIGRAM FRESH LIVER PER HOUR IN THE PRESENCE OF 0.05M SODIUM ASCORBATE, 8 X 10^{-5}M CYTOCHROME C AND 0.53M DMSO WITH VARYING CONCENTRATIONS OF DDE

DDE Concentration (M)	0	6.67 X 10^{-3}
Q_{O_2} (Duplicates)	27.0	15.0
	29.7	13.8
	18.6	16.8
	21.0	12.0

Figure 1. Log $\frac{\text{(initial } [H_2O_2])}{[H_2O_2] \text{ at time t}}$ vs. time using fresh muscle tissue of *Micropterus salmoides*.

■ ——— Catalase assay in 5.5% dioxane (control)

o --- --- Catalase assay in 5.5% dioxane-2.4 X 10^{-3}M 2,4-D (sodium salt)

Δ------- Catalase assay in 5.5% dioxane-2.4 X 10^{-5}M DDT

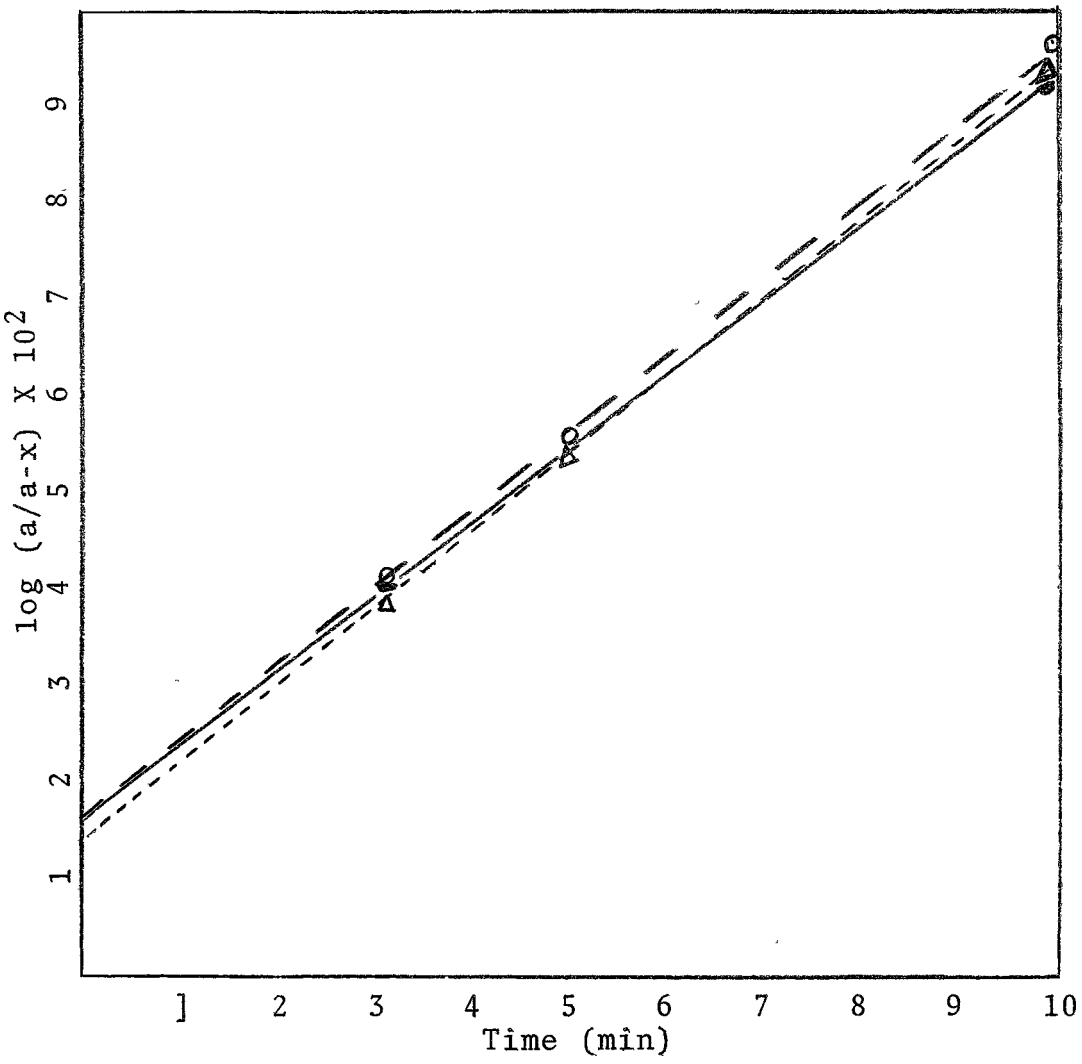

FIGURE I

EFFECT OF DDT AND 2,4-D ON CATALASE ACTIVITY

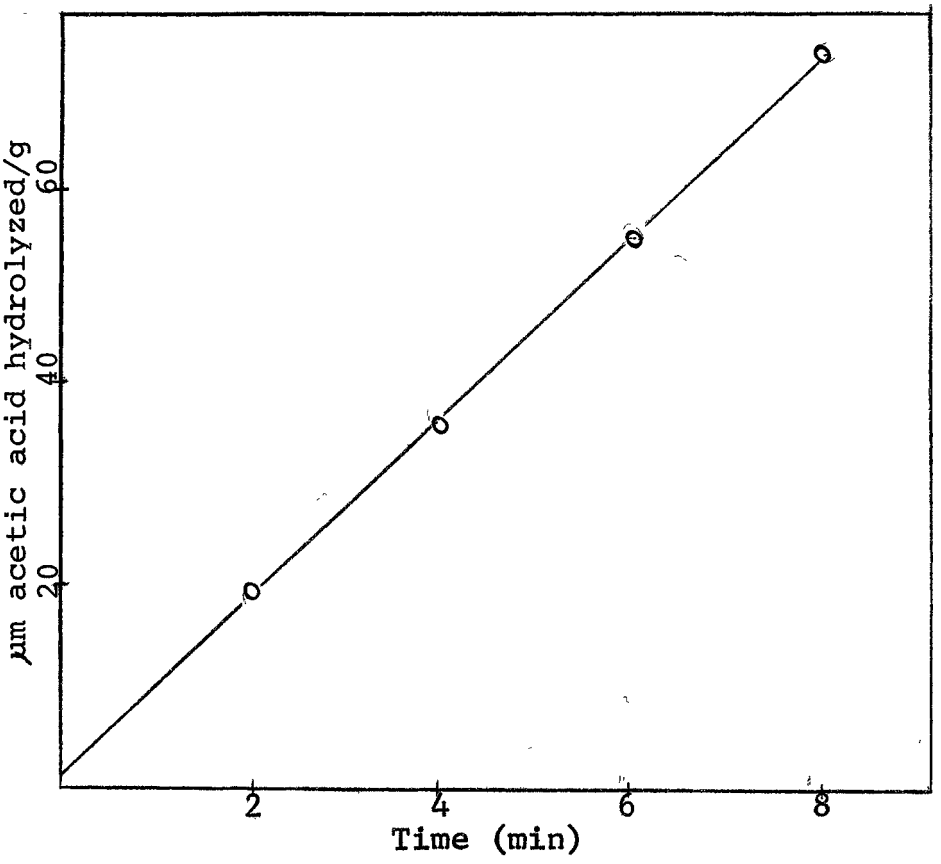

FIGURE 2

ACETYLCHOLINESTERASE ACTIVITY OF

BASS MUSCLE HOMOGENATE

Figure 2. Micromoles of acetic acid hydrolyzed per gram of fresh muscle tissue homogenate of Micropterus salmoides vs. time in the presence of 0.0733M acetylcholine chloride, in aqueous medium.

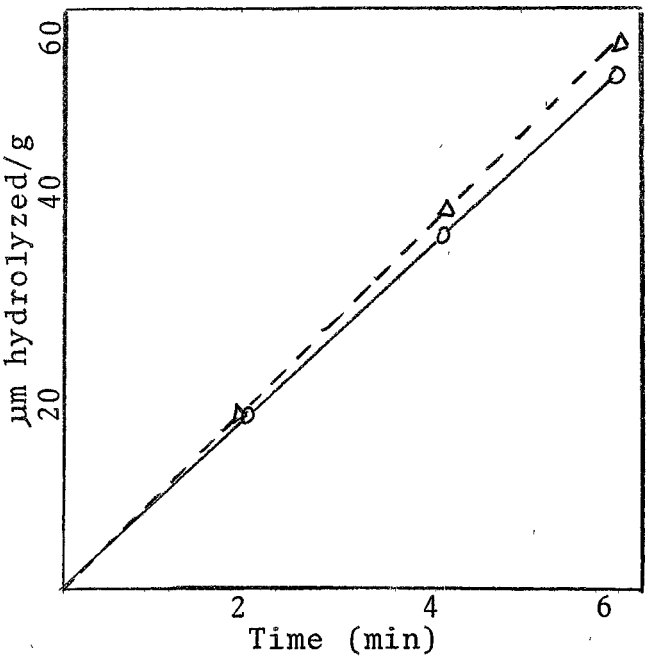

FIGURE 3

EFFECT OF 0.6M ETHANOL ON ACETYLCHOLINESTERASE

Figure 3. Micromoles of acetic acid hydrolyzed per gram of fresh muscle homogenate in the presence of 0.0733M acetylcholinechloride.

o —— Acetylcholinesterase assay in aqueous solution of 0.0733M acetylcholinechloride.

Δ ---- Acetylcholinesterase assay in 0.6M ethanol medium with 0.0733M acetylcholinchloride.

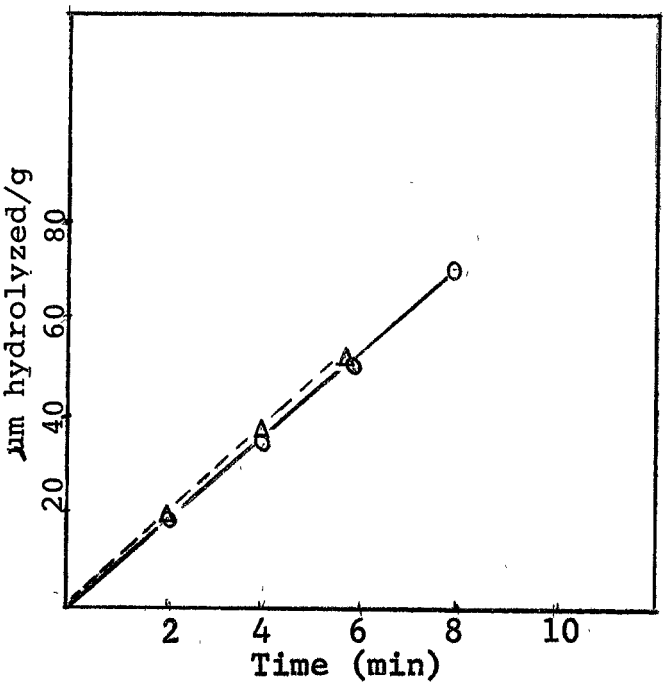

FIGURE 4

EFFECT OF DDT ON ACETYLCHOLINESTERASE

Figure 4. Micromoles of acetic acid hydrolyzed per gram of fresh muscle tissue of Micropterus salmoides vs. time in the presence of 0.0733M acetylcholine chloride, 0.3×10^{-4}M DDT and 0.6 M ethanol.

Δ ----- Acetylcholinesterase assay in 0.6 M ethanol - control.

o _____ Acetylcholinesterase assay in 0.3×10^{-4}M DDT in 0.6M ethanol.

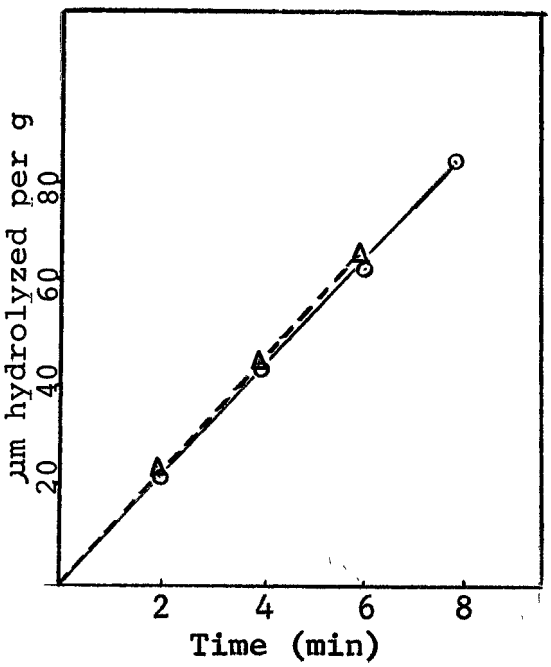

FIGURE 5

EFFECT OF 2,4-D ON ACETYLCHOLINESTERASE

Figure 5. Micromoles of acetic acid hydrolyzed per gram of fresh muscle tissue of Micropterus salamoides in the presence of 0.0733M acetylcholine chloride and 0.3 X 10^{-4}M 2,4-D Na^{+} vs. time.

Δ ---- Acetylcholinesterase assay in water solvent (control).

o —— Acetylcholinesterase assay in 0.3 X 10^{-4}M 2,4-D Na^{+} in aqueous medium.

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