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Lawrence Curtis

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Roger Hoopingarner
Major professor

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ABSTRACT

THE PURIFICATION OF DDT-DEHYDROCHLORINASE AND ITS LOCALIZATION IN TISSUES OF THE HOUSE FLY (MUSCA DOMESTICA L.) BY THE FLUORESCENT ANTIBODY TECHNIQUE

By

Lawrence Curtis Besaw

DDT-dehydrochlorinase (E.C. .4.5.1.1) from Musca domestica L. has been purified to homogeneity as judged by disc gel electrophoresis, Ouchterlony double diffusion, disc gel immunoelectrophoresis, and sucrose gradient centrifugation. An improved yield of pure enzyme was obtained by a purification procedure consisting of ammonium sulfate precipitation, molecular exclusion chromatography (Bio-Gel P-10 and P-150 polyacrylamide gel column chromatography) and preparative disc gel electrophoresis.

Rabbit serum containing antibodies against DDT-dehydrochlorinase was prepared and employed in the indirect fluorescent antibody staining of this enzyme in various tissues of the adult house fly. While specific fluorescent staining demonstrated that DDT-dehydrochlorinase was more or less ubiquitously distributed throughout the DDT-resistant house fly, high titers of the enzyme were localized in the epidermal and endocuticle layers of the integument, along the chorionic membrane surrounding egg cells and the neural lamella region of nerves.

It was concluded that these "barriers" of DDT-dehydrochlorinase play an important role in protecting vital sites of the house fly against the toxic action of DDT.

In addition, the fluorescent antibody technique was used to demonstrate the induction of DDT-dehydrochlorinase in DDT-treated house flies. Quantitation by a light sensitive photocell, attached to a fluorescent microscope, indicated a significant difference between levels of fluorescence in identical tissues sectioned from DDT-treated and non-treated house flies. Of the tissues tested, fat body demonstrated the highest degree of DDT-dehydrochlorinase synthesis. These results would seem to support the theory that resistance to DDT in strains of house flies may be a combination of preadaptation and induction. These results providing evidence that the fluorescent antibody technique was successful in demonstrating enzyme induction, suggest another unique and valuable application of this increasingly popular histochemical tool.

THE PURIFICATION OF DDT-DEHYDROCHLORINASE AND ITS LOCALIZATION IN TISSUES OF THE HOUSE FLY (MUSCA DOMESTICA L.) BY THE FLUORESCENT ANTIBODY TECHNIQUE

By

Lawrence Curtis Besaw

A THESIS

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To my parents

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INTRODUCTION

The development of resistance of arthropods to insecticides, which has been recognized for more than 50 years, poses one of the most serious problems in the chemical control of insects. Perhaps the most realistic commentary on the resistance problem was made in 1915 by Stephen A. Forbes when he said: "The struggle between man and insects began long before the dawn of civilization, has continued without cessation to the present time, and will continue, no doubt, as long as the human race endures ... We commonly think of ourselves as the lords and conquerors of nature, but insects had thoroughly mastered the world and taken full possession of it long before man began the attempt... We cannot even protect our very persons from their annoying and pestiferous attacks, and since the world began, we have never yet exterminated - we probably shall never exterminate - so much as a single species" (quoted by Decker, 1958).

In terms of the geometric growth of the human population, and the corresponding need for increased agricultural production, the resistance of insects to chemical control is becoming even more important, and if solutions are to be found, researchers must investigate all aspects of the problem.

This thesis study was undertaken to further study the enzymatic role of DDT-dehydrochlorinase in the dehydrochlorination of DDT to DDE

in house flies. The purpose of this work was to develop a purification procedure which would yield a quantity of pure enzyme adequate for the production of a specific, high-titered antibody, which then could be used to localize DDT-dehydrochlorinase in various tissues of the house fly via the fluorescent antibody technique. This information should facilitate our understanding of the factors involved in the functions of this key enzyme in the metabolic degradation of DDT in the house fly.

LITERATURE REVIEW

I. Nature of Resistance

The phenomemon of insecticide resistance has been defined by the World Health Organization as follows: "the development of an ability in a strain of insects to tolerate doses of toxicants which would prove lethal to a majority of individuals in a normal population of the same species" (Hoskins, 1963). The first known case of insecticide resistance was in 1908, when it was found that strains of the San Jose scale, Aspidiotus perniciosus, in Washington state had become resistant to lime-sulfur sprays (Melander, 1914). In 1946, DDT-resistant house flies were first discovered in Sweden. Since that time insecticide resistance is known to have developed in strains of 224 species of insects and acarines. Of these, 105 are of public health and veterinary importance, and 119 are pests of stored products of field or forest crops (Brown, 1969).

Resistance in insects is not generalized to give protection to all insecticides, but is specific to a particular group of compounds. At the present time resistance can be categorized into four principal types: (1) to DDT and related compounds; (2) to cyclodiene derivatives; (3) to organophorus compounds; and (4) to carbamate insecticides. Cross-resistance has been reported between compounds within each group, but does not occur between compounds of different groups. A number of cases of insect resistance to inorganic and botanical insecticides have also been reported to occur (Brown and Pal, 1971).

The failure of a given dosage which previously has been effective in control is due to selection of those individuals in a population which possess the ability to survive this dosage. The traits giving resistance are not mutagenically produced by an insecticide (King, 1954; Merrell and Underhill, 1956; Crow, 1957), or inducible by habituation during the lifetime of an insect (Beard, 1952a, 1952b; Harrison, 1951; Cole et al., 1957). Thus, resistance is not postadaptive but preadaptive, resulting from selection of those naturally occurring genotypes in a gene pool which give an adaptive advantage according to Darwinian principles operating on a microevolutionary level (Brown and Pal, 1971).

The various mechanisms of resistance may be generally categorized as physiological and behavioristic. In the majority of organisms reported to have and ability to survive a toxicant, physiological mechanisms have been shown to play a dominant role. The multiplicity of mechanisms, under these two categories have been summarized by Georghiou, (1965), as follows:

1. Physiological Factors

   a. Penetration, transport, and activation of the insecticide.

      Decreased cuticular penetration of the insecticide.

      Reduction in rate of transport of an inhibitor to a susceptible enzyme.

      Decreased cellular or neural permeability to an inhibitor.

      Decreased conversion of an administered compound to an active (or more active) form.

   b. Decreased sensitivity of site of action

   c. Destruction or inactivation of the insecticide

      Increased rate of inactivation of the compound prior to or after its conversion to an active inhibitor.

      Greater availability of enzyme with ability to destroy or inactivate the insecticide.

d. Increased storage of administered compound or its more active metabolite in less sensitive cells.

e. Increased elimination of the compound.

2. Behavioral Factors

a. Decreased period of contact with an insecticide treated surface.

Increased irritability to contact with the treated surface.

b. Avoidance of treated areas.

Increased discrimination of treated areas.

Selection of exophilous population.

Numerous reviews have been published on the many ramifications of insecticide resistance. A comprehensive review covering various aspects as it affects the public health field has been compiled by Brown and Pal (1971). Additional reviews covering specific items and discussing the resistance phenomenon from different points of view are as follows:

(a) Physiology and Biochemistry - Brown (1961, 1963); Chadwick (1957); Perry (1974); O'Brien (1966, 1967); Oppenoorth (1965a); Winteringham (1969); Lipke and Kearns (1960).

(b) Genetics - Brown (1967a, 1967b); Crow (1957); Davison and Mason (1963); Georghiou (1965, 1969); Milani (1963); Tsukamoto (1969).

(c) General Aspects - Brooks (1968); Brown (1969); Busvine (1968); Hoskins (1963); Toppozada (1966).

(d) Behavioristic Factors - Mattingly (1962).

II. DDT

A. Introduction

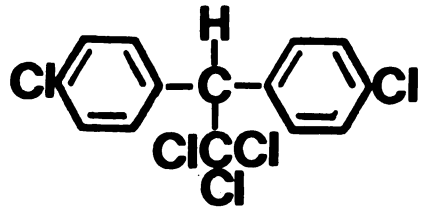
In 1874, Othmar Zeidler, a German chemist, synthesized the compound dichlorodiphenyltrichloroethane (DDT) as part of his doctoral

research. This easily synthesized compound then lay forgotten until 1939, when Paul Mueller discovered its very effective insecticidal properties.

DDT has had an unprecedented development as a synthetic insecticide because of a combination of physical and chemical properties promoting a wide spectrum of insecticidal action, low manufacturing cost, prolonged stability resulting in persistent residual activity, and relatively low mammalian toxicity (Metcalf, 1955). The use of DDT spread world wide during and after World War II, and was the principal agent responsible for controlling epidemics of malaria, yellow fever and typhus in a number of countries. Its use on agricultural pests brought about increased crop yields which provided economic benefits for the farmer, and the additional food needed for the world's growing population. Although it still remains an important insecticide in the control of certain pests throughout the world, the use of DDT has dropped considerably. The reasons for this are summarized by O'Brien (1967), as the following: (1) increasing numbers of DDT-resistant insect species; (2) its extreme persistence, which caused DDT to spread from target areas and ultimately throughout the environment; (3) an ignorance of its specific mode of action, which makes the effects of the persistent DDT residues difficult to determine; and (4) the deleterious effect DDT residues may have on the reproductive success of certain animals. These problems and others have stimulated basic research on a variety of parameters involved with DDT, other chlorinated hydrocarbons, as well as the organophosphorus, carbamate, inorganic and botanical insecticides. A knowledge of the complex processes involved in the chemistry, mode of action and metabolism of pesticides is essential to the development of more highly selective biodegradable pest control chemicals.

B. Chemistry and Structure of DDT

The proper chemical designation of DDT is 2,2-bis (p-chlorophenyl)-1,1,1-trichloroethane, and its structure is as follows:



Pure DDT has a m.p. of 109-10°C, and a vapor pressure of 1.5×10^{-7}mm at 20°C (Balsom, 1947). DDT is extremely apolar, having a high oil-water partition coefficient and a low water solubility. The latter value has been reported to be approximately 1.2 ppb (3.4×10^{-10}M) at 25°C (Bowman et al., 1960).

Under alkaline conditions DDT is dehydrochlorinated to form 2,2-bis (P-chlorophenyl)-1,1-dichloroethylene (DDE); or it can be oxidized to dicofol and DDA (2,2-bis(p-chlorophenyl) acetate) and thence to p,p'-dichlorobenzophenone, reactions which account for much of the decomposition of DDT residues (Metcalf, 1955). These reactions and others of DDT are summarized in Figure 1 (Hoskins, 1964).

C. Mode of Action of DDT

Although there had been extensive research on the subject, the precise mode of action of DDT still remains unclear. However, there is general agreement that it primarily affects the nervous system. The evidence for this view has been briefly summarized by O'Brien (1967) as follows: (1) The symptoms of poisoning suggest nervous impairment. Studies with various insect and mammalian species have shown abnormal neuronal activity resulting in tremors hyperactivity, ataxia and eventual paralysis. (2) When DDT is applied to isolated tissues and enzymes, only

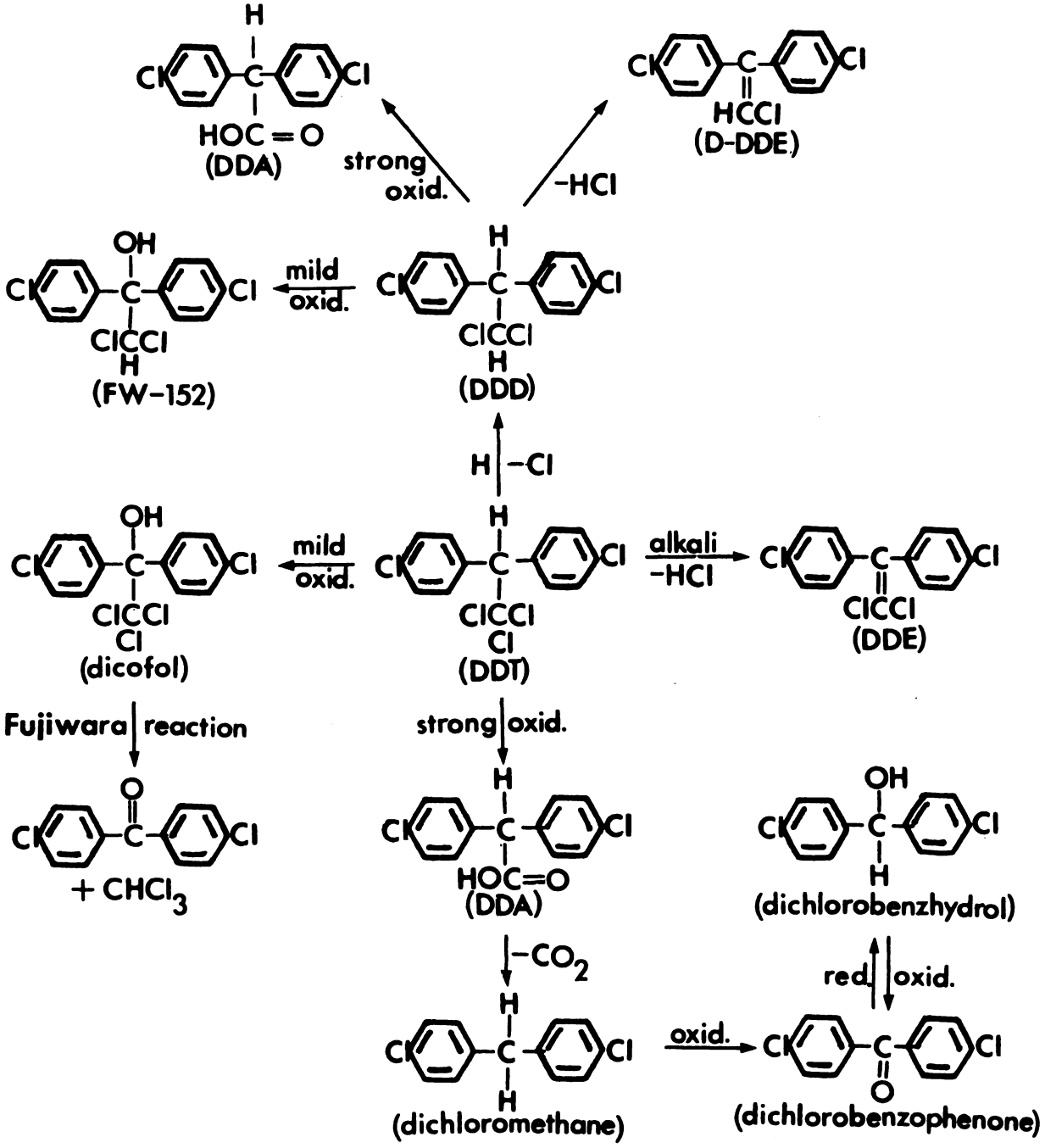


Fig. 1. Principle reactions of the DDT family.

nervous tissue is sensitive to very low concentrations. (3) An excellent correlation has been found in DDT treated rats between levels of DDT in the central nervous system and the intensity of symptoms.

Where does DDT disrupt the nervous system, and what is the nature of this disruption, are questions still being answered. One hypothesis that is gaining acceptance is that DDT has a general effect on membranes, and certain membrane functions. Specifically, evidence seems to indicate that DDT forms a charge-transfer complex with one or more components of the nerve membrane, causing an interference with ionic movement in and out of the axon (Holan, 1969, 1971), the observation that DDT is more active at low temperatures than at high would seem to support this hypothesis. An additional effect of DDT has been the observation that DDT poisoning may release "toxins" into the insect system, an effect which causes change from specific to general symptoms of stress.

For detailed information on the mode of action of DDT and related subjects, the following reviews by O'Brien (1966, 1967), Narahashi (1971), and Corbett (1974) are suggested.

D. Metabolism of DDT

The metabolism of DDT has been extensively investigated, and a number of comprehensive reviews on this subject have been published (Kearns, 1955; Metcalf, 1955; Winteringham and Barnens, 1955; Brown, 1960; Hayes, 1965; O'Brien, 1967).

There are five principal routes of DDT metabolism in various organisms as summarized in Figure 2: Oxidation to DDA (2,2-bis(p-chlorophenyl)acetate) (route 1) or to dicofol (1,1-bis(p-chlorophenyl) 2,2,2-trichloroethanol) (route 2) of p,p'-dichlorobenzophenone (route 3); dehydrochlorination to DDE (1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene)

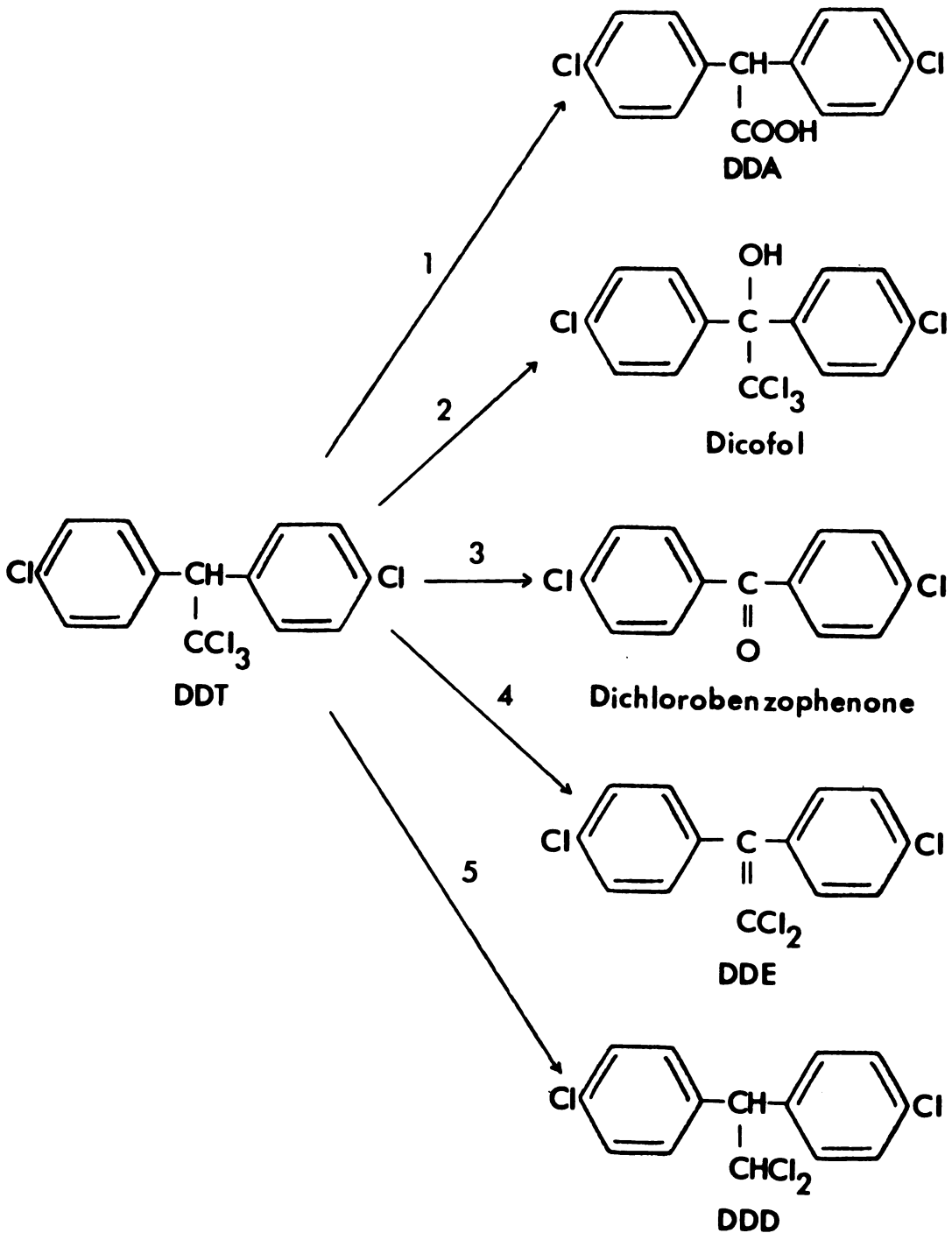


Fig. 2. Routes of DDT metabolism

(route 4); or reductive dechlorination to DDD (1,1-dichloro-2,2-bis(p-chlorophenyl)ethane) (route 5) (O'Brien, 1967).

The oxidative metabolism of DDT to DDA has been reported as common in vertebrates. Early investigations with DDT orally administered to rabbits demonstrated that DDA was excreted in the urine (White, et al., 1945) and in the feces (Judah, 1949). Similar studies done with rats also showed that DDA was the principle metabolite (Burns et al., 1957; Jenson et al., 1957). More recently, Peterson and Robinson (1964) proposed the following pathway of DDT metabolism in rats: DDT→DDD→DDMU (1-chloro-2,2-bis(p-chlorophenyl)ethylene)→DDMS (1-chloro-2,2-bis(p-chlorophenyl)ethane)→DDNU (unsym-bis(p-chlorophenyl)ethylene)→DDA. The evidence for this sequence is that the feeding of each compound to rats gave the subsequent intermediate as the major metabolite in liver. While it has been found that DDA is primarily a vertebrate metabolite, Perry (1963), reported that homogenates of the body louse, Pedicus humanus, degraded DDT to DDA, dichlorobenzophenone and DDE in the ratio of 2:2:1.

The oxidation of DDT to dicofol has been reported by Matsumura and Boush (1968) to have occurred in 8 soil-isolated variants of the fungus Trichoderma viride, incubated anaerobically for 3 days in a liquid medium containing C^{14} - labeled DDT. This metabolic route has also been demonstrated to occur in certain insects. Tsukamoto (1959, 1961) and Menzel et al. (1961), reported that adults of the pomace fly, Drosophila melanogaster, metabolized C^{14} - labeled DDT to dicofol and to two unidentified metabolites; they also reported that oxidative metabolism in the larvae produced dichlorobenzophenone as a major metabolite. Agosin (1961) reported that the German cockroach, Blattella germanica, had the ability to convert DDT to dicofol via a microsomal enzyme system,

and this same metabolic route has also been reported to occur in the house fly (Agosin et al., Tsukamoto and Casida, 1967).

Probably the most extensively researched degrative pathway of DDT in insects is dehydrochlorination to DDE. It was first reported independently by Sternburg et al., (1950); and by Perry and Hoskins, (1950), to occur rapidly in DDT-resistant house flies, Musca domestica, and only to a minor extent in DDT-susceptible flies. Sternburg et al. (1954) demonstrated that an enzyme, which they called "DDT-dehydrochlorinase" (hereafter referred to as DDT-ase), catalyzed the dehydrochlorination reaction.

Degradation to DDE is also a major pathway in some insects other than house flies, including the Mexican bean beetle (Chattoraj and Kearns, 1958), pink bollworm (Bull and Adkisson, 1963), the Aedes aegypti mosquito (Abedi, et al., 1963), and others (Perry, 1960). The purification and characterization of DDT-ase and other related topics are dealt with at greater length in section II-E of this review.

Refinements in chromatographic techniques brought about the discovery that reductive dechlorination of DDT to DDD occurred in various organisms. Prior to this time, DDD was not separable from DDT by available analytical methods. The first report of this conversion route was that of Kallman and Andrews, (1963), who demonstrated that commercial yeast could metabolize DDT to DDD under anaerobic conditions. In the same year, Finely and Pillmore (1963), reported the presence of DDD in samples of water, soil, plant, and animal tissues collected from areas sprayed with DDT.

Several species of bacteria isolated from animals, soil and laboratory cultures convert DDT to DDD when cultured anaerobically (Baker

and Morrison, 1965; Stenersen, 1965; Chacko et al., 1966; Wedemeyer, 1966; Johnson and Goodman, 1967; Plimmer et al., 1968; French and Hoopingarner, 1970). One example of a fungus, having the ability to dechlorinate DDT to DDD, namely Trichoderma viride, has been reported by Matsumura and Boush (1968).

Non enzymatic degradation of DDT to DDD has been reported to occur in such diverse conditions as lake water, stagnant rumen fluid, and hemoglobin and hematin solutions (Miskus et al., 1965). It has also been reported to occur in repeatedly frozed and thawed chicken blood (Ecobicon and Saschenbrecker, 1967) and in anaerobically incubated pigeon and rat liver homogenates (Bunyan et al., 1966).

E. Resistance in the house fly (Musca domestica)

The study of DDT resistance, particularly in house flies, serves as a model for the understanding of insecticide resistance in general. The history, global occurrence and various mechanisms of DDT resistance in the house fly have been completely reviewed by Brown and Pal (1971). Reference should also be made to the outline of reviews mentioned previously in Section I.

In addition to the dehydrochlorinative resistance in the house fly, a number of other mechanisms have also been reported to occur. These mechanisms, reported to differentiate susceptible from resistant strains, include: (1) avoidance behavior to treated areas (King and Gahan, 1949; Bruce and Decker, 1950; Morrison, 1950); (2) morphological differences in cuticular penetration (Tahori and Hoskins, 1953; Perry and Sacktor, 1955; Perry, 1958; Sanchez and Sherman, 1966); increased lipoid content of the fat body and tarsi (Reiff, 1956; Wiesmann, 1957), nerve sensitivity (Pratt and Babers, 1953; LeRoux and Morrison, 1954;

Wiesmann, 1955; Weiant, 1955; Smyth and Roys, 1955), and titres of certain enzymes, such as cytochrome oxidase (Sacktor, 1950, 1951; Perry and Sacktor, 1955), and microsomal hydroglases (Oppenoorth, 1965b; Schonbrod et al., 1965). Since these mechanisms have been demonstrated to occur in only a few specific resistant strains, it is probably they play a minor role in the total picture of house fly resistance to DDT.

1. Inheritance of Resistant Types

Research on the inheritance of various resistance types have shown that most are due to allelism in a single principal gene. Monofactorial inheritance has been reported for DDT-resistance in 13 species, for dieldrin-resistance in 19 species and for organophosphate resistance in 6 species. Poly.factorial inheritance was found only in arsenic-resistance and low-order tolerances to carbamates, malathion and DDT in 4 species (Brown, 1972).

The chromosomal localization of resistance genes in the house fly has been accomplished by linkage studies with crosses between resistant strains and mutant marker strains. These studies have shown that the genes responsible for 4 mechanisms of resistance to DDT in the house fly are located on chromosomes II, III, and V (Linkage groups follow those of Wagoner (1967)). The recessive gene kdr on chromosome III is responsible for knockdown resistance, and determines nerve sensitivity to DDT (Milani and Travaglino, 1957; Tskumoto et al., 1965; Narahashi, 1971). The 5th chromosomal gene, responsible for conversion of both DDT and DDE to polar metabolites, is present in the Danish Fc strain (Oppenoorth, 1965a) and probably in the SKA strain (El-Basheir, 1967). The dominant gene, Deh, which controls the production of DDT-ase, located on chromosome II (Lovell and Kearns, 1959; Tsukamoto and Suzuki,

1964; Hoyer and Plapp, 1966).

2. Pro and Cons of the Dehydrochlorination Hypothesis

The general acceptance that dehydrochlorination is the chief mechanism of DDT resistance in the house fly is supported by the fact that marked DDT-ase activity has been demonstrated to be present in most DDT-resistant strains, while it was relatively absent in susceptible strains. It has been also reported that the titre of this enzyme in resistant strains is directly proportional to their respective levels of DDT tolerance (Kearns, 1955).

Support for the dehydrochlorination mechanism of resistance also comes from investigations of DDT synergists. A number of these compounds have been shown to inhibit the detoxification of DDT to DDE *in vivo* and the action of DDT-ase *in vitro* (Perry and Hoskins, 1950; Speroni *et al.*, 1953; Perry *et al.*, 1953; Moorefield and Kearns, 1955; Reuter *et al.*, 1956). Some of the more effective DDT synergists include: piperonyl cyclonene, chlorofenethol, MS-60 (Chlorobis(p-chlorophenyl)methane), Mr-30 (4,4'-dichloro-ethynylbenzhydrol), DMC (bis-(p-chlorophenyl)methyl carbinol), and WARF antiresistant (N,N-di-n-butyl-p-chloro-benzene-sulfonanide).

While the type of inhibition caused by synergists is not entirely clear, it does appear plausible that those synergists structurally related to DDT block the action of DDT-ase by competing for the active site of the enzyme (Perry, 1964). Most of the compounds listed above do not synergize the action of DDT in susceptible strains, and it was concluded by Moorefield and Kearns (1955) that this was due to the lack of DDT-ase to be inhibited in these strains. Comprehensive reviews on the subject of insecticide synergism have been published by Sumerford (1954); Metcalf (1955); Kearns (1956); Veldstra (1956); O'Brien (1967);

Brown and Pal (1971); and Perry and Agosin (1974).

While the evidence is strong supporting enzymatic dehydrochlorination as the primary mechanism of DDT resistance in house flies, Perry and Agosin (1974) have suggested the following as anomalies in this hypothesis: (1) Some DDT-resistant flies have cross resistance to compounds which cannot be dehydrochlorinated, such as Prolan (Perry and Buckner, 1959) and dianisyl neopentane (Brown and Rogers, 1950). (2) Certain insect species, such as the milkweed bug, Oncopeltus fasciatus. can metabolize DDT as rapidly as resistant flies, yet are susceptible to DDT poisoning (Perry and Agosin, 1974). (3) Large quantities of unchanged DDT sufficient to kill several susceptible flies remain in the tissues of surviving resistant flies (Babers and Pratt, 1953; Perry and Hoskins, 1951b; Tahori and Hoskins, 1953). (4) Differences exist between resistant and susceptible strains in the innate sensitivity of their nerves to DDT (Pratt and Babers, 1953; LeRoux and Morrison, 1954; Smyth and Roys, 1955; Wiessmann, 1955).

While these counter-arguments may suggest that dehydrochlorination is not the only mechanism providing resistance to house flies, they are not convincing enough to alter the acceptance of its primary role. In fact, logical explanations of these so-called "anomalies" have been proposed.

A Prolan-resistant strain has been reported that can dehydrochlorinate DDT and metabolize Prolan to neutral and acidic metabolites which are excreted. This would indicate the presence of two distinct metabolic processes operating independently of each other (Brown and Rodgers, 1950; Perry and Buckner, 1959).

Arguing against dehydrochlorination in the house fly by analogy with other insect species is probably inappropriate. The fact that some species, such as the milkweed bug, can rapidly metabolize DDT, yet are still susceptible to its action, may simply indicate their higher innate sensitivity to the compound (Perry and Agosin, 1974). Other insect species, such as the Khapra beetle, Trogoderma granarium, cannot appreciably metabolize DDT, yet are insensitive to it (Winteringham, 1952). This is probably due to the presence of some non-metabolic defense mechanism (O'Brien, 1967).

Reports of substantial amounts of unchanged DDT in resistant house flies do not exclude the possibility of enzymatic dehydrochlorination. Miyake et al. (1957) determined the level of DDT-ase activity in various excised tissues of DDT resistant flies on a soluble protein basis. They demonstrated the enzyme was more or less ubiquitously distributed throughout the organism with the highest titres found in the brain and fat body, intermediate amounts in the cuticle, muscle and haemolymph, and lowest amounts in the ovary and intestinal tract. From these data the authors concluded that undegraded DDT in resistant flies (cf. Babers and Pratt, 1953) is probably extracellular, or retained in a system where it is neither capable of acting physiologically nor liable to degradation. In addition, they speculated that the nerve sensitivity differences between DDT resistant and susceptible strains could, at least partially, reflect differences between nerves protected by DDT-ase and those that are not.

3. Purification and Properties of DDT-ase

The demonstration that DDT-susceptible strains dehydrochlorinated small amounts of DDT to DDE in vivo (Perry and Hoskins,

1951a; Linquist et al., 1951; March, 1952; Tahori and Hoskins, 1953) logically raised the question whether detoxication was the cause or result of survival (Chadwick, 1952). The answer to this question came after the successful isolation and quantification of DDT-ase under conditions where in vivo intoxication by DDT could not be a complicating factor. The fact that this enzyme can only be isolated in significant quantities from DDT-resistant house flies is one of the strongest arguments supporting the hypothesis that the presence of DDT-ase is the principal cause of resistance to DDT in this species (Sternburg et al., 1954; Lipke and Kearns, 1959a).

The isolation and subsequent analysis of the physicochemical properties of DDT-ase required the solution of problems quite unique to the field of enzymology (Lipke and Kearns, 1960). The most difficult of these were the following: (a) In order to isolate DDT-ase in practicable amounts it was necessary to continually rear and harvest kilogram quantities of DDT-selected house flies of high enzyme titre, a problem alleviated to some extent when Lipke and Kearns (1959a) reported that resistant flies could be stored at -20°C for up to 4 months with only minor losses in enzymatic activity; (b) The solubilization of DDT in aqueous systems had to be perfected before the activity of purified DDT-ase preparations could be accurately assayed, or various properties of the enzyme, such as kinetic parameters, analyzed; (c) Although it was earlier reported by Sternburg et al. (1954) that crude preparations of DDT-ase had a fair degree of stability, it was later reported that the purified enzyme was extremely unstable, and any analysis of its chemical or physical properties had to be completed within 48 hours of its purification (Lipke and Kearns, 1959a); (d) Finally, the isolation

of DDT-ase required unusually high purity indices due to the fact that whole flies had to be used as the source of the enzyme, rather than some specific tissue as is the usual case in enzyme purification.

Sternburg et al. (1954) were the first to report a method of concentrating the DDT-ase enzyme via an ammonium sulfate precipitation procedure. The extract produced by this method contained the equivalent in DDT-ase of 50 flies per ml. DDT-ase activity was assayed by incorporating the DDT substrate into the reaction system in a particulate form, either in an ethanol-water emulsion or by absorption on to the surfaces of glass beads 15 to 60 microns in diameter. The enzyme reaction products were measured spectrophotometrically by absorbance at 241 and 260 nm. An increase in DDT-ase activity per unit weight of protein amounting to 120-fold was reported by Moorefield (1956) when they added either charcoal absorption or freezing-thawing fractionation techniques to the above ammonium sulfate precipitation procedure. The freezing-thawing step was reported to be susceptible to the activity of oxidases, and the addition of thiourea to the preparation was required to inhibit their action.

With the crude DDT-ase extract, Sternburg et al. (1954) were able to demonstrate a number of parameters of the enzyme which have been validated in subsequent literature on the subject. The enzyme catalyzed the conversion of DDT to DDE only, and required glutathione (GSH) for activation. Maximum activity occurred at a temperature of 37°C, at pH 7.4, and was sustained for longer periods under an atmosphere of nitrogen. DDT-ase was also shown to dehydrochlorinate DDD, methoxychlor (2,2,-bis-(p-methoxyphenyl)-1,1,1-trichloroethane), and DIDT (1,1,1-trichloro-2,2-bis(p-iodophenyl)ethane) and DBDT (1,1,1-trichloro-2,2-

bis(p-bromophenyl ethane)), but not the o,p'-isomer of DDT.

Lipke and Kearns (1959a) were the first to report the isolation of DDT-ase in a pure state. This achievement was accomplished with a fractionation scheme which included in order; ammonium sulfate precipitation, methanol precipitation, flash heating (48°C for 4 minutes), cation-exchange chromatography, calcium phosphate gel treatment, and a final precipitation with methanol. This procedure resulted in a purification of the enzyme by about 570-fold, and a yield of .25 mg of total protein. The final product was judged homogeneous by the analytical ultracentrifuge, and reported to be a small protein with an approximate molecular weight of 36,000.

A difficult problem was resolved in this study, when the authors reported a direct spectrophotometric assaying technique using egg yolk lipoprotein as a solubilizing agent for DDT. This method was devised when it was found that the glass bead assaying technique, described previously, could not accurately measure initial reaction rates in kinetic studies, or give a linear response when measuring DDT-ase activity versus protein concentration. It was also demonstrated that the use of egg yolk lipoprotein enhanced the activity of DDT-ase by rendering the DDT substrate more accessible to the action of the enzyme. The only disadvantage encountered with the lipoprotein assay procedure was that it could not accurately measure DDT-ase activity in the initial crude fractions due to color interference with the spectrophotometer.

Despite the fact that the purified enzyme was very unstable and the yield low, Lipke and Kearns (1959b) were able to determine a number of properties of DDT-ase. The specificity of the enzyme for various analogues of DDT was investigated and it was reported that the

p,p'-diBr compound was degraded at the same rate, and the 1,1-dichloroethane derivative 4 times as fast, as DDT. It was reported that the enzyme could be activated by cysteinyl-glycine and the reaction maintained at a rate of 60 per cent of an equivalent amount of GSH. It was observed that the enzyme was not sensitive to SH inhibitors or metal-binding agents. The purification procedure and the sensitivity of the lipoprotein assay method permitted for the first time the demonstration of DDT-ase in susceptible flies. It was reported that the CSMA strain contained about 0.05 percent of that present in an equivalent number of resistant flies.

The most comprehensive studies on DDT-ase were reported by Dinamarca et al. in 1961 and 1971. The earlier paper described an improved purification procedure and a new enzymatic assay. A purification of 224-fold and a final yield of 2.94 mg total protein was achieved with a fractionation scheme involving ammonium sulfate precipitation, hydroxylapatite absorption and elution, ethanol precipitation, followed by Sephadex and DEAE-Sephadex column chromatography. The enzyme was assayed by a procedure which substituted ethylene glycol for lipoprotein as the DDT solubilizer, and was reported to give more consistent results than the earlier method. A significant contribution to the study of DDT-ase was made when it was reported that the stability and activity of the enzyme could be maintained by simply adding GSH to the purification preparation.

The latter paper reported in detail the structure of DDT-ase. The enzyme was found to have a molecular weight of 120,000 and to be formed by four monomers of a molecular weight of 30,000 each. The four monomers were reported to have identical physical and chemical properties.

It was observed that DDT converted monomers into tetramers which could be stabilized by GSH, but dissociated by either B-mercaptoethanol or dithiothreitol. The enzyme was reported to contain 34 cysteinyl residues per tetramer, but no disulfide bonds. None of the sulfhydryls appeared to be associated with the active site, which contradicted the theory that GSH was involved in maintaining enzyme sulfhydryls in a reduced state. This paper also raised the question as to the validity of the results of Lipke and Kearns (1959a,b). It was reported that when monomer, dimer, trimer and tetramer were assayed for dehydrochlorinating activity against DDT, it was found that only the trimer and tetramer were active. Lipke and Kearns' report of a final product having a molecular weight of approximately 36,000 would seem to indicate that this corresponds to the monomer and not to the whole enzyme.

Oppenoorth and Voerman (1965) have reported a simple and very sensitive method of assaying DDT-ase activity. In this procedure the enzyme was allowed to react with DDT solubilized by dimethylsulfoxide, and the final reaction products were quantitated by gas-liquid chromatography. It was demonstrated to be sensitive enough to estimate DDT-ase in single susceptible and resistant house flies. Although not conducive to studies of DDT-ase where the reaction has to be continuously monitored, this assay technique is probably the method of choice when only quantification of total enzyme activity is required.

F. Induction of DDT-ase

It has been demonstrated that DDT stimulates protein synthesis in insects (Agosin et al., 1966; Balazs and Agosin, 1968; Litvak and Agosin, 1968) and in mammals (Morello, 1964, 1965; Remmer et al., 1968). This effect is blocked by puromycin and it has been

suggested that it corresponds to enzyme induction (Morello, 1965). The fact that these observations may be related to the resistance mechanisms to the insecticide are supported by reports of an increase in microsomal activities after DDT-treatment in Triatoma infestans (Morello, 1964), house flies (Gil et al., 1968) and rat liver (Morello, 1965). DDT-ase levels have been reported to increase in house flies after treatment with phenobarbital (Yu and Terriere, 1973), juvenile hormone analogs (Terriere and Yu, 1973), and cyclodiene insecticides (Yu and Terriere, 1972).

The first demonstration of induction of DDT-ase in DDT-treated house flies has been recently reported by Capdevila et al, (1973). An increase in DDT-ase activity of approximately 50 percent was found in resistant flies topically treated with 10 µg of DDT per fly, after a lag period of 9 hours. Additionally, the stimulatory effect on the incorporation of labeled precursors into purified DDT-ase, and the fact that this incorporation is inhibited by actinomycin D and puromycin, suggested that a de novo synthesis of the enzyme was responsible for the increased activity. This report would seem to indicate that resistance, at least in certain house fly strains, is a combination of preadaptation and induction.

III. Fluorescent Antibody Techniques

Fluorescent antibody techniques have been employed with increasing frequency in immunohistological investigations within a variety of biological and medical disciplines. The number of references to papers in the field of applied immunohistology surpasses those of any other single cytochemical technique (Pearse, 1968). Some of the

more comprehensive books and reviews on the subject published since 1958 include the following: "Fluorescent antibody techniques" (Coons, 1958); "Immunofluorescent staining: The Fluorescent antibody technique" (Beuter, 1951); "Immunological Methods" (Ackroyd, 1964); "Fluorescent protein tracing" (Nairn, 1964); "Fluorescent antibody methods" (Goldman, 1968); "Fluorescent antibody techniques and their applications" (Kawamura, 1969): and "Defined immunofluorescent staining" (Beutner, 1971). Several brief but informative brochures on immunofluorescence have been produced by microscopic and antisera suppliers, and these are usually available on request.

The underlying principle of the fluorescent antibody technique is based on the fact that dye molecule can be attached to a protein without hindering its function as an antibody. One of the first demonstrations of this was reported by Marrack (1934) who coupled R-salt-azo-benzidine to antityphoid and anticholera sera which reacted with the homologous organisms and colored them pink. The first successful coupling of a fluorescent dye to an antibody was reported by Coons et al. (1941). These authors conjugated beta-anthrylisocyanate with antipneumococcal III serum. Their product agglutinated Type III pneumococci only and the clumped organisms showed macroscopic and microscopic fluorescense when exposed to UV light of wave length between 300 and 400 nm. However, since the beta-anthrylisocyanate conjugate fluoresced blue, it did not provide a distinction from the normal blue autofluorescence of the tissue and was therefore not suitable for localizing pneumococci in infected host cells. To overcome this problem, Coons et al. (1942) substituted the greenish-yellow fluorescent dye, fluorescein-4-isocyanate, which made it possible to observe the

distribution of the pneumococcal organism in various stained tissues of infected mice.

After a suspension of research during the period of World War II, Coons et al. (1950 et seq.), published five papers which served as a vade mecum for workers in the field. The contributions from these papers to fluorescent antibody methodology was essentially as follows: (1) the production of fluorescein conjugated antibodies specifically staining a variety of antigens; (2) the demonstration that fluorescent antisera could effectively stain soluble as well as particulate antigens; (3) technical improvements in ultraviolet microscopy, tissue sectioning, and in the control of nonspecific staining.

Although the groundwork for the successful application of the fluorescent antibody technique to various cytochemical problems had been laid down, inherent technical difficulties restrict its use to a limited number of laboratories. Riggs et al. (1958) solved one of the major problems when they described the synthesis and use of isothiocyanate derivatives of both fluorescein and tetramethylrhodamine B. These compounds were much easier and safer to synthesize in the laboratory than fluorescein isocyanate, and had the additional advantages of greater stability during storage and of higher intensity of fluorescence. Rhodamine B, a fluorescentred, provided a contrasting dye to the yellow-green of fluorescein and the blue autofluorescent tissues. Silverstein (1957) reported the utilization of tetramethylrhodamine B as a second label for the simultaneous identification of two antigens. Chadwick et al. (1958) prepared a stable sulfonyl chloride derivative of lissamine rhodamine B 200, which also fluoresced red. The use of this dye as a

nonspecific counterstain applied in conjunction with fluorescein labeled specific serum was reported by Smith *et al*. (1959).

Another difficulty which has persisted since the introduction of the fluorescent antibody is the non-specific fluorescent staining of tissue sections. Before accepting any fluorescent staining as specific for some antigen it is necessary that this problem be adequately controlled. Details of causes of non-specific staining and their solutions have been reported by a number of authors. Complete reviews on this subject are included in most of the publications referred to at the beginning of this section.

There are two basic procedures for the fluorescent antibody staining of tissue antigens, which can be accomplished either directly with labeled specific antiserum, or indirectly by one of the "sandwich" techniques (Fig. 3). The direct method was described by Coons (1958) and involves the conjugation of a specific antiserum with the fluorochrome and reacting this directly with the specimen to be examined. It is the simpler and the most reliable of the two methods, but has the disadvantage that before application each specific antiserum must be labeled with the fluorochrome. The indirect method was first described in detail by Weller and Coons (1954). In this procedure unlabeled (primary) antibody is placed on the homologous antigen and then fluorochrome-labeled secondary antibody, specific against the gamma globulin deposited *in vitro*, is brought into contact with the antigen-primary antibody complex. The advantages of this method are its increased sensitivity, the requirement of small amounts of primary antibody, and the possibility of using a single labeled antiglobulin for a number of unlabeled antisera. It has the disadvantage of increasing the potential

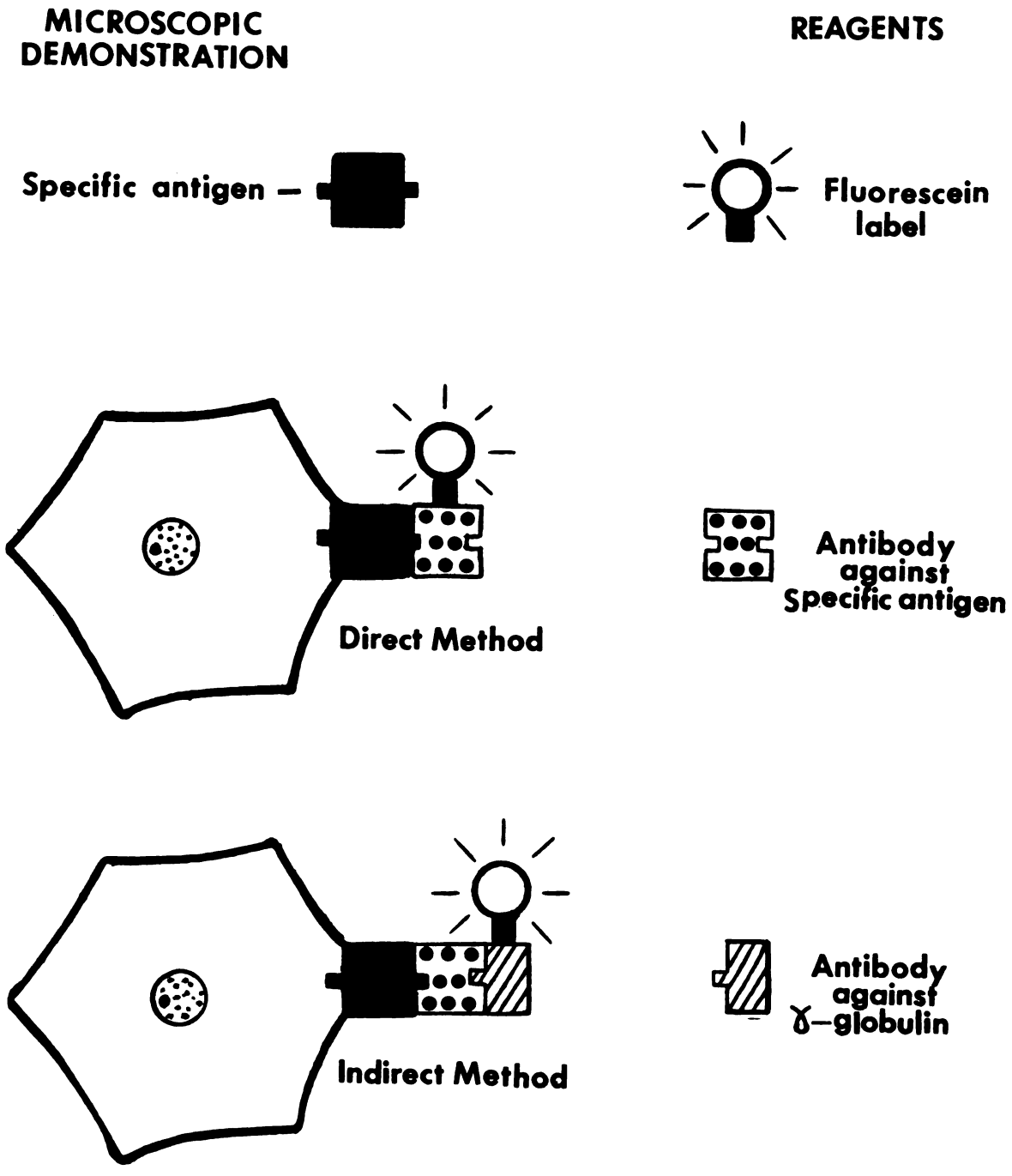


Fig. 3. Symbolic representation of the Direct and Indirect techniques of the fluorescent antibody method for the detection of specific cellular antigen.

for non-specific reactions and thus increasing the need for including more controls in the test procedure.

As soon as a satisfactory standardization of reagents used in immunofluorescence is achieved, standardization of the evaluation of the fluorescent image seen with the UV microscope will be possible. At this time it is difficult to use the fluorescent antibody technique as a quantitative procedure for the detection of antigen, although objective measurements of the relative intensity of specific fluorescence under specified conditions by means of photographic or photoelectric photometry have been made (Mellors, 1955; Ehrlich and Ehrmantrant, 1955; Birge, 1959; Curtain, 1961; Kunz, 1964; Goldman, 1967; Cherry et al., 1969; Irvine, et al., 1969; Hijmans, et al., 1970; Ploem, 1970). The extremely refined antigenic studies that have made possible the photometric measurement of as little as 10^{-7} µg of labeled antibody on individual cells (Goldman and Carver (1961) would seem to offer opportunities in immunohistochemical research that have, as yet, been hardly explored.

The application of fluorescent antibody techniques to the field of entomology has been mostly limited to studies on the detection of plant viruses in insect vectors (eg. Shepard and Boldwasser, 1960; Sinha and Black, 1963; Sinha, 1965; Chiu et al., 1970). One of the few exceptions to this is the report of the fluorescent antibody localization of glyceraldehyde-3-phosphate dehydrogenase in the muscle of the cockroach Periplaneta americana (Emmart et al., 1961).

MATERIALS AND METHODS

INSECT MATERIAL

House Flies (musca domestica L.) were reared in a humidified growth chamber (Sherer Inc.) kept at 30°C. Larvae were grown by placing 0.3 ml of eggs in a #10 can containing a mixture of 1000 ml of CSMA media, 25 ml malt, 75 ml yeast, and 1200 ml water. The life cycle took about 14 days.

Non-sexed adults used for enzyme purification were fed on 40% sucrose for four days prior to harvesting. The flies were stored at -20°C before processing for periods up to three months with minor losses in enzyme activity.

The following house fly strains were used:

1. NAIDM - Susceptible strain, obtained from Michigan State Department of Agriculture.
2. DDT-Orlando - Resistance beyond accurate measurement for DDT. Adults were routinely pressured with 2% DDT (w/w) evaporated from acetone on to powdered milk/sugar (1:1) diet.
3. WHO-SRS - Susceptible to DDT, obtained from WHO Musca Stock Center, Zoology Institute, University of Pavia, Italy.
4. 17 f - Originally a DDT resistant strain, but reverted after selection for DDT susceptibility.
5. 17 bb - DDT resistant. These last two strains were obtained through the courtesy of J. Keiding, Danish Pest Infestation Laboratory, Lyngby, Denmark.

DDT-ase Assay

DDT-ase was estimated by a combination and modification of two methods previously described by Ilivicky et al. (1964) and Oppenoorth et al. (1964). All reactions were carried out in 25 ml erlenmeyer flasks modified with lateral ports to accept gas-liquid chromatographic rubber injection septa (Supelco Inc.). Each flask contained 400 µmoles KH_2PO_4 buffer pH 7.4, 20 µg of DDT dissolved in 20 µl dimethylsulfoxide, and 2.0 ml of the enzyme solution to be assayed. After equilibration for 15 minutes in a 37° water bath, the reaction was started by injecting into each flask 0.5 ml of 0.1 M KH_2PO_4 buffer, pH 7.4, containing 20 µmoles of reduced glutathione (GSH), also equilibrated to 37°. The final volume of the assay mixture was 3.0 ml. Incubation was continued for time periods varying from 30-120 minutes with constant shaking. The reaction was stopped by the addition of 2 ml of a saturated Na_2SO_4 solution. Ten ml of a 2:1 mixture of redistilled cyclohexane and isopropanol was then added, and the flask shaken vigorously for fifteen minutes. The contents were then transferred to a 60 ml separatory funnel, and after separation of the layers, the lower layer was drained off. Approximately 10 ml of distilled H_2O was added to the separatory funnel. After gentle shaking, the layers were allowed to separate, and the lower layer was drained off. The remaining layer was filtered through anhydrous $NaSO_4$, and was ready for analysis by gas-liquid chromatography.

Quantitation was accomplished by a Packard Model 85 gas chromatograph equipped with a nickel foil (N^{63}), electron capture detector. The pyrex column of 6' x ¼" was loaded with 10% DC-200 on 60/80 Gas Chrom Z. Nitrogen was used as the carrier gas at a rate of 170 ml/min.

The temperature of the column was 190°C. Relative quantitations were made by measuring and comparing the areas of the peaks obtained from enzyme assay extracts with peaks from a series of standards of DDE and DDT.

The protein content of the enzyme solutions was estimated by the method of Lowry et al. (1951), and the micro-biuret method of Itzhaki and Gil (1964). Bovine serum albumen was used as a standard.

The enzyme unit is defined as the amount catalyzing the formation of 1 mμmole of DDE in 60 minutes under the specified conditions. Specific activity is expressed in units/mg of protein.

Enzyme-Purification

The homogenation procedure and first four fractionation steps were similar, but slightly modified, to that described by Dinamarca et al. (1971). Unless otherwise stated, all manipulations were carried out at 2-4°C. Buffers routinely contained 0.01 M GSH, except those used for dialysis. Glass distilled water was used throughout. Dialysis tubing was purified according to Klotz (1956). Each step of the purification procedure was analyzed by analytical disc electrophoresis and assayed for specific DDT-ase activity.

A. Preparation of Homogenate

Frozen flies (DDT-Orlando) in 50 gm batches were homogenized for 3 minutes in 150 ml of 0.02 M KH_2PO_4 buffer, pH 7.4, at full speed in a Lourde's homogenizer. Heat produced by the homogenization process was controlled by chopped ice around the stainless steel homogenization vessel. The material was centrifuged at 20,000 x g/15 minutes and the precipitate discarded.

B. Fractions I and II

The supernatant fluid was filtered through cheesecloth to remove the floating fatty layer. Solid ammonium sulfate was added to the filtrate in consistent increments over a time span of 30 minutes, with constant mechanical stirring, until 40 percent saturation was reached (24 gm per 100 ml). When the ammonium sulfate was completely dissolved, the solution was allowed to stir slowly for 15 minutes and stand for 15 minutes. The solution was centrifuged at 15,000 x g/20 minutes and the precipitate discarded. Solid ammonium sulfate was added to the supernatant to bring it to 80 percent saturation (28 gm per 100 ml), following the above procedure, and again centrifuged at 15,000 x g/20 minutes. The precipitate was weighed and dissolved in 0.01 M KH_2PO_4 buffer, pH 7.4, to make a 30 percent solution (Fraction I).

This fraction was fractionated with solid ammonium sulfate as described above. The precipitate obtained between 40 and 80 percent of saturation was weighed and dissolved in a minimal volume of 0.01 M KH_2PO_4 buffer, pH 7.4, and dialyzed overnight against 100 vol. of 0.001 M KH_2PO_4 buffer, pH 7.4, containing 0.6 µmoles of neutralized EDTA/liter. Any precipitate was removed by centrifugation at 15,000 x g/15 minutes (Fraction II).

C. Fraction III

The fraction II dialysate was fractionated by molecular exclusion chromatography using intermediate mesh (74-149 µ bead diameters) Bio-Gel P-10 polyacrylamide gel beads (Bio-Rad Laboratories). Dry beads were hydrated by slowly adding them to 0.01 M KH_2PO_4 buffer, pH 7.4, with constant stirring, and allowing them to stand for at least eight hours

before use. Excess "fines" were removed by decanting the supernatant fluid after allowing the hydrated beads to settle one hour in replenished starting buffer solution.

A jacketed Sephadex laboratory column (Pharmacia Fine Chemical, Inc.) 2.5 x 100 cm was coated with 1% solution of dimethyl-dichlorosilane prior to packing. The coating solution was heated to approximately 60°C and poured into the clean, dry column and allowed to stand for several minutes. The column was emptied and dried in a hot air oven. This process was repeated. The hydrated gel was deaerated in a vacumn flask and gradually poured into the column until the gel column was slightly above the desired bed level. The gel bed was washed for 12 hours with 0.01 M KH_2PO_4 elution buffer, pH 7.4.

Prior to sample application, the void volume of the column and homogeneity of the gel packing was determined using 0.3% Blue Dextran 2000 (Pharmacia Fine Chemicals, Inc.). Up to fifteen ml of the Fraction II dialysate was carefully placed in the sample applicator at the top of the column and allowed to drain into the bed. The sample applicator and sides of the column were washed with several 2-4 ml portions of elution buffer before the elution buffer reservoir was connected to the column for continuous operation. The flow rate was adjusted to 90 ml/hour. Chromatography was performed with the jacketed column cooled with running 4°C distilled water. Fractions of 7.5 ml were collected on a refrigerated, rotating drum fraction collector fitted with a photoelectric volumetric collection unit (Buchler Instruments Inc.). The eluate was analyzed for absorption at 280 mμ by an Isco, Model UA-2, ultraviolet analyzer (Instrumentation Specialties Co.), and recorded continuously. The fractions with enzyme activity, corresponding to the

void volume of the chromatographic bed, were combined, dialyzed against 0.001 M KH_2PO_4, 10^{-6} M EDTA buffer, pH 7.4, and lyophilized (Fraction III).

D. Fraction IV

Up to three hundred and fifty mg of the Fraction III lyophilized eluate were dissolved in 5.0 ml of 0.01 M KH_2PO_4 buffer, pH 7.4, and applied to a 5 x 100 cm column of Bio-Gel P-150 (200-400 mesh). The procedures followed in setting up and packing the column were basically the same as those described above under C - Fraction III, with the exception that the Bio-Gel P-150 beads required 24 hours of hydration time. Ten ml fractions were collected at a flow rate of 0.5 ml/minutes. The fractions demonstrating enzyme activity were combined, dialyzed against 0.001 M KH_2PO_4, 10^{-6} M EDTA buffer, pH 7.4, and lyophilized (Fraction IV).

E. Fraction V

The final purification of the enzyme was done by preparative disc electrophoresis using a Canalco Prep Disc apparatus equipped with a Pd-2/320 upper column (Canal Industrial Corp.), and a 250 ma constant current power supply (Buchler Instrument Inc.). The temperature of the system was regulated by pumping $4^{o}C$ water through the jacketed cooling chamber and interior core of the column. The upper column, with its' bottom covered with Saran Wrap, was supported vertically in the clamp supplied with the apparatus. Sufficient 6.0 percent acrylamide gel solution was added to give a 5.0 cm lower gel and this was overlayed with water. The standard polyacrylamide gel solutions were used, but the composition of stock solution CN was adjusted to give a

6.0 percent separating gel. The compositions of stocks and working solutions are given in Table 1. After the polymerization was complete (approximately 40 min.), the water was drawn off, sufficient spacer gel solution was added to give a 2.0 cm spacer gel and this was overlayed with water. Photopolymerization was completed in approximately 30 minutes in front of a fluorescent light. The unit was completely assembled, and the lower and upper buffer reservoirs filled with standard Tris-glycine buffer containing 0.01 M GSH. Up to 250 mg of the lyophilized Fraction IV material was electrophoresed by dissolving the desired amount of this material in 1 ml of 50% prep/cryl solution. A few drops of .005% bromphenol blue solution were added and the mixture was carefully layered on the spacer gel by displacement of the anodic buffer. The cooling water and the elution buffer (Tris-glycine running buffer diluted 1:4 with distilled water) were started and the slit was adjusted to 1-2 mm. Finally, the electrodes were connected, the current was turned on and adjusted to 5 ma until stacking was complete and the proteins had entered the spacer gel (approximately 50 minutes). The current was then increased to 10 ma. The elution buffer was pumped through at approximately 1 ml per minute; 10 ml fractions were collected until just before the tracking dye front came off the gel (Approximately fraction 21) when the rate was increased to 4 ml per minute and 4 ml fractions were collected. During the run adjustments of the slit were made to maintain it at 0.5-1.5 mm. The appropriate fractions were assayed for enzyme and protein, and those with the highest specific activity were combined, dialyzed against 0.01 M KH_2PO_4, 10^{-6} EDTA buffer, pH 7.4, and lyophilized to yield the final pure enzyme for the studies described.

Table 1. Stock and Working solutions for preparative-disc electrophoresis

STOCK SOLUTIONS

Stock A

1N HCL	48	ml
TRIS	36.3	gm
TEMED	0.23	ml
DHOH to	100.0	ml

Stock CN

Prep/Cryl	24	gm
BIS	0.41	gm
DHOH to	100.0	ml

Stock E

Riboflavin	4.0	mg
DHOH to	100.0	ml

Stock B

1N HCL	48	ml
TRIS	5.98	gm
TEMED	0.46	ml
DHOH to	100.0	ml
pH 6.6 - 6.8[1]		

Stock DN

Prep/Cryl	14.0	gm
BIS	0.25	gm
DHOH to	100.0	ml

TRIS buffer

TRIS	3.0	gm
Glycine	14.4	gm
DHOH to	100.0	ml
pH 8.2		

[1] pH adjusted by titrating with 1N HCL
TRIS--Tris (hydroxymethyl) aminomethane
TEMED--N,N,N',N'-tetramethylethylenediamine
BIS--N,N'-methylene-bis-acrylamide
Prep/Cryl--Preparative grade acrylamide (Canal Industrial Corp.)
DHOH--Distilled water

WORKING SOLUTIONS

Small Pore (separation) solution - 6%

1 part A
2 parts CN
1 part DHOH
4 parts catalyst
(Ammonium persulfate)
0.14 gm/100 ml DHOH

Elution Buffer

1 part Tris buffer
4 parts DHOH

Large Pore (spacer) solution

1 part B
2 parts DN
1 part E
4 parts DHOH

Analytical disc electrophoresis

Disc electrophoresis was performed using the procedure described by Ornstein and Davis (1961), and later modified by David (1964). The apparatus used was constructed of Plexiglass (Fig. 4), and has been described previously (Besaw et al., 1972). The compositions of stock and working solutions are given in Table 2. A spacer gel 1 cm in length was layered over the 5.1 cm separation gel. From 0.05-0.2 ml of the enzyme purification fractionation samples, containing approximately 0.25 mg of protein in 0.05-0.2 ml of 40% sucrose, were layered on the spacer gel by buffer displacement. Bromphenol blue marking dye was added to the cathodic buffer, and the fun started by applying a constant current of 3 ma per tube at room temperature until the migrating schlieren boundary (interface), indicated by the dye marker, moved a distance of 42 mm into the separation gel. The constant current was applied by a 250 ma DC power supply (Buchler Instruments Inc.).

Gel columns were stained for proteins by immersion in a 0.5% solution of Amido Black for 0.5-2 hours at room temperature. Excess stain was removed electrophoretically in 7.5% acetic acid. The stained gel columns were observed under indirect fluorescent light and diagrammatic representation of line patterns were drawn as composited from several columns run simultaneously. Rf values were compiled as the ratios of the migration distance of individual lines to the schlieren front. Quantitative densitometry was done on the stained gel columns using a Model E Microdensitometer (Canal Industrial Co.).

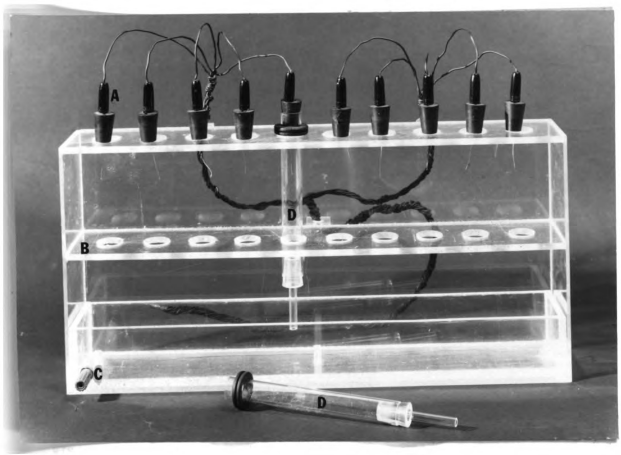


Figure 4. Disc Gel Electrophoretic Apparatus

(A) Cathodic electrode.

(B) Plexiglass stand (8½" x 3¼" x 17½").

(C) Anodic buffer reservoir.

(D) Cathodic buffer reservoir - with a tapered silicon adapter and glass gel tube in place.

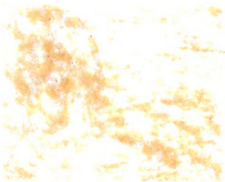


Table 2. Stock and working solutions used for disc electrophoresis.

STOCK SOLUTIONS

Stock A

1N HCL	48	ml
TRIS	36.6	gm
TEMED	0.23	ml
DHOH to	100.0	ml
pH 8.9		

Stock B

1N HCL	approximately 48.0	ml[1]
TRIS	5.98	gm
TEMED	0.46	ml
DHOH to	100.0	ml

Stock C

Acrylamide	28.0	gm
BIS	0.735	gm
DHOH to	100.0	ml

Stock D

Acrylamide	10.0	
BIS	2.5	gm
DHOH to	100.0	ml

Stock E

Riboflavin	4.0	mg
DHOH to	100.0	ml

Stock F

Sucrose	40.0	gm
DHOH to	100.0	ml

[1]pH adjusted by titrating with 1N HCL
TRIS--Tris (hydroxymethyl)aminomethane
TEMED--N,N,N',N'-tetramethylethylenediamine
BIS--N,N'-methylene-bis-acrylamide
DHOH--distilled water

WORKING SOLUTIONS

Small pore (separation) solution

1 part A
2 parts C
1 part DHOH
4 parts catalyst
(Ammonium persulfate)
0.14 gm/100 ml DHOH
pH-(8.8-9.0)

Large pore (spacer) solution

1 part B
2 parts D
1 part E
4 parts F
pH-(6.6-6.8)

Stock buffer solution for electrode reservoirs

TRIS	6.0	gm
Glycine	28.8	gm
DHOH to	1	liter
pH 8.2		

Electrodialysis

Localization and identification of DDT-ase in unstained disc electrophoresis gels was accomplished by a combination of electrodialysis and enzyme assay procedures. Ten disc electrophoresis columns, containing identical protein samples, were run simultaneously until the schlieren front in each column migrated 42 mm into the separation gel. After the migration was completed, each gel was cut into sections, 2 mm wide, using a slicer constructed from single-edged razor blades, separated by pieces of Plexiglass 2 mm thick, glued together with epoxy cement (Borden Chemical Co.). The gel sections were numbered sequentially, with section #1 measured from the schlieren front.

The pieces of gel anodic to section #1 and cathodic to the last numbered section were discarded. Like numbered sections were collated and placed in small beakers on ice.

Caps were screwed on ten electrodialysis vials, which were constructed (Michigan State Univ. glassblowing shop) from scintillation vials modified to accept screw-caps on both ends (Fig. 5). Six ml of small-pore gel solution (Table 2) was placed in each vial and allowed to polymerize for thirty minutes. The polymerized gel surfaces were rinsed with spacer buffer (Table 2), and one group of collated sections was placed on top of this gel in each vial and arranged so they all laid on the same horizontal plane. Five ml of large-pore gel solution (Table 2) was carefully pipetted on top of the sections and allowed to photopolymerize in front of a fluorescent light. The polymerized gel surfaces were rinsed, and the remaining volume of each vial was filled with approximately 2 ml of spacer buffer. Small pieces of wetted dialysis tubing, single thickness, were laid over the threaded ends of

the vials, and caps, with 1/2 inch drilled center holes, were screwed on tight. The original caps were removed, and the vials screwed into the bottom of the anodic buffer reservoir. The entire assembly was placed on a Plexiglass frame with the cathodic ends of the vials (i.e. holed capped ends with dialysis tubing barriers) extending one-half inch into the cathodic buffer (Fig. 5). Electrodialysis was carried out at 4°C at 4 ma per vial for 12 hours using a 250 ma DC power supply. After electrodialysis was completed, the volume of buffer in each vial was analyzed for DDT-dehydrochlorinase activity according to the assay procedure already described.

Sucrose Density Gradient Centrifugation

The molecular weight and sedimentation coefficient of purified DDT-ase was estimated using sucrose density gradient centrifugation follwoing the procedure of Martin and Ames (1961). Linear 5 - 20% sucrose gradients (total volume, 4.6 ml) containing 0.01 M potassium phosphate, pH 7.4, and 0.01 M GSH were made in ½" x 2" beckman cellulose nitrate tubes. Gradients were allowed to stand at least 4 hours at 4°C before use.

A sample containing 2 mg of purified enzyme and varying amounts of marker proteins in a volume of 0.1 ml was carefully applied to the top of the gradient. Hemoglobin (10 mg/ml), lipoic dehydrogenase (10 mg/ml) and lactate dehydrogenase (10 mg/ml) were used as marker proteins. All proteins were made up in the same buffer as the sucrose gradient.

Centrifugation was at 40,000 rpm for 18 hours at 4°C in a Spinco Model L centrifuge with a SW-50 rotor. After centrifugation, contents

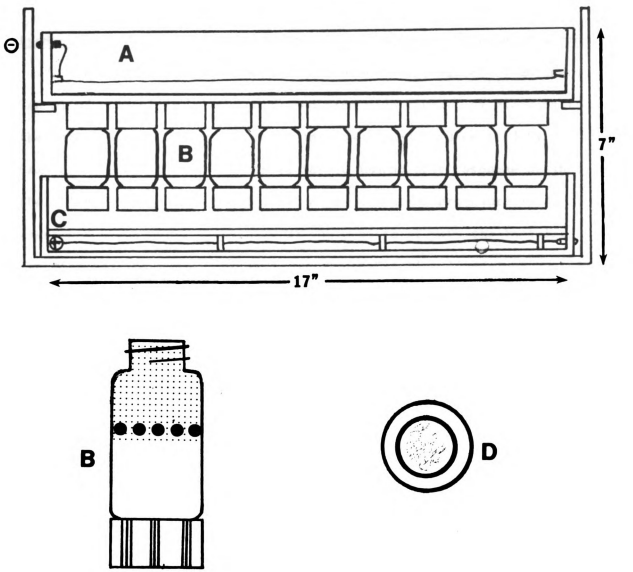


Figure 5. Electrodialysis Apparatus

(A) Cathodic buffer reservoir with scintillation vial screw-caps cemented to the bottom.

(B) Scintillation vial modified to accept a screw-cap at both ends. Large view shows disc gel sections held in polymerized gel, above elution buffer solution.

(C) Anodic buffer reservoir.

(D) Screw-cap showing drilled hole and dialysis tubing barrier.

of the tube were collected in 10-drop fractions and assayed for DDT-ase activity and marker proteins.

Production of Antisera and Isolation of the Immunoglobulin (Ig) Fraction

Adult Dutch Belted rabbits were used as the source of antisera. Ten ml of control sera were collected from each rabbit, by bleeding from the marginal vein of the ear following the procedure described by Campbell et al. (1970), and tested for an immunologic response against the DDT-ase antigen. A modified alum precipitation procedure as described by Campbell et al. (1970) was used to prepare the inocula. Two solutions were made, using 35 mg of the Fraction II product, obtained form the DDT-ase purification procedure, and 35 mg of the Fraction V product, each dissolved in 35 ml of phosphate buffered saline (PBS) (Kwapinski, 1972), pH 7.3, containing 0.01 M GSH. To each of these solutions 14 ml of a 10% $AlK(SO_4)_2 \cdot 12\ H_2O$ was slowly added with constant stirring. The two mixtures were adjusted to pH 7.0 with dropwise addition, with stirring, of 20% NaOH. The solutions stood for 30 minutes and then were centrifuged at 1000 x g for 10 minutes. The supernatants were discarded, and each of the precipitates was washed twice in PBS, recovering them each time by centrifugation at 1000 x g for 10 minutes. Each precipitate was finally resuspended in 16.7 ml of PBS containing 0.01 M GSH, and stored at -20°C.

Three rabbits were inoculated with the alum-precipitated Fraction II suspension and three rabbits with the Fraction V suspension. Each rabbit was inoculated with the antigen-adjuvant suspension (10 mg of protein/ml) according to the following schedule: two intramuscular injections of 0.5 ml, two intramuscular injections of 0.25 ml

and two intraperitoneal injections of 0.25 ml of the suspension, spaced by 7-day periods (Kwapinski, 1972). After a rest period of period of fourteen days, each rabbit was bled (approximately 50 ml) from the marginal vein of the ear. The freshly drawn blood was allowed to stand for 2 hours at room temperature for clot formation. It was then refrigerated overnight to permit clot contraction. The serum was decanted and centrifuged at 1000 x g for 30 minutes.

Quantitation of the various antisera for DDT-ase antibody was performed using the quantitative precipitation antibody test as described by Kabat and Mayer (1964). Analysis of the antiserum was also done with the Ouchterlony micro-double diffusion and disc immunodiffusion techniques described in the next two sections of the Materials and Methods.

The sera from rabbits inoculated with the alum-precipitated Fraction II suspension, showing comparable antibody titres, were pooled, merthiolate (Lilly) added (1:10,000), and stored at -20°C. This antisera is referred to hereafter as Fraction II antiserum. The same procedure was followed with sera from rabbits inoculated with the alum-precipitated Fraction V suspension. This pooled antisera is referred hereafter as Fraction V antiserum.

Serum albumins were removed from the Fraction II and V antisera and from the control sera by three successive 50 percent ammonium sulfate precipitations of the immunoglobulin (Ig) protein fractions. All centrifugations were done at 15,000 x g for 15 minutes. The final precipitates were redissolved in a minimal volume of PBS, pH 7.3, and dialyzed for 18 hours in one change of PBS, pH 7.3. After dialysis, an appropriate amount of PBS was added to each serum dialysate to restore the original serum volume. Merthiolate was added (1:10,000), and the antisera divided

into 1 ml aliquots and stored at -20°C.

Ouchterlony Micro-Immunodiffusion

A microtechnique of the double immunodiffusion test was performed using a modification of the procedure described by Wadsworth (1957). Thoroughly cleaned microscope slides were uniformly coated with 2.5 ml of melted 1.0% Ionagar No. 2 (Consolidated Laboratories, Inc.) in 0.15 M PBS, pH 7.3, containing 1:10,000 merthiolate. After the agar was allowed to solidify for 30 minutes, wells were cut in the gel using a metal template (Template No. 71692 - Gelman Instruments). The pattern cut consisted of six outer wells and one inner well. All wells were 3 mm in diameter and spaced 6 mm apart. Agar was removed from the wells by using a Pasteur pipette attached to tubing on a water aspirator. The center well was filled with Fraction V antisera and outer wells with the Fraction II and V antigens obtained from the DDT-ase purification procedure. Appropriate dilutions of antigens and antisera were made based on the optimal proportions of each as determined by the quantitative precipitating antibody test (Kabat and Mayer, 1964). The diffusion slides were incubated for 24-36 hours at 37°C. After incubation the slides were washed for three days in daily changes of 0.15 M PBS, pH 7.3, to remove unreacted protein. They were rinsed in distilled water, dried to a thin film by air evaporation, and stained in an Amido Black 10B solution containing 1.0 gm of the dye in 1 liter of a 9:1 methanol-glacial acetic acid solution. After staining for 5 minutes, the slides were washed in a 9:1 methanol-acetic acid solution, distilled water, and air dried.

Disc Immunoelectrophoresis

Two dimensional disc immunoelectrophoresis was performed using the agar embedding method described by Darcy (1968). In this procedure, the pure DDT-ase antigen was subjected to disc gel electrophoresis. The electrophoresed gel was then squeezed into a 3 x 55 mm trough cut into a 2 mm thick agar layer covering the bottom of a petri dish (the composition of the agar is the same as that used in the micro-immunodiffusion technique). Two parallel antibody troughs, 2 x 55 mm, were cut in the agar 6 mm from each side of the polyacrylamide gel. After the agar plugs were removed from the troughs, Fraction II antiserum was placed in one trough and Fraction V antiserum in the other. The gels were incubated for five days at 37°C, and observed daily. After incubation, the polyacrylamide gel was removed, and the agar layer washed, dried and stained in the same manner as the Ouchterlony micro-immunodiffusion slides.

Absorption of Fraction II Antiserum

Two absorption procedures were used to remove antibodies from Fraction II antisera not specific to the DDT-ase antigen:

Method A: In this method the absorption technique described by Kopeloff and Kopeloff (1949) was performed. Two ml of the Fraction II antiserum (Ig fraction), diluted 1:4, was mixed with an equal volume of a two percent dialysate from the NAIDM - susceptible strain of house flies (analagous to the Fraction II dialysate from the DDT-Orlando strain). The mixture was incubated for 1 hour at 37°C, and centrifuged at 20,000 x g for 30 minutes. The clear supernatant containing the absorbed serum was decanted off, perevaporated to a volume of 2 ml, and stored at -20°C.

Method B: In this absorptive technique, 2 ml of the Fraction II antiserum, diluted 1:4, were mixed with an equal volume of a two percent solution of the Fraction II dialysate from the DDT-Orlando strain, which was first reacted for 1 hour (37°C) with 20 µg of DDT dissolved in 20 µl of dimethylsulfoxide, and 20 µmoles of GSH. The absorption mixture was incubated for 1 hour at 37°C, and centrifuged at 20,000 x g for 30 minutes. The supernatant was perevaporated to a volume of 2 ml and stored at -20°C.

House fly Tissue Sectioning

Small pieces of unfixed house fly tissues and whole flies (DDT-Orlando and NAIDM-susceptible were sectioned using an IEC CTF Microtome-Cryostat (International Equipment Co.) maintained at -20°C. A tape cylinder, approximately 13 x 15 mm, was centrally placed on a wetted, sectioning chuck, and filled with embedding medium (Tissue-Tek - Ames, Co.). Various excised tissues or whole flies, with wings and legs removed, were oriented in the medium and snap-frozen by placing the bottom of the chuck in liquid nitrogen until the embedding medium was uniformly white. Tissue sections were cut at 4 µ, after allowing the embedded tissues to equilibrate to the temperature of the cryostat for one hour, and after the removal of the outside layer of tape. Tissue sections were affixed to coverslips by gently touching the tissue section with a coverslip kept at room temperature, and air drying for 10 minutes at room temperature.

Indirect Fluorescent Antibody Staining

The indirect method of fluorescent antibody staining was performed following the basic procedures outlined by Kawamura (1969). The air-dried tissue sections were fixed in 95% ethanol for 10 minutes at room temperature, and then thoroughly rinsed with PBS, pH 7.4. Two drops of one specific DDT-ase antiserum was placed on each test section and incubated for 30 minutes at 37°C in a humidified chamber.[1] After incubation the antisera was gently washed off the tissue sections with PBS using a Pasteur pipette. The coverslips were then placed in a glass staining dish filled with PBS and shook on a mechanical shaker for 30 minutes with three changes of PBS. The coverslips were removed from the staining dish, excess PBS taken off, and the final staining step continued without allowing the tissue sections to dry.

Two drops of a 1:1o PBS diluted sheep anti-rabbit globulin, conjugated with fluorescein isothiocyanate (FITC)[2], was placed on each test section and incubated as before for 30 minutes. In order to reduce nonspecific fluorescence, the FITC conjugate was absorbed with an acetone powder of NAIDM-susceptible flies according to the procedure described by Coons and Kaplan (1950). After incubation the conjugated antiserum was rinsed off, and the tissue sections again washed on the shaker for 30 minutes. The tissue sections were finally rinsed with distilled water, and covered with a drop of buffered glycerol (10% PBS). Each coverslip was inverted on a slide, avoiding trapped air bubbles, and

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[1] The following specific antisera was used: (1) Fraction II antiserum (Ig fraction) absorbed by Method A, dil. 1:4. (2) Fraction II antiserum (Ig fraction) absorbed by Method B, dil. 1:4. (3) Fraction V antiserum (Ig fraction), dil. 1:8.

[2] Nutritional Biochemical Corp., Control #4343. Preserved with 1:10,000 merthiolate.

sealed with clear nail polish.

Several trial and error experiments were done using a series of dilutions of antisera concentrations, incubation and washing times, before selecting the optimal experimental parameters described above.

Fluorescent Microscopy, Photography and Quantitation

A Wild M-20 microscope equipped with a 200 watt mercury-vapor very-high pressure lamp (Osram HBO) was used with appropriate exciter and barrier filters for observation of the FITC-stained tissue sections. Since FITC absorbs visible blue light at 495nm and re-emits this energy as green fluorescence radiation at 520nm, most of the observations and photography of the sections were performed using blue-light fluorescence. The optimal filter combination consisted of a BG 12/4mm blue-light fluorescence exciting filter, which transmits short-wave radiation up to 500nm, and an OG 1/2mm barrier filter, which inhibits blue radiation and transmits light of 510nm and above. Observations were made using a darkfield condensor and a magnification of 150-300x. Photographs were taken using a Wild MKa 1 attachable camera, 35mm magazine, and Kodak Ektachrome film (High Speed-ASA 125). Exposure times of the various tissue sections were electronically controlled by a light sensitive photocell in a Wild MKa 5 Photoautomat. Additionally, the individual exposure times for all sections were measured with a stop-watch and recorded.

Quantitative estimates of fluorescence intensity in the various sections were made using the Wild Photoautomat. The inverse proportion between the level of fluorescent brightness of each section and length of exposure time, sensitively regulated by the Photoautomat

attachment, was used to estimate the relative amounts of non-specific staining, autofluorescence and DDT-ase antigen between control and experimental sections.

Induction of DDT-ase

The fluorescent antibody staining technique was used to demonstrate the induction of DDT-ase in house fly tissues following the topical application of DDT.

Ten micrograms of DDT dissolved in acetone was applied topically to the dorsal region of adult flies under CO_2 anaesthesia (Busvine, 1972). The control flies were treated with acetone under the same conditions. After standing at 27°C for 9 hours, the insects were sectioned and stained with the fluorescent antibody. The experimental parameters used are the same as those reported by Capdevila et al. (1973), which produced maximum DDT-ase induction. Quantitation of the DDT-ase levels in the experimental and control sections was performed by measuring the relative exposure times controlled by the light sensitive photocell in a Wild MKa 5 Photoautomat, attached to the fluorescent microscope.

RESULTS AND DISCUSSION

GENERAL REMARKS

The feasibility of utilizing the fluorescent antibody technique for localizing DDT-ase in various house fly tissues required the development of a method of isolation which could yield a quantity of pure enzyme adequate for the production of a specific, high-titered antibody. At the onset of this study there were two fractionation procedures reported in the literature (Lipke and Kearns, 1959a; Dinamarca et al., 1969), however, both were limited in their ability to isolate a practical quantity of pure enzyme. The relatively low yield of these two purification procedures was probably due, at least in part, to the inclusion of such fractionation steps as flash-heating, calcium phosphate gel treatment and alcoholic precipitation. Although these techniques have been successfully applied to a number of problems in enzymology, they are not the methods of choice when isolating an enzyme having the degree of unstability characteristic of DDT-ase.

Therefore, the logic behind the purification procedure reported in this thesis study, was based on the selection of fractionation techniques which would keep chemical and physical denaturation of the DDT-ase enzyme to a minimum. Preliminary experiments demonstrated that molecular exclusion chromatography in combination with preparative disc electrophoresis satisfied these conditions, while at the same time providing optimal resolving and concentrating capabilities. It was at this time that Dinamarca et al. (1971) published their second paper

reporting an improved procedure for the isolation of DDT-ase. It is apparent that they arrived at the same conclusion, in that by utilizing molecular exclusion and anion-exchange chromatography, a two-three fold increase in yield of pure enzyme was achieved over their previously published procedure (Dinamarca et al., 1969).

One of the most significant contributions of both of these publications was the report that the addition of 0.01 M GSH to the buffers used in the fractionation procedures stabilized DDT-ase in its tetrameric form and maintained activity. This technique not only improved the yield of active enzyme isolated by the procedures used in this thesis study, but it also allowed time for collating the quantities of DDT-ase, purified from four successive isolations, necessary for the production of its high-titered antibody.

PURIFICATION OF HOUSE FLY DDT-DEHYDROCHLORINASE

A summary of the major steps in the purification procedure is shown in Table 3. DDT-ase enzyme with an approximate specific activity of 8961 mμmoles of DDT dehydrochlorinated per mg protein/hour was obtained each time the purification procedure was run. The preparation takes four days, with the homogenization and ammonium sulfate concentration steps on the first day, the Bio-Gel p-10 filtration chromatography on the second, the Bio-Gel P-150 filtration chromatography on the third and the preparative disc gel electrophoresis on the final day. This preparation uses 100 g of whole flies and yields approximately 9.1 mg of pure enzyme.

TABLE 3

PURIFICATION OF DDT-DEHYDROCHLORINASE

Fraction		Total Protein (mg)	Total Units	Specific Activity	Yield
	Homogenization supernatant	9352	392,064	41.9	100
I	40-80% ammonium sulfate	2993	305,810	102	78
II	40-80% ammonium sulfate	778	214,067	275	56
III	Molecular exclusion chromatography (Bio-Gel P-10 polyacrylamide gel)	201	183,094	911	47
IV	Molecular exclusion chromatography (Bio-Gel P-150)	49	133,302	2720	34
V	Preparative Disc Electrophoresis	9.1	81,545	8961	20.8

Homogenization -- Optimal solubilization of DDT-ase was accomplished by homogenizing the house flies in 0.02 M KH_2PO_4 buffer, which is consistant with the results reported by Dinamarca et al. (1969). The total amount of protein solubilized after homogenization is 9352 mg with a specific activity of 41.9, which is an increase of 0.09 percent over that previously reported (ibid.). This increase in specific activity can possible be attributed to a higher titre of DDT-ase present in the DDT-Orlando house fly strain used in this study, and to the care undertaken to prevent denaturation during homogenization. The enzyme is not only very heat labile, but extremely sensitive to mechanical effects as well. Goodchild and Smith (1970) reported that DDT-ase was very susceptible to frothing, even in crude preparations. It was reported that gently shaking or bubbling air through a solution containing 1 mg of protein/ml caused a loss of 80% of the activity in 20 seconds. Through trial and error it was noted in this study that homogenizing for three minutes at full speed in the Lourdes homogenizer produced optimum solubilization, while at the same time maintaining maximum enzyme activity. Attention to small details such as storing the homogenizing vessel at -20°C prior to use, using refrigerated buffer, partially frozen house flies, in addition to the typical packing of ice around the homogenizing vessel, assured protection of the homogenate from the effects of overheating.

Ammonium Sulfate Precipitation (Fractions I and II) -- Fractionation on the basis of differential solubilities in ammonium sulfate solutions was employed to achieve an initial fractionation of the enzyme from the crude homogenate supernatant. The precipitate obtained between 40 and 80

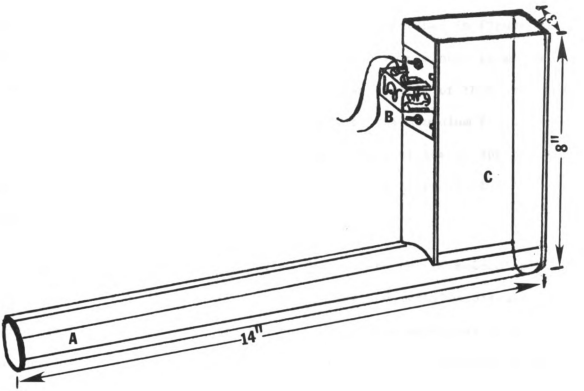


Figure 6. Ammonium Sulphate Vibrating Dispenser

(A) Plexiglass dispensing tube (14" x 1" o.d.).

(B) Edward's 10 volt vibrating door buzzer.

(C) Container for measured amount of ammonium sulphate to be dispenced.

percent of saturation was removed by centrifugation to give Fraction I. The precipitate was redissolved in 0.01 M KH_2PO_4 buffer, pH 7.4, and the identical fractionation process was repeated on this solution to give Fraction II.

Powdered ammonium sulfate was consistently added over a time span of 30 minutes using a vibrating apparatus described in Figure 6. The solutions were maintained at 4°C and stirred very slowly to minimize heat and mechanical denaturation. The precise control of these physical factors gave very reproducible results between the ammonium fractionation steps of successive enzyme isolations. An increase of approximately 6.5 fold in specific activity was achieved with a recovery of 56% (Fraction II), as shown in Table 3.

Bio-Gel P-10 Gel Filtration (Fraction III) -- The use of molecular exclusion chromatography utilizing acrylamide and methylenebis-acrylamide copolymer bead filtration, which fractionates molecules primarily on the basis of difference in size, was adopted to the further purification of DDT-ase.

The Bio-Gel P-10 beads have a fractionation range of 1,500 - 20,000 M.W., which served to eliminate a number of low molecular weight proteins, including various colored pigments. Dinamarca et al. (1971) reported similar results using a Sephadex G-50 column. This fractionation range excluded DDT-ase from the Bio-Gel P-10 pores, and the eluted fractions demonstrating enzyme activity corresponded to the void volume of the column.

The Bio-Gel P-10 filtration step provided a 3.3 fold increase in specific activity over that of the preparation obtained by the

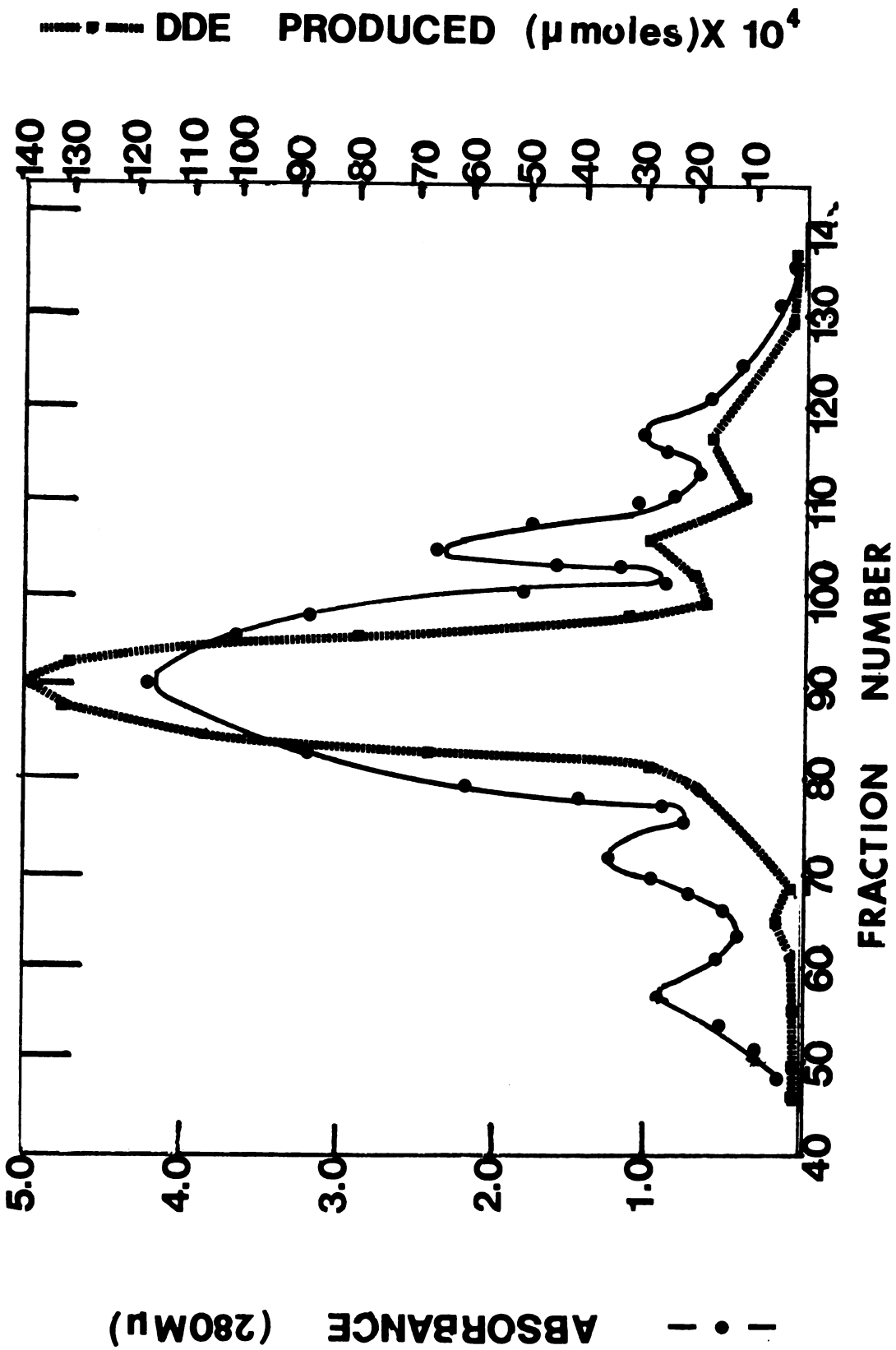
ammonium sulfate fractionation with a total yield to this point of 47%. (Table 3)

Bio-Gel P-150 Gel Filtration (Fraction IV) -- The fraction III product was applied to a 5 x 100 cm column of Bio-Gel P-150 polyacrylamide beads (fractionation range of 15,000-150,000 M.W.) equilibrated with 0.01 M KH_2PO_4 buffer, pH 7.4, containing 0.01 M GSH, and eluted with the same buffer. The elution was performed descendingly at a flow rate of 25 ml per hour. A volume of 10 ml per fraction was collected and aliquots of fractions were assayed for DDT-ase activity. The peak of enzyme activity was localized at the region of eluate volume from 820 to 970 ml (Figure 7). Fractions of this region were pooled and concentrated either by pressure dialysis against the same elution buffer or by lyophilization.

This purification step provided a 3.0 fold increase in specific activity over that of the preparation obtained by the Bio-Gel P-10 fractionation with a total yield of 34% (Table 3).

Preparative Disc Gel Electrophoresis -- Preliminary experimentation on the Fraction IV product indicated that preparative disc gel electrophoresis was a logical choice for the final step in the purification of DDT-ase. These experiments demonstrated that the following prerequisites, required for the successful application of this technique, were fulfilled: (1) preparative disc gel electrophoresis is best adapted for the isolation of a protein from a simple complex of proteins. It is also necessary that the desired protein be reasonably well separated from the contaminating components after electrophoresis, and without the smearing effect of disassociation. Results of analytical

Figure 7. Elution diagram for separation of the Fraction III purification product on a Bio-Gel P-150 Column (5 x 100cm) equilibrated with 0.01 M KH_2PO_4 buffer, pH 7.4, containing 0.01 M GSH. Experimental details are given in the text.


DDE PRODUCED (μmoles)X 10⁴
140
130
120
110
100
90
80
70
60
50
40
30
20
10
5.0
4.0
3.0
2.0
1.0
40
50
60
70
80
90
100
110
120
130
FRACTION NUMBER
ABSORBANCE (280mμ)

disc gel electrophoresis of the Fraction IV product demonstrates a simple, and cleanly resolved mixture of seven components, which adequately meets the above conditions (Figure 8A). (2) An enzyme must be able to tolerate the physical and chemical effects of electrophoretic separation without a significant loss of activity. This requirement was tested by first performing analytical disc electrophoresis on the Fraction IV product, and then electrodialysing out the proteins present in sequentially cut, collated sections from ten identically electrophoresed gels. The results of assaying the electrodialysate from each group of sections demonstrates DDT-ase activity in that group containing the major electrophoretic component of the Fraction IV product (Figure 9). DDT-ase, therefore, can withstand electrophoretic separation with only a minor loss of activity.

Two things are important for the success of the preparative disc electrophoretic step: the use of GSH in the preparative and running buffers and the use of a 6.0 percent acrylamide gel. The presence of 0.01 M GSH in the buffers helped to maintain DDT-ase in its tetrameric form until its elution off the end of the preparative gel. The 6.0 percent acrylamide gel gave the desired resolution required for distinct separation of the enzyme from the few contaminating proteins present after the Fraction IV purification step (Figure 8A).

Electrophoresis was started at 5 ma until the sample entered into the stacking (spacer) gel. The current was then increased to 10 ma for approximately 16 hours. The electrode reservoirs were filled with the standard Tris-glycine buffer, containing 0.01 M GSH. The elution buffer (Tris-glycine running buffer diluted 1:4 with distilled water, containing 0.01 M GSH) was pumped through at 4 ml per minute

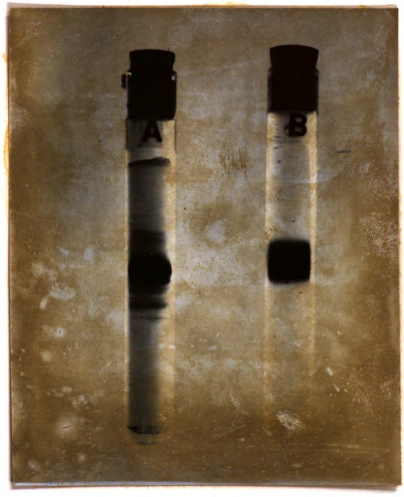
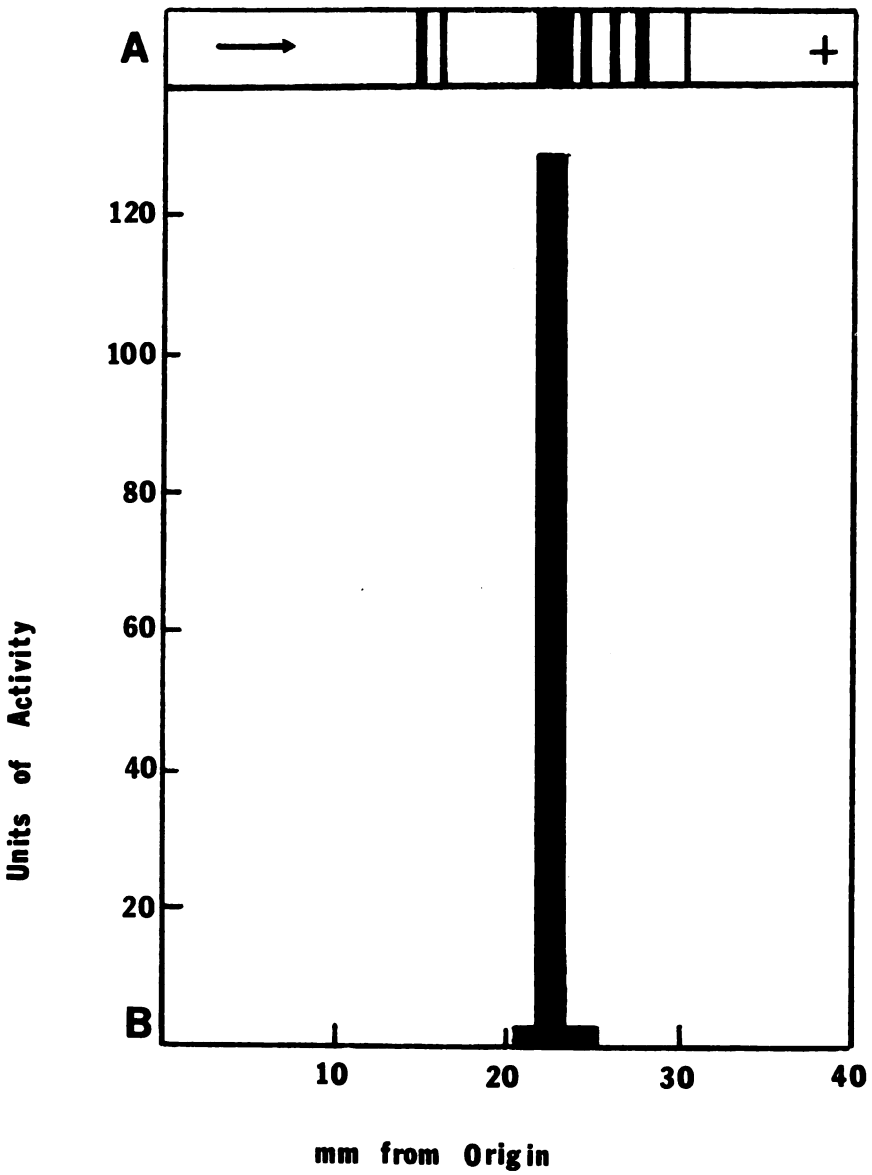


Figure 8A. Disc Gel Electrophoresis of the Fraction IV product (200 µg) was electrophoresed and the gel stained as described in the text. The direction of electrophoresis was from the Top to the Bottom of the Gel.

Figure 8B. Electrophoretic Homogeneity of Purified DDT-ase. A sample (200 µg) of the purified enzyme was electrophoresed and the gel stained as described in the text. Increasing the sample to 400 µg did not show any additional bands.

Figure 9. Disc Gel Electrophoresis of the Fraction IV product. The Fraction IV product (200 µg) was electrophoresed and the gels either stained for protein, or sliced into 2 mm segments, and eluted by electrodialysis for activity determination. The direction of electrophoresis is indicated by the arrow. (A) Schematic reproduction of the electrophoresed Fraction IV product (the actual gel is shown in Figure 8A). (B) Activity eluted from ten sectioned unstained gels, which were identically electrophoresed.

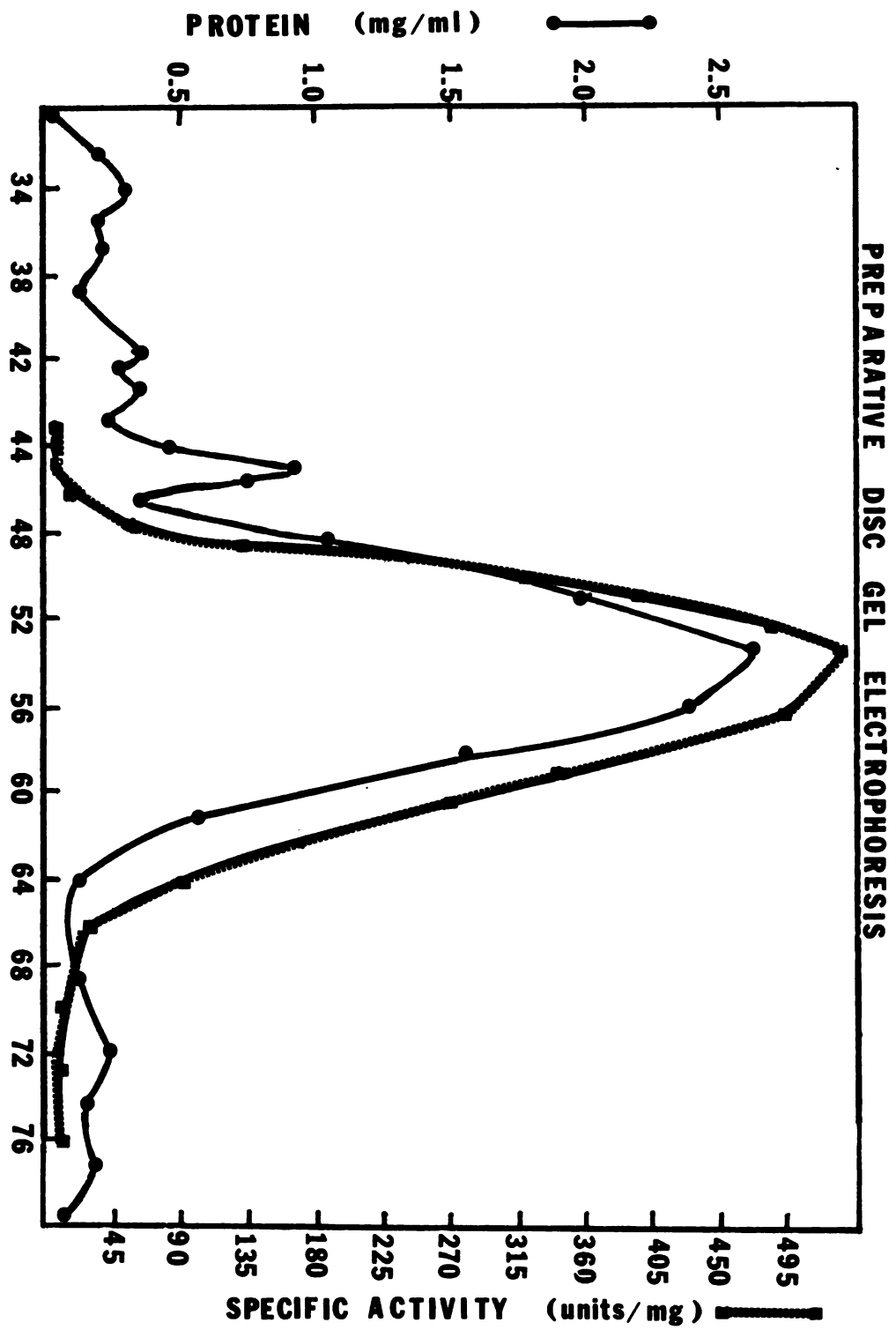

A
+
120
100
80
60
40
20
B
10
20
30
40
Units of Activity
mm from Origin

(after the tracking dye came off the gel) and 4 ml fractions were collected. The appropriate fractions were assayed for enzyme and protein, and those with the highest specific activity were combined (fractions 48 to 64), dialyzed, and lyophilized to yield the final pure enzyme.

This purification step yielded a total of 9.1 mg of DDT-ase with a specific activity of 8,961 (Table 3), which is a 3.3 fold increase in specific activity over that of the Fraction IV product, and a final purification of about 214 fold. The results from a typical preparative disc gel electrophoresis run are shown in Figure 10.

The degree of purification and specific activity of the pure enzyme are consistant with the results reported by Dinamarca et al. (1969, 1971). However, the final yield of DDT-ase reported in this study is a three fold increase over the amount obtained from the purification procedure reported in their first paper, and a one fold increase over the amount reported purified in their second paper. This higher yield can probably be attributed to a number of minor reasons, but most logically to the use of preparative columns in the molecular exclusion purification steps, and to the use of preparative disc gel electrophoresis in the final step. The high resolving power of this latter technique, based on the combined mechanisms of the concentrating effect, physical sieving effect and differential electrophoretic mobilities of proteins in polyacrylamide gel, made it as a very successful tool in the procedure for purifying DDT-ase.

Figure 10. Preparative Disc Gel Electrophoresis. Up to 250 mg of the Fraction IV product was dissolved in 1 ml of 50% prep/cryl solution and applied to a 6% acrylamide gel column and electrophoresed in a Canalco Prep Disc apparatus with a Pd-2/320 upper Column. The running buffer was the analytical Tris-glycine buffer (Table 2) diluted 1:4 with distilled water. Fractions 48 - 64 were combined and concentrated.

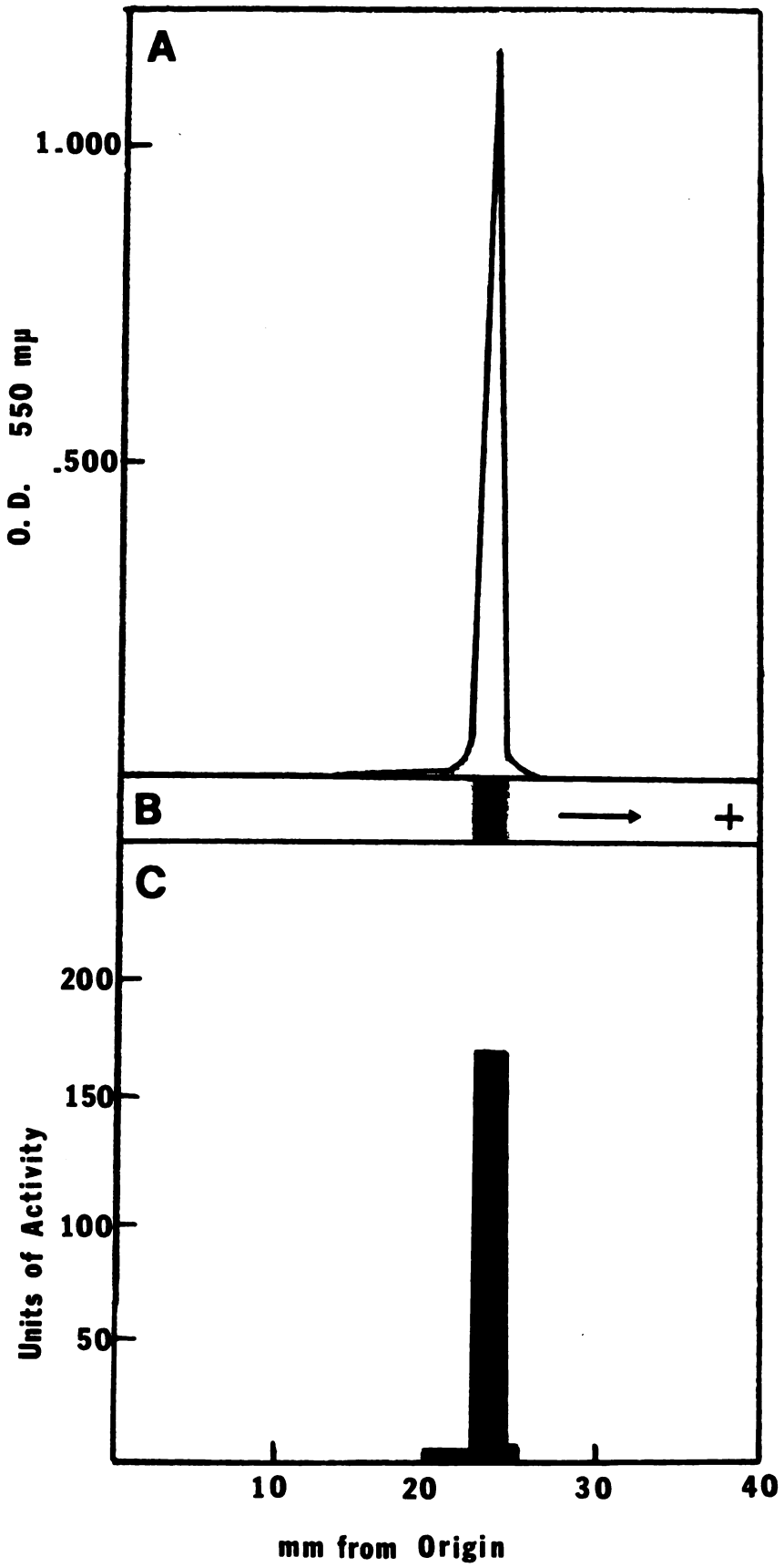

PROTEIN (mg/ml)
0.5
1.0
1.5
2.0
2.5
34
38
42
44
48
52
56
60
64
68
72
76
PREPARATIVE DISC GEL ELECTROPHORESIS
45
90
135
180
225
270
315
360
405
450
495
SPECIFIC ACTIVITY (units/mg)

HOMOGENEITY OF THE HOUSE FLY DDT-ase PREPARATION

Polyacrylamide Disc Gel Electrophoresis -- The purified DDT-ase (200 mg of protein) showed a single protein band when examined by polyacrylamide gel electrophoresis (Figure 8B and Figure 11A). Increasing the sample up to 400 mg of protein did not show any additional bands. The demonstration of DDT-ase activity of this single band was performed by electrodialyzing it our of appropriate sections of gels and assaying the resulting eluate. The results of this experiment are depicted in Figure 11C, which demonstrates that this protein band possesses DDT-ase activity.

Ouchterlony Double Diffusion -- The pooled serum of rabbits injected with purified house fly DDT-ase (Fraction V antiserum) was found to contain antibodies to DDT-ase. Reacting the Fraction V antiserum against the Fraction II and V purification products showed the formation of a single precipitin band indicating the existence of a single antigen-antibody system (Figure 12). Furthermore, the precipitin band formed by the impure Fraction II product and by the purified DDT-ase showed no "spurs", indicating the presence of a single identical antigen-antibody system in both fractions. The combining ratio of antigen and antiserum was varied to determine the precipitin pattern in the zones of antigen-excess, antibody-excess and equivalence. Although the resolution of the precipitin pattern was altered, there was no indication of any additional antigen-antibody systems present other than the one shown in Figure 12.

Figure 11. Disc Gel Electrophoresis of the Purified DDT-ase. DDT-ase (200 µg) was electrophoresed and the gels either stained for protein or sliced into 2 mm segments, and eluted by electrodialysis for activity determination. The direction of electrophoresis is indicated by the arrow. (A) A tracing of an optical density recording, produced by a Model E Microdensitometer (Canalco Corp.), of a Amido Black stained gel. (B) Schematic reproduction of the electrophoresed purified DDT-ase (the actual gel is shown in Figure 8B). (C) Activity eluted from ten sectioned gels, which were identically electrophoresed.


A
1.000
.500
O. D. 550 mμ
B
+
C
200
150
100
50
Units of Activity
10
20
30
40
mm from Origin

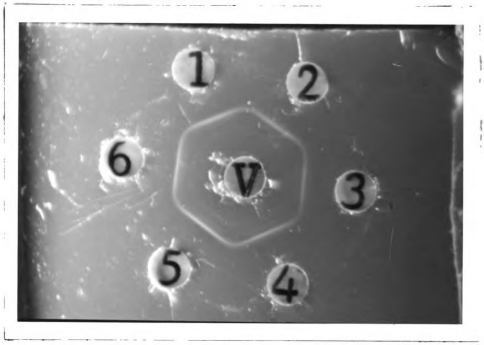


Figure 12. Ouchterlony Double Diffusion Pattern of House fly DDT-ase against Rabbit Antiserum. Rabbit antiserum was placed in the center well. Purified house fly DDT-ase (15 µl, containing 60 µg protein) was placed in outer wells 1,3, and 5; crude Fraction II preparation (15 µl, containing 180 µg protein) in wells 2,4, and 6. Diffusion was carried out at 37° for 24 hours before photographing under diffuse light.

Disc Gel Immunoelectrophoresis -- The homogeneity of purified DDT-ase was also analyzed by two-dimensional gel immynoelectrophoresis. In this procedure, 200 µg of the Fraction V product was subjected first to disc gel electrophoresis (first dimension) and then to immunodiffusion (second dimension). The precipitin pattern formed by the reaction between the electrophoresed antigen and the perpendicularly diffused antiserum is shown in Figure 13. The results demonstrate the formation of only one antigen-antibody system between the separated DDT-ase antigen and both the Fraction II and V antisera. No additional precipitin bands were formed by varying the pH of the agar diffusion media, serially diluting the antisera or electrophoresing increased amounts of antigen (up to 400 µg).

MOLECULAR WEIGHT DETERMINATION

Sucrose Density Gradient Centrifugation -- The molecular weight of purified DDT-ase was determined by sucrose gradient centrifugation. The distributions of DDT-ase and marker proteins on sucrose density gradients are shown in Figure 14. Martin and Ames (1961) have reported that the ratio (R) of the distances travelled from the meniscus by any two substances is constant during centrifugation in a sucrose density gradient. Therefore,

$$R = \frac{\text{Distance travelled from meniscus by unknown}}{\text{Distance travelled from meniscus by standard}} = \frac{S_{20,w \text{ of unknown}}}{S_{20,w}^{0.725} \text{ of standard}}$$

In addition, Martin and Ames (1961) pointed out that

$$R \simeq \left(\frac{\text{Mol. wt. of unknown}}{\text{Mol. wt. of standard}}\right)^{2/3}$$

thus, a linear relationship between distance travelled from the meniscus and (Mol. wt.)$^{2/3}$ should be obtained.

A molecular weight of 120,000 was estimated for DDT-ase from the linear relationship derived by plotting the sedimentation velocity against the molecular weight of standard proteins to the two-third power (Figure 15). This value is in excellent agreement with the molecular weight calculated for DDT-ase, previously reported by Dinamarca et al. (1969).

Sedimentation Coefficient -- The sedimentation coefficient of purified DDT-ase was also estimated from the results of sucrose density centrifugation, and a $S_{20,w}$ value of 6.3 for the enzyme was obtained (Figure 16). This value is identical to the sedimentation coefficient reported for the tetrameric form of DDT-ase by Dinamarca et al. (1969).

IMMUNOLOGICAL STUDIES

Production of Rabbit Antisera -- The rationale for the injection sequence and type of adjuvant used to produce antibodies against DDT-ase was based on the extreme instability of the enzyme. It was theorized that a series of small injections would minimize denaturation effects and, therefore, assure the development of antibodies against the tetrameric form of DDT-ase. Depots of antigen, resulting from a few large injections, would logically be more susceptible to denaturation before the completion of antibody formation, resulting in the possible

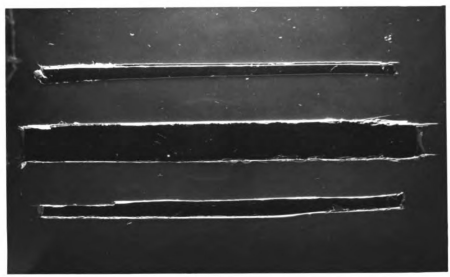


Figure 13. Disc Gel Immunoelectrophoresis of the purified House fly DDT-ase. Purified DDT-ase (200 µg) was electrophoresed (first dimension) and then inserted into a 3 x 55 mm trough cut into a 2 mm thick agar layer covering the bottom of a petri dish. Fraction II antiserum was placed in the top parallel trough and Fraction V antiserum in the bottom trough. Diffusion was carried out at 37°C for 24 hours before photographing under diffuse light.

Figure 14. Sucrose Density Gradient Centrifugation of Purified House fly DDT-ase and Marker Proteins. The procedure is described in the text. Fraction numbers increase from the bottom (20% sucrose) to the top (5% sucrose) of the gradient.

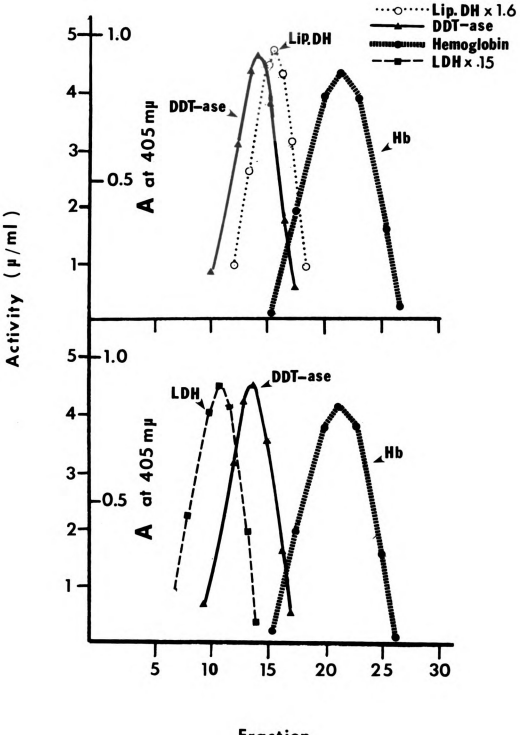
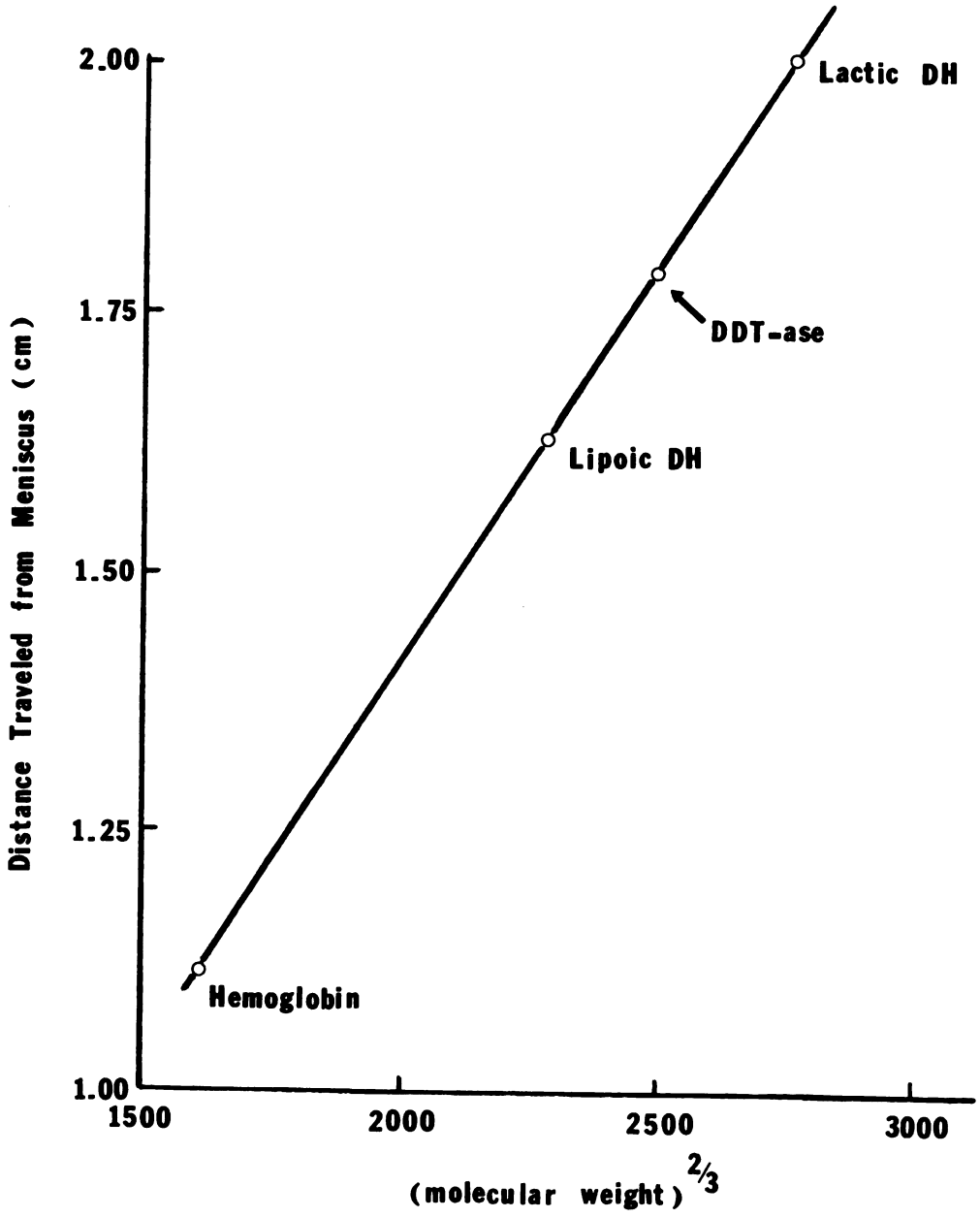

Lip. DH x 1.6
DDT-ase
Hemoglobin
LDH x .15
Lip. DH
DDT-ase
Hb
A at 405 mμ
1.0
0.5
LDH
DDT-ase
Hb
A at 405 mμ
Activity (μ/ml)
Fraction
5
10
15
20
25
30

Figure 15. Molecular Weight of Purified House fly DDT-ase from Sucrose Density Centrifugation. The position of the peaks (Figure 14) are plotted against molecular weight to the 2/3 power, giving a straight line relationship (Martin and Ames, 1961). From this plot, the molecular weight of DDT-ase is estimated to be 120,000.


2.00
1.75
1.50
1.25
1.00
Distance Traveled from Meniscus (cm)
Lactic DH
DDT-ase
Lipoic DH
Hemoglobin
1500
2000
2500
3000
(molecular weight) 2/3

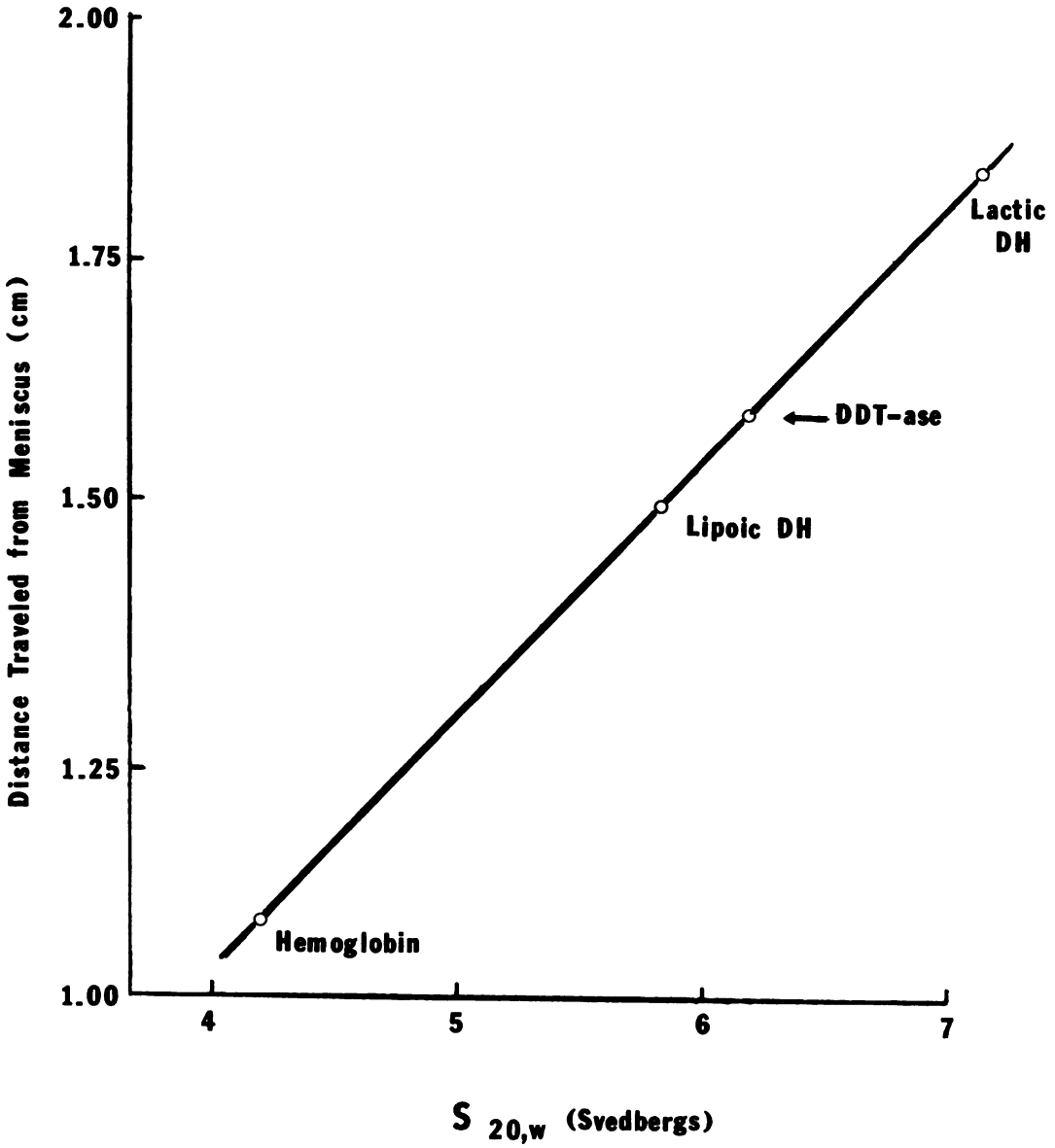
production of antibodies against the monomeric forms of the enzyme.

The decision to use alum as an adjuvant for enhancing antibody production, as opposed to the more widely used Fruend's mineral oil adjuvant, was based on the vigorous emulsification requirement of this latter technique. The susceptibility of DDT-ase to mechanical effects (Goodchild and Smith, 1969) precluded the use of this adjuvant in order that the tetrameric structure of the enzyme would not be altered prior to injection.

Quantitative Precipitin Reaction -- The quantitative precipitin reaction was performed as described by Kabat and Mayer (1964). Undiluted Fraction V antiserum (0.5 ml) was added to the purified DDT-ase antigen serially diluted with 0.01 M KH_2PO_4 buffer, containing 0.01 M GSH, at pH 7.4. The mixture was allowed to react for 1 hour at 37°C followed by 20 hours at 4°. After centrifugation, the precipitates were washed twice with cold 0.9% sodium chloride and resuspended in 0.5 ml of the same solution. Protein concentration was estimated by the microbiuret method of Itzhaki and Gil (1964).

The quanitative precipitin curve of Fraction V antiserum with the purified enzyme is shown in Figure 17. The typical bell shaped precipitin curve was obtained, and at the equilivance point on this curve it was calculated that each ml of undiluted antiserum contains 1.56 mg antibody to the DDT-ase antigen. The antibody-antigen molar ratio was also calculated, based on the assumptions that DDT-ase has a molecular weight of 120,000 and the antibody is the immunoglobin G (Ig G) type with a molecular weight of 150,000. Figure 17 shows an

Figure 16. Determination of Sedimentation Coefficient of Purified House fly DDT-ase by Sucrose Density Gradient Centrifugation. The positions of the peaks are plotted against the S-values of the marker proteins. From this plot, the sedimentation coefficient of purified house fly DDT-ase was found to be 6.3 S.


2.00
1.75
1.50
1.25
1.00
Distance Traveled from Meniscus (cm)
Lactic DH
DDT-ase
Lipoic DH
Hemoglobin
4
5
6
7
S 20,w (Svedbergs)

antigen-antibody mole ratio of 3.06 at the equivalence point, and indicates that at this region of the precipitin curve approximately three molecules of antibody combined with one molecule of antigen.

The data obtained from the quantitative precipitin curve of the Fraction V antiserum was used to determine the optimal proportions of antigen and antibody required for the Ouchterlony double diffusion, disc gel immunoelectrophoretic and fluorescent antibody techniques.

Inhibition of DDT-ase Activity by the Fraction V Antiserum -- Inhibition of DDT-ase activity by rabbit antiserum was determined by incubating a constant amount of enzyme (200 μg) with increasing amounts of undiluted Fraction V antiserum (50 μl - 700 ul) in 0.5 ml 0.01 M phosphate buffer, pH 7.4, containing 0.1 M GSH. After incubation for 30 minutes at 37°C, each antigen-antiserum mixture was assayed for DDT-ase activity.

Figure 18 shows the results of this experiment, and indicates a progressive reduction of DDT-ase activity with the addition of increasing amounts of specific antiserum. At the equivalence point (Ab/DDT-ase = 3.06), the enzyme incurred a 100% loss of activity. When normal rabbit serum was substituted for the Fraction V antiserum, there was no effect on enzyme activity demonstrating that antibodies induced by DDT-ase were responsible for the observed inhibition.

Inhibition of the Antigen-Antibody Reaction by the Chemical Dissociation of the DDT-ase Antigen -- This experiment was performed to determine whether or not the antibody induced by DDT-ase was specific for just the tetrameric form of the enzyme or if it also had the ability

Figure 17. Quantitative Precipitin Reaction of the Purified House fly DDT-ase with Rabbit Antiserum. Undiluted Fraction V antiserum (0.5 ml) was added to purified DDT-ase antigen serially diluted with 0.01 M KH_2PO_4 buffer, containing 0.01 M GSH, at pH 7.4. The mixture was reacted for 1 hour at 37°C and then for 20 hours at 4°. The precipitates were collected by centrifugation and protein concentration measured by the microbiuret method.

Molar ratio (R) of antibody to DDT-ase in the precipitate was calculated by the following equation:

$$R = \frac{\dfrac{\text{Total protein precipitated (µg) - total DDT-ase added (µg)}}{150{,}000}}{\dfrac{\text{Total DDT-ase added (ug)}}{120{,}000}}$$

The open symbols (-o-o-) indicate precipitated protein; the closed symbols (-●-●-) the molar ratio of antibody: DDT-ase in the precipitate. The arrows indicate the equivalence point, and zones of antibody and antigen excess.

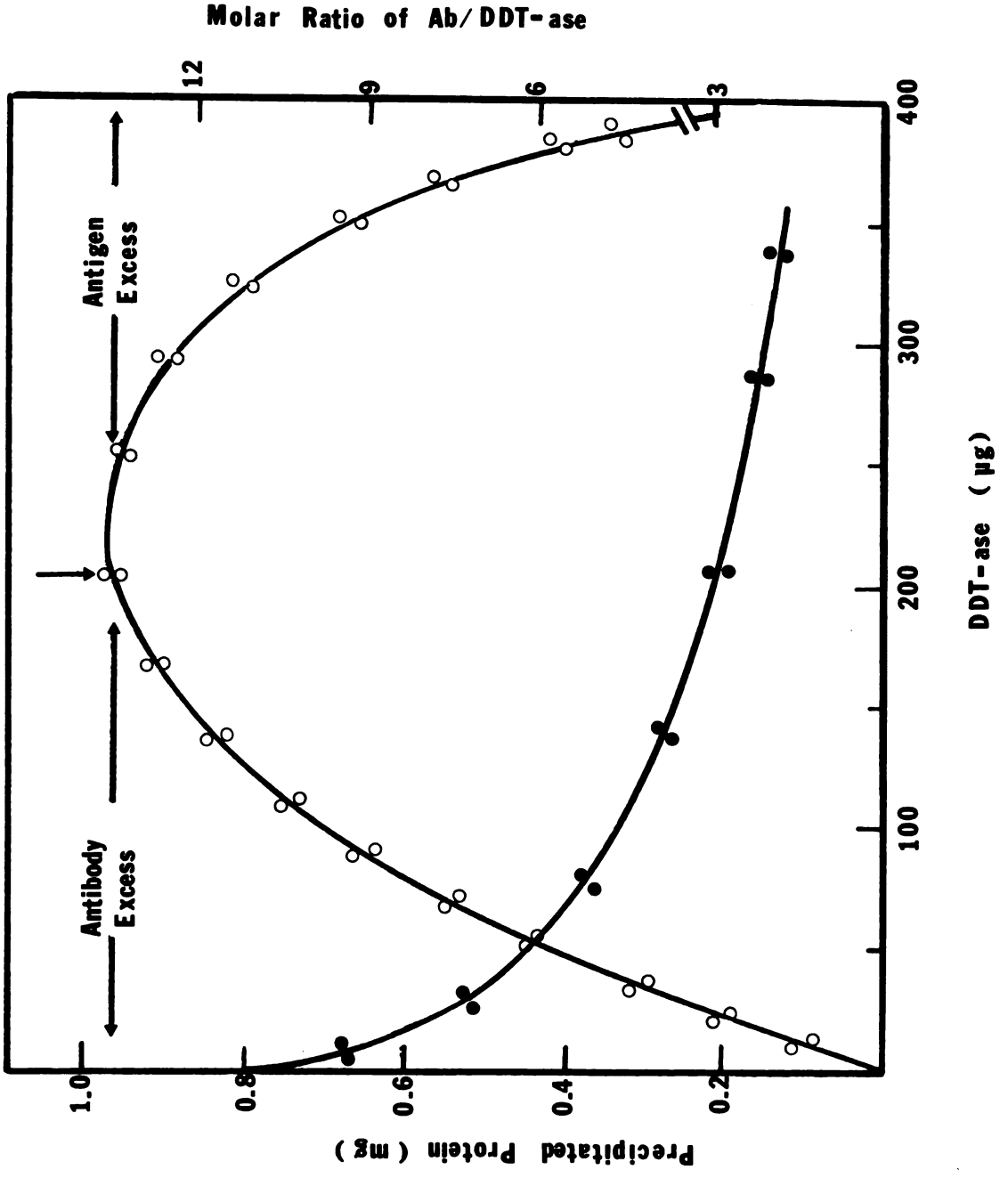
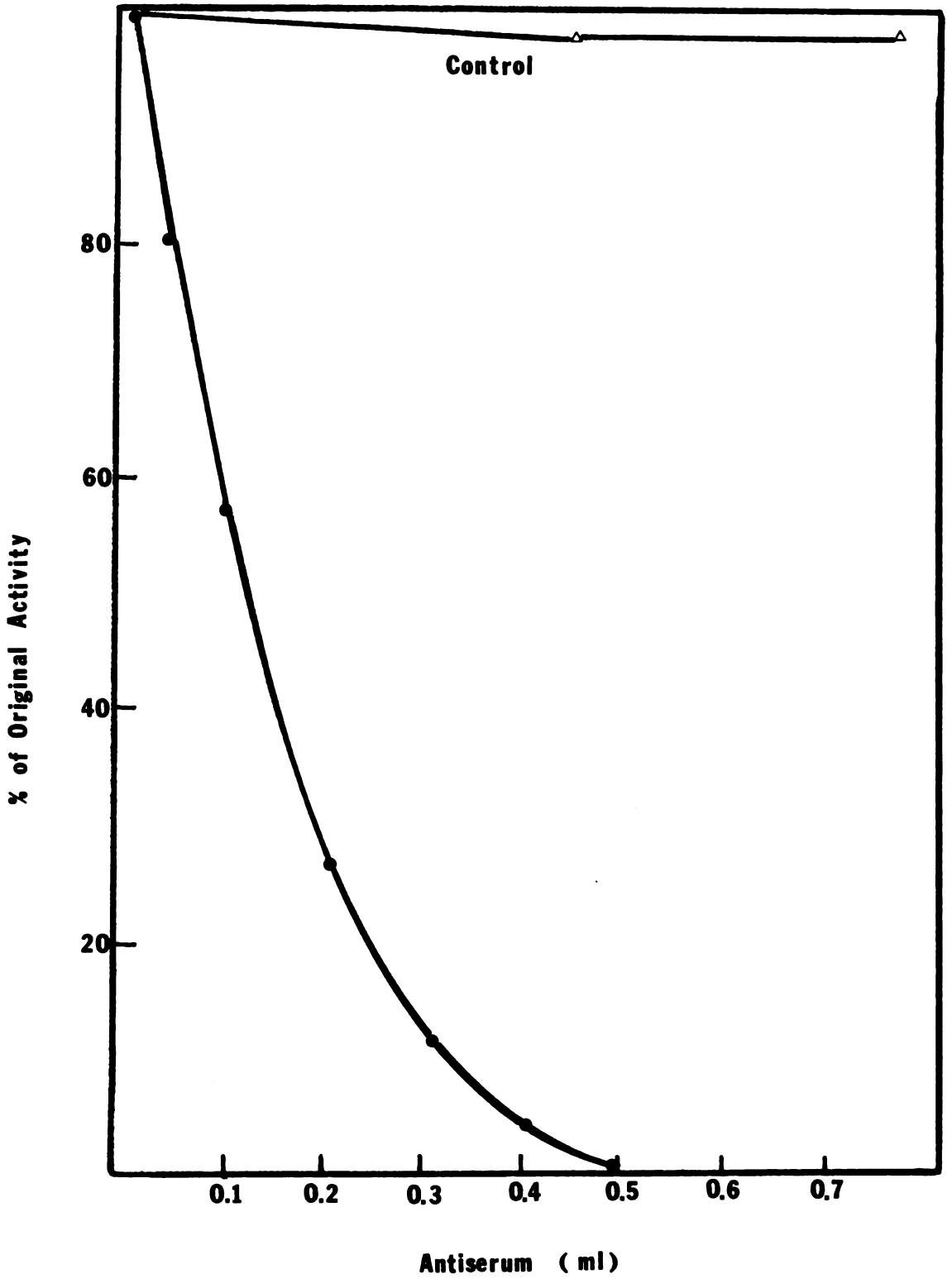

Molar Ratio of Ab/DDT-ase
12
9
6
3
Antigen Excess
Antibody Excess
1.0
0.8
0.6
0.4
0.2
Precipitated Protein (mg)
400
300
200
100
DDT-ase (µg)

Figure 18. Inhibition of Purified House fly DDT-ase by Rabbit Serum and Antiserum. Reaction mixtures contained a constant amount of enzyme (200 µg) with the indicated amounts of rabbit serum or antiserum in 0.5 ml 0.01 M KH_2PO_4 buffer, pH 7.4, containing 0.1 M GSH. After incubating for 30 minutes at 37°C each antigen-antibody mixture was assayed for DDT-ase activity.

—▲— represents the purified DDT-ase with normal serum; —●— represents the purified DDT-ase with antiserum.


Control
80
60
40
20
% of Original Activity
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Antiserum (ml)

to combine with the monomeric form.

Complete dissociation of DDT-ase into the monomeric form was accomplished by adding 0.1 M mercaptoethanol to a solution of the purified enzyme (600 μg in 1.5 ml 0.01 M KH_2PO_4 buffer, pH 7.4). Disc gel electrophoresis of this solution revealed a single protein band of a higher mobility than what was resolved from electrophoresing the Fraction V product in the presence of 0.01 M GSH (Figure 8B). Dinamarca et al. (1971) reported a similar protein band resolved after electrophoresing DDT-ase in the presence of 0.1 M mercaptoethanol in the cathodic buffer reservoir and sample solution. The molecular weight of this fast migrating band was demonstrated to be 30,000, which is the value expected for the monomeric form of the enzyme.

Ouchterlony double diffusion was performed by reacting the Fraction V antiserum with the associated and dissociated forms of purified DDT-ase. Figure 19 shows the results of this experiment, which indicates a precipitin band forming only between the antiserum and the associated form of the enzyme. It can be concluded from these results that the purification procedure yielded the pure enzyme in its tetrameric form, and that the injection sequence was successful in producing an antibody specific only to this form of the enzyme.

It can also be deduced that the enzyme is in vivo in the active tetrameric form. Dinamarca et al. (1969) reported that while GSH could not promote monomeric polymerization, it could be effectively done by the substrate DDT. For this reason they speculated that the enzyme is in vivo in the inactive form and becomes active only

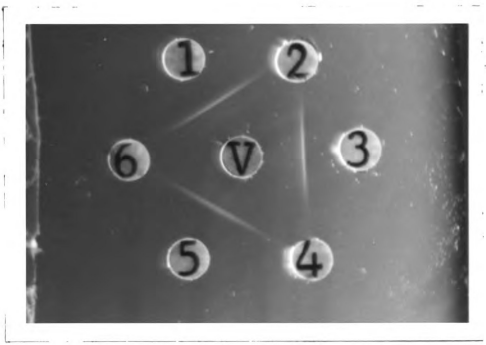


Figure 19. Inhibition of the Antigen-antibody Reaction by the chemical dissociation of the DDT-ase Antigen. Complete dissociation of DDT-ase was accomplished by adding 0.1 M mercaptoethanol to a solution of the purified enzyme. Ouchterlony double diffusion was performed by reacting the associated enzyme (tetramer) (wells 1, 3, and 5) and dissociated (momomer) (wells 2, 4, and 6) enzyme with the Fraction V antiserum. The gels were incubated at 37^{o} for 24 hours before photographing under diffuse light.

when DDT is present. However, the results demonstrated in Figure 19 indicate that this is probably not the case. Further proof is also provided by the specific tissue staining obtained by the fluorescent antibody technique discussed later in this section. Since the Fraction V antiserum has the ability to react only with the tetrameric structure, specific staining could not have occurred it the enzyme present in house fly tissues was in the dissociated monomeric form.

FLUORESCENT ANTIBODY STUDIES

Control of Non-Specific Staining -- Although serologic studies with labeled antibodies are simple to perform and can offer significant advantages, failure to recognize the numerous factors that govern the observable outcome of a reaction sequence restricts their effective utilization. Adequate controls must be employed in order to yield reproducible and interpretable and results.

Non-specific staining is one of the major causes of difficulty in interpreting immunofluorescence. This problem is amplified when the indirect method is utilized (Wellor and Coons, 1954), because of its greater complexity and sensitivity. The series of control reactions, summarized in Table 3, were routinely performed in order to establish that the fluorescent staining observed in the tissue sections were indeed due to the presence of DDT-ase.

TABLE 4

Controls for Establishing Specificity
Of Staining by
The Indirect (Antiglobulin) Method

Antigen	Reagent for Step 1	Reagent for Step 2	Result
Homologous	None or saline	Labeled antiglobulin	No fluorescence
	Normal serum	Labeled antiglobulin	No fluorescence
	Absorbed specific antiserum	Labeled antiglobulin	No fluorescence
	Specific antiserum	Labeled normal serum	No fluorescence
Heterologous	Specific antiserum-against NAIDM susceptible housefly strain	Labeled antiglobulin	No fluorescence

To eliminate additional causes of non-specific staining the following procedures were also undertaken: (1) Unconjugated free dye present in the labeled sheep antirabbit gamaglobulin (Nutritional Biochemical Corp.) was removed by absorption with tissue powder. In this procedure 100 mg/ml of dry acetone powder, prepared from NAIDM-susceptible house flies, was added to the solution of conjugated antibody, and mixed thoroughly. After standing for 1 hour at room temperature, the mixture was centrifuged at 10,000 x g for 20 minutes. A second portion of 50 mg/ml of powder was added to the supernatant, and the procedure repeated. The supernatant was collected and stored at $0^{o}C$ for future use (Kawamura, 1969). (2) The highly reactive, negatively charged albumen fraction was removed from the Fraction II and V antiserum by the ammonium sulfate procedure discussed previously in the Materials and Methods section. (3) Finally, a series of trial and error experiments

was done to determine the maximum dilution point for the labeled and specific antisera, which minimized non-specific staining, yet still produced discernible staining of the antigen in the tissue sections. The optimal dilution ratio was found to be 10:1 for the labeled antiserum, 8:1 for the Fraction V antiserum, and 4:1 for the Fraction II antiserum.

Absorption of the Fraction II Antiserum -- It was originally theorized that specific staining might be obtained by absorbing out contaminating antibodies not specific to the DDT-ase antigen from the Fraction II antiserum, and therefore saving the time and effort required in the complete purification of DDT-ase. The following two procedures described in detail in the Materials and Methods section, were used in an attempt to achieve this end:

Method A -- The rationale for this method was based on the theory that the NAIDM-susceptible strain of house flies was identical to the resistant DDT-Orlando strain, except for their relative lack of DDT-ase. Therefore, reacting a solution of a Fraction II preparation of NAIDM-susceptible house flies with the Fraction II antiserum (induced form a Fraction II preparation of DDT-Orlando house flies) should absorb out the non-specific antibodies from this antiserum, while leaving the DDT-ase specific antibody intact.

The absorbed Fraction II antiserum was reacted against the Fraction II preparation in an Ouchterlony double diffusion test and the results demonstrated a complete absence of precipitating antibodies.

In addition, when this absorbed antiserum was used for fluorescent antibody staining of tissue sections, there was very little fluorescent staining present in the sections. The fluorescence that was observed was definitely non-specific and proved that this absorption method was not adaptible for this study.

The failure of this technique can probably be attributed to the fact that the two strains of house flies are different in more ways than just their respective abilities to dehydrochlorinate DDT. The loss of the specific DDT-ase antibody from the absorbed antiserum could be the result of it forming some type of complex with other proteins in the system, which were then precipitated out of the solution during centrifugation.

Method B -- The rationale for this absorption method was based on the observation that the specific DDT-ase antibody does not form a complex with the enzyme, which was first reacted with the DDT substrate and GSH cofactor.[1] Therefore, mixing this "reacted" enzyme solution (Fraction II Preparation) with the Fraction II antiserum should result in the complexing of the various contaminating antibodies with their respective antigens. After centrifugation, these complexes should precipitate out of the antiserum, leaving a supernatant containing only the specific DDT-ase antibody.

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[1] It is interesting to note that this observation would seem to indicate some type of conformational change in the structure of DDT-ase during the dehydrochlorination process. A speculation also made by Dinamarca et al., 1971 in an attempt to explain the role of GSH in this enzymatic reaction.

Figure 20 shows the results of reacting this absorbed Fraction II antiserum with the crude Fraction II preparation in an Ouchterlony double diffusion test. The appearance of a single chevron indicates the existence of a single precipitating antigen-antibody system.

The use of this absorbed Fraction II antiserum for fluorescent antibody staining is demonstrated in Figure 21. The tissue represented in this photomicrograph is a cross-section of a 4 day old resistant house fly larva. Specific staining occurred and indicates a significant amount of DDT-ase present in the imaginal discs. However, the control section demonstrated that part of this fluorescence was due to non-specific staining. The use of this absorbed antiserum for the fluorescent staining of other house fly tissues gave inconsistent results. For this reason it was decided that optimal results with the fluorescent antibody technique could only be achieved by producing an antiserum containing antibodies specific to just the purified DDT-ase antigen.

Specific Fluorescent Antibody Staining of DDT-ase in Various House Fly Tissues -- The following photomicrographs demonstrate the localization of DDT-ase in various house fly tissues by the indirect fluorescent antibody staining technique. Although there are varying degrees of non-specific staining present in these tissue sections, the various control sections indicate that it does not interfere with the demonstration of specific staining of DDT-ase. The procedures followed in the indirect fluorescent staining of the various house fly tissues are described in

Figure 20. The Absorption of Non-Specific Antibodies Out of the Fraction II Antiserum. Fraction II preparation was reacted with DDT and GSH, and then mixed with the Fraction II antiserum (See text for details). This absorbed mixture was reacted in an Ouchterlony double diffusion test (center well) against the Fraction II preparation (all outer wells). Diffusion was carried out at 37°C for 24 hours before photographing under diffuse light.

Figure 21. Cross section of a four day old DDT-resistant larva. Specific fluorescent antibody staining demonstrated in the imaginal discs. Magnification 700x.

Figure 20. See opposite page.

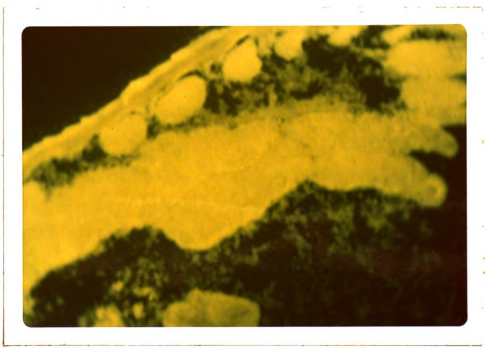

Figure 21. See opposite page.

detail in the Materials and Methods section. It should also be pointed out that the colors represented in the photomicrographs are only an approximation of what was seen through the microscope. This is due to inaccuracies in the processing of the Ektachrome slides (Kodak) into enlarge prints. The UV light source and long exposure times altered the color emulsion layers of the transparencies in such a way that accurate color reproduction in the prints could have only been accomplished by prohibitively costly "custom" processing.

(A) Integument -- Figure 22 shows that DDT-ase is located in relatively high amounts in the epidermal layer and to a lesser degree in the lamellae of the endocuticle. A significant amount of DDT would, therefore, dehydrochlorinate as it penetrates through these regions of the integument.. The cuticulin is sharply defined due to its natural autofluorescence.

(B) Ovary -- While the total amount of DDT-ase in the house fly ovary is quite low, there is a significant amount localized along the chorionic membrane of the egg (Figures 23, 24, and 25). This enzymatic barrier would logically inhibit DDT from entering and becoming solubilized in the lipid-rich yolk material. Distinct autofluorescing tracheae can be seen paralleling the perimeters of the egg cells visible in Figures 23 and 25. Figure 26 shows the conspicuous absence of fluorescent staining in an ovarian section dissected from a NAIDM-susceptible house fly. The structure barely visible in this control section is an autofluorescent trachea.

(C) Thoracic Flight Muscle -- DDT-ase is ubiquitously present in discernible amounts in this house fly tissue (Figure 27). However,

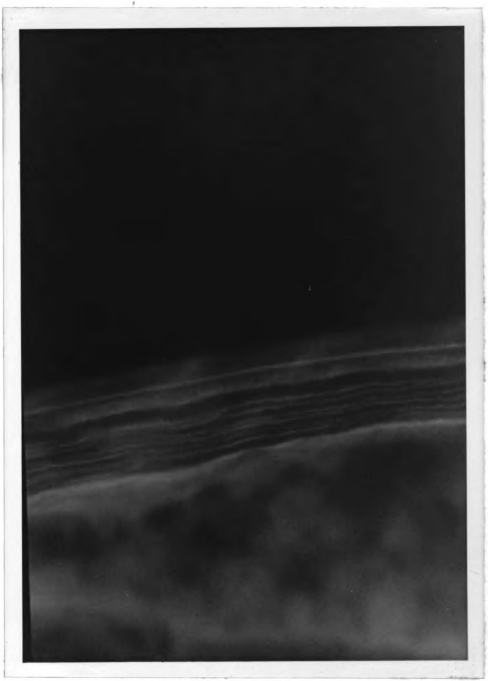

Figure 22. Cross section of the integument of an adult DDT-resistant house fly. Magnification 860x.

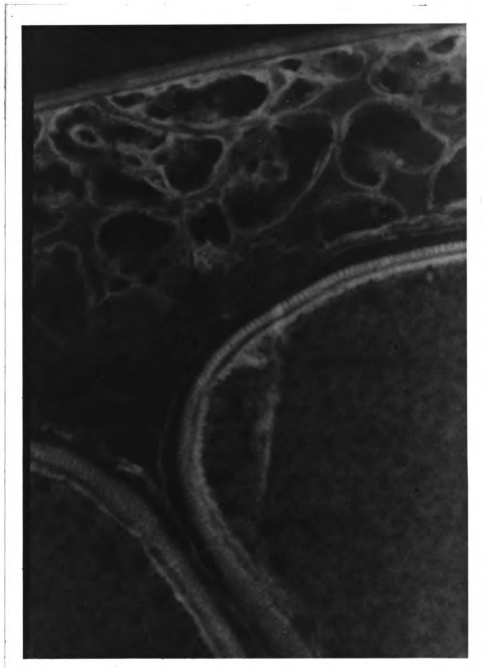

Figure 23. Cross section through the ovary of an adult DDT-resistant house fly. Specific fluorescent antibody staining of DDT-ase is demonstrated along the chorionic membrane of the two egg cells observable in the lower half of the picture, and in the fat body tissue near the top of the picture. Magnification 700x.

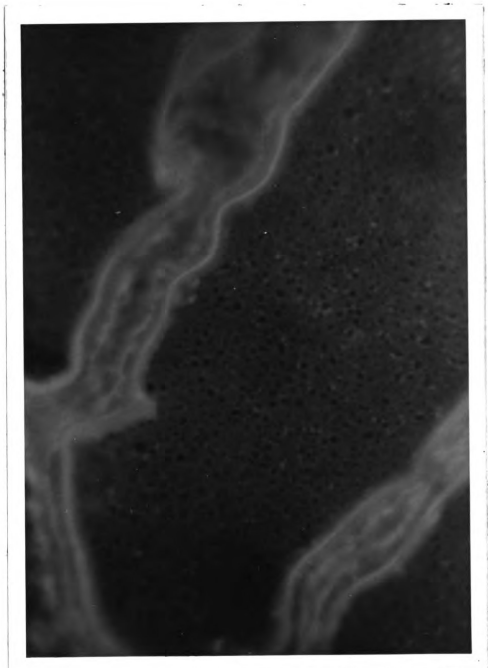

Figure 24. Cross section of an egg in the ovary of an adult DDT-resistant house fly. Specific flourescent antibody staining of DDT-ase is demonstrated along the chorionic membrane surrounding the egg cell. Magnification 860x.

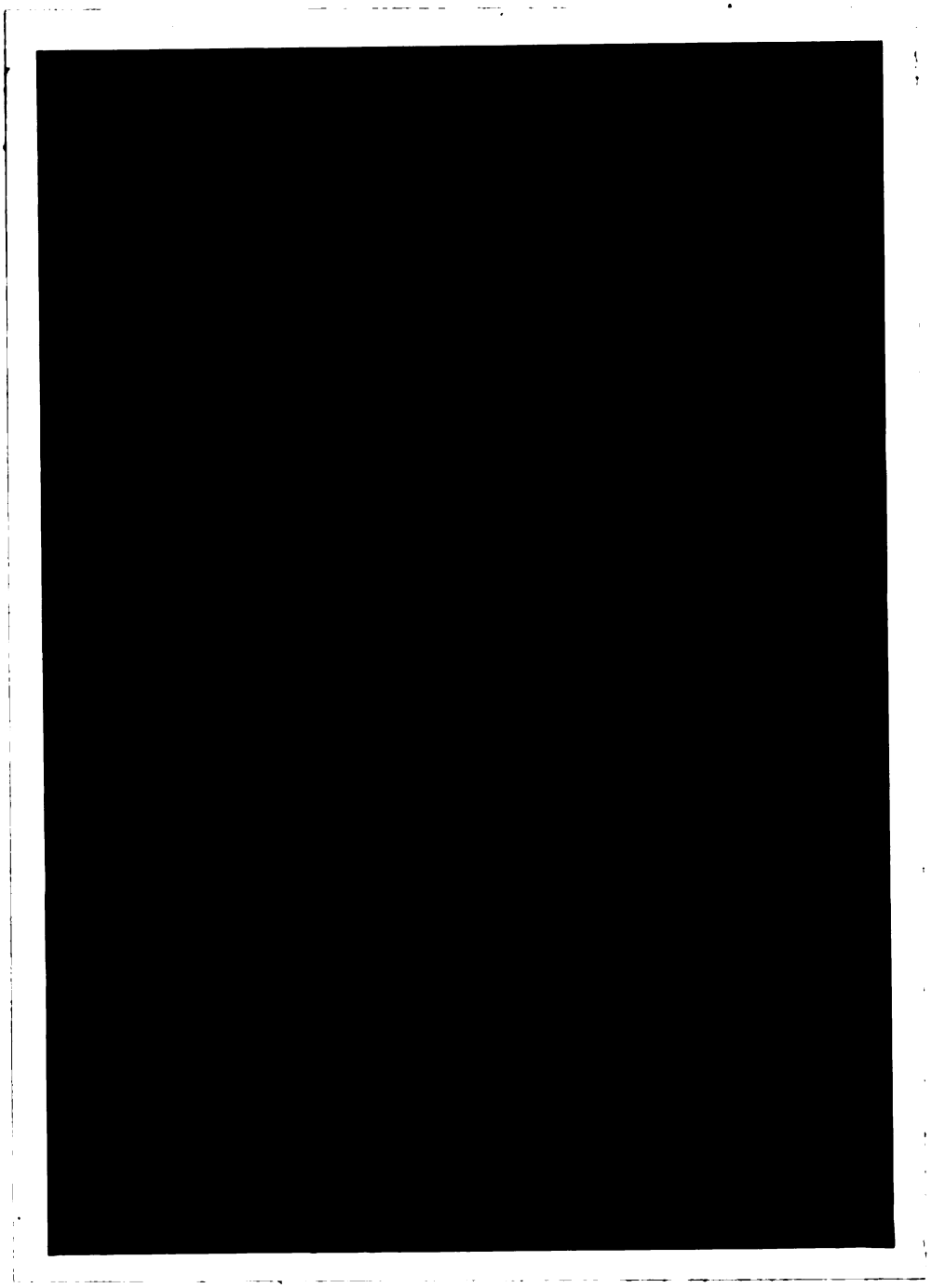

Figure 25. Saggital section through egg cells of an adult DDT-resistant house fly. Specific fluorescent antibody staining of DDT-ase can be seen along the chorionic membrane on the perimeter of the egg cells. Magnification 860x.

Figure 26. Cross section of an ovary of an adult non-resistant house fly (NAIDM-susceptible). This control section shows the absence of DDT-ase typical of most control sections. The structure barely visible is an autofluorescent trachae. Magnification 860x.

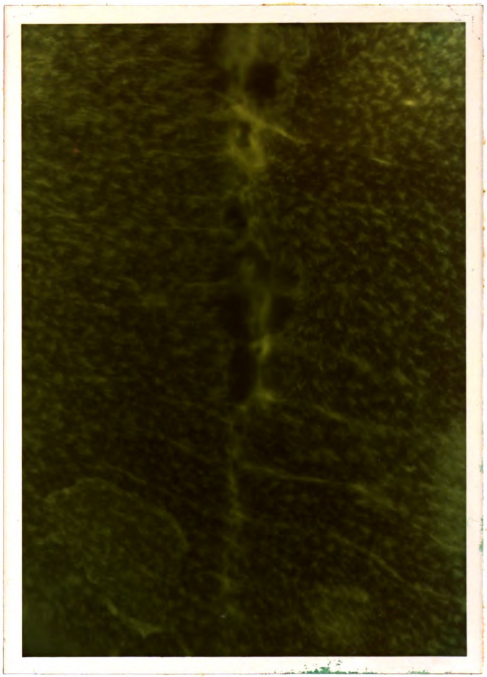

Figure 27. Cross section of thoracic flight muscle of an adult DDT-resistant house fly. Magnification 700x.

Figure 28. Cross section through the proximal intestine of an adult DDT-resistant house fly. Magnification 860x.

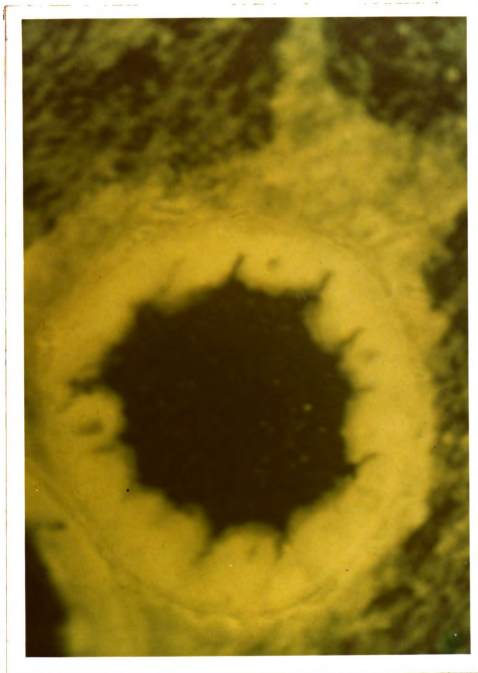

Figure 29. Cross section through the mid-gut of a third stage larva, demonstrating significant fluorescent antibody staining of DDT-ase in this tissue. Magnification 860x.

much of the "typical" yellow-green (FITC) fluorescence is masked by the natural blue autofluorescence of this tissue, resulting in the observed uncharacteristic "kelly" green color.

(D) Intestinal Tract -- Figure 28 demonstrates the titre of DDT-ase present in the epithelial wall of the proximal intestine of the adult house fly. After subjectively subtracting the amount of non-specific staining observed in the control sections, it is obvious that DDT-ase is not present in any appreciable amount in this tissue.

This is not the case, however, with the intestinal tract of the house fly larvae. Figure 29 shows a cross-section through the mid-gut region of a third-stage larva, and demonstrates a significant titre of DDT-ase located in this tissue. Although the control sections indicate that there is approximately 30 percent non-specific staining, it accounts for only a portion of the fluorescence observed in this tissue.

(E) Fat Body --Figures 30 and 31 show that this tissue contains a very high titre of uniformly distributed DDT-ase. Unfortunately, the problems in color processing discussed previously, are especially apparent in these photomicrographs. The fluorescence observed through the microscope, and recorded on the Ektachrome transparencies, is much more intense than what can be observed in the enlarged prints. Figure 32 is a control showing the minor quantity of DDT-ase present in the fat body sectioned from a non-resistant house fly (NAIDM-susceptible). The purplish color of the print is the result of the color processor attempting to enhance the barely visible fluorescence recorded in the dark transparency.

(F) Nervous Tissue -- The high degree of fluorescent staining observed in nerve tissue indicates that it contains the highest titre of DDT-ase of any of the house fly tissues tested. Figure 33 is a section through an optic peduncle of the procerebrum region of the central nervous system, and the bright specific fluorescent staining displayed, denotes the significant amount of DDT-ase present. Figure 34 represents a section through a dorsal mesothoracic nerve, and Figure 35 a section through a mesothoracic ganglion (West, 1951). In both photomicrographs, DDT-ase can be observed to be concentrated in the neural lamella surrounding the nerve cord. In Figure 35 the presence of DDT-ase actually "outlines" the ganglionic tissue from the non-nervous tissue around it. It is apparent that the strategic location of DDT-ase observed in these nerve tissues, operates as a "last line of protection" against the toxic action of DDT on the vulnerable nervous system of DDT-resistant house flies.

In summary, the localization of DDT-ase by the fluorescent antibody technique successfully demonstrates that this enzyme is more or less ubiquitously distributed throughout the DDT-resistant house fly. This substantiates the earlier report of Miyake et al. (1957), in which they measured DDT-ase activity in different excised resistant house fly tissues. Additionally, a comparison of the specific fluorescent staining observed in the photomicrographs of the various house fly tissues, indicates that nerve and fat body tissue contain the highest titres of enzyme, the integument and muscle, intermediate levels, while the ovary and intestinal tract contain the lowest amounts of DDT-ase.

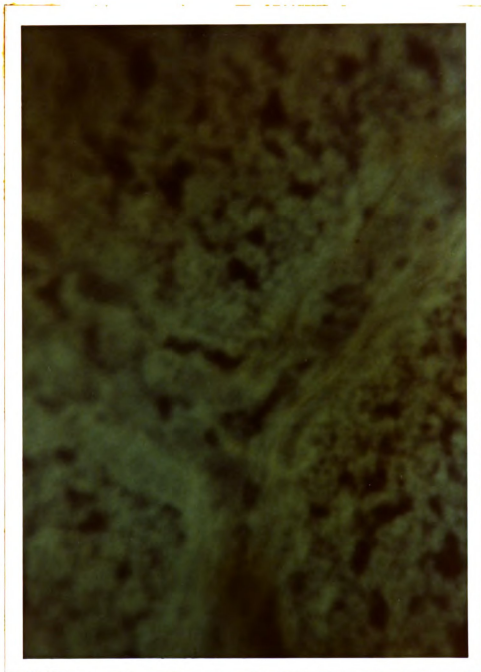

Figure 30. Cross section through the fat body of an adult DDT-resistant house fly. Magnification 280x.

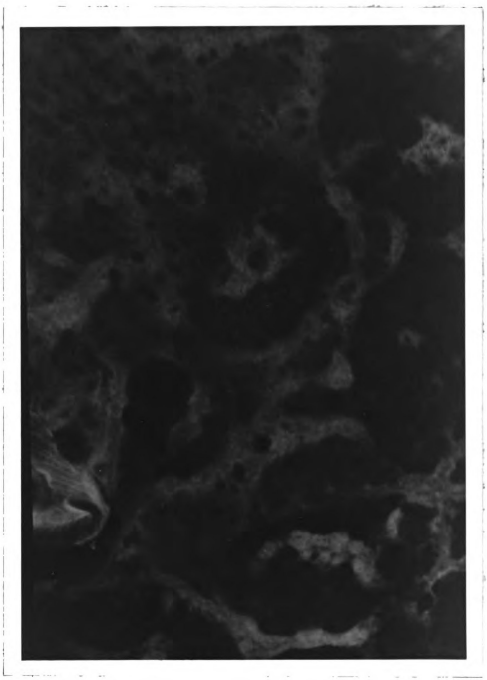

Figure 31. Cross section through the fat body of an adult DDT-resistant house fly. Magnification 860x.

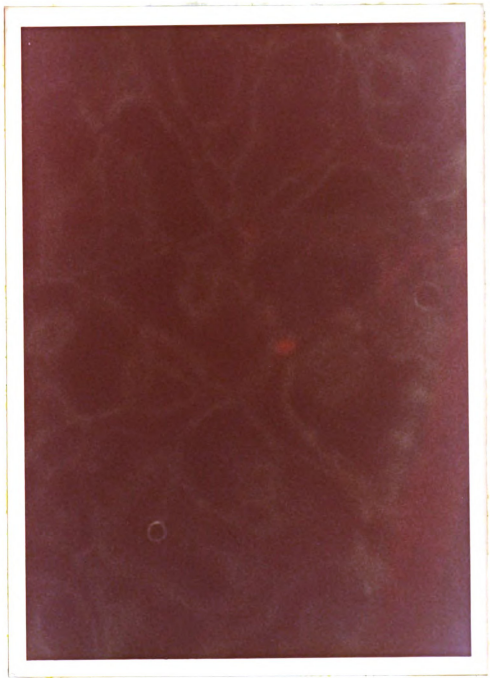

Figure 32. A control section through the fat body of an adult non-resistant house fly. Magnification 860x.

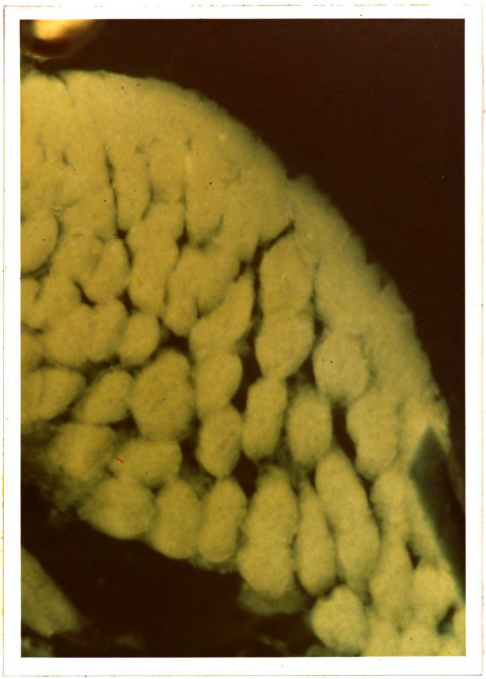

Figure 33. Cross section through the optic peduncle of the procerebrum region of an adult DDT-resistant house fly. The bright specific fluorescent staining displayed, denotes the significant amount of DDT-ase present.

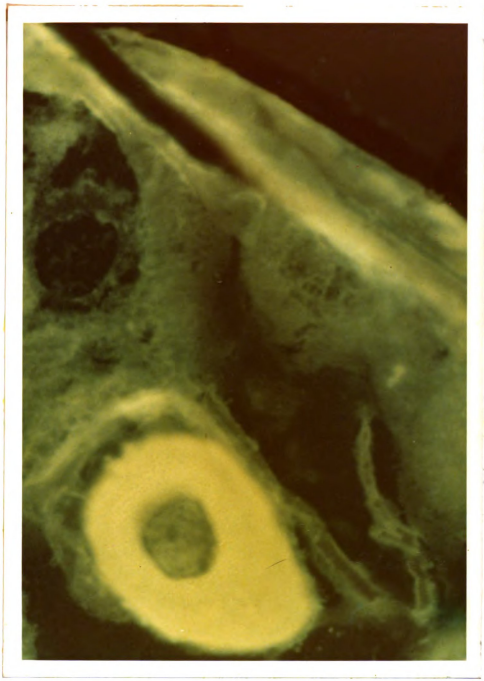

Figure 34. Cross section through a dorsal mesothoracic nerve of a DDT-resistant house fly. Specific fluorescent staining shows that DDT-ase is concentrated in the neural lamella of the nerve.

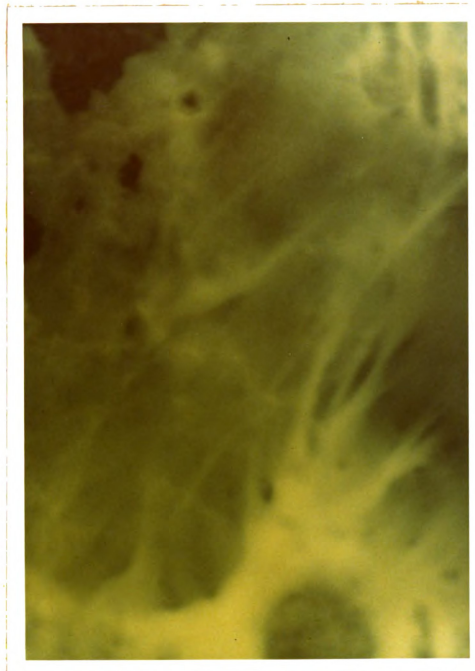

Figure 35. Cross section through a mesothoracic ganglion of an adult DDT-resistant house fly. Specific fluorescent antibody staining shows the presence of DDT-ase "outlining" the ganglionic tissue.

This also parallels the results reported by Miyake et al. (1957).

The most significant contributions of the localization of DDT-ase by the fluorescent antibody technique are as follows:

(1) The distribution of DDT-ase in the epidermal and endocuticle layers of the integument (Fig. 22) definitively supports the speculation by Sternburg and Kearns (1950), and Tahori and Hoskins (1953) that significant detoxification of DDT occurred in the integument before it reached a vital site.

(2) While the total level of DDT-ase contained in ovarian tissue is relatively low, the presence of the enzyme along the chorionic membrane is quite significant (Figures 23, 24, and 25). Since the amount of DDT-ase in house fly eggs is negligible (Moorefield and Kearns, 1957), this enzymatic barrier could provide important protection to the maturing egg prior to ovipositing.

(3) The high titre of DDT-ase localized in the fat body (Figures 30 and 31), give additional support for the important role this organ plays in the detoxification of pesticides (Perry and Agosin, 1974).

(4) The demonstration of significant amounts of DDT-ase in the larval intestinal tract (Figure 29), would appear to be an important survival mechanism for this stage of the house fly. When one considers the feeding habits of house fly larvae, its understandable how the presence of DDT-ase in the walls of the long alimentary canal would give a selective advantage for their successful development.

(5) Finally, the strategic localization of DDT-ase in nervous tissue provides evidence against the theory that differences between resistant and susceptible house flies are due to differences in the inate sensitivity of their nerves to DDT (Pratt and Babers, 1973; LeRoux and Morrison, 1954; Smyth and Roys, 1955; Wiessmann, 1955). The presence of DDT-ase in the neural lamella (Figures 33, 34, and 35), logically provides protection against DDT poisoning and, therefore, the maintainance of normal nervous activity. The abnormal nervous patterns manifested in poisoned susceptible flies can then be attributed to their nervous system lacking this enzymatic protection.

The fact that resistant flies have also been shown to contain large quantities of undegraded DDT, sufficient to kill several susceptible flies (Babers and Pratt, 1973; Perry and Hoskins, 1951b; Tahori and Hoskins, 1953), can also be explained by barriers of DDT-ase protecting vital sites against the toxic action of DDT.

Demonstration of DDT-ase Induction by the Fluorescent Antibody Technique

This experiment was performed to determine the feasibility of using the fluorescent antibody technique to demonstrate the induction of DDT-ase.

The results of this experiment are shown in Table 4, and indicate that there is a significant difference between the exposure times of the control and experimental sections. While all the tissues tested show statistically that the variations obtained are probably not due to chance, the fat body is the most impressive, demonstrating a high degree of metabolic synthesis of DDT-ase in this organ. Not only does this

experiment support the results reported by Capdevila et al. (1973), proving that DDT can induce the in vivo synthesis of DDT-ase, but it may also be the first time the fluorescent antibody technique has been employed to demonstrate enzyme induction.

TABLE 5

The Effect of DDT Treatment
On the Level of DDT-ase
Present in Various House Fly Tissues

	Tissue	Treatment	Exposure Time + S.D.[1]	T value	P
1)	Ovary	Control (acetone only)	229 ± 19.3	3.08	.04
		Experimental (DDT treated)	185 ± 15.5		
2)	Muscle	Control	285 ± 13.2	3.9	.02
		Exptl.	250 ± 8.0		
3)	Cuticle	Control	193 ± 13.4	2.7	.05
		Exptl.	161 ± 15.1		
4)	Brain	Control	85 ± 11.3	2.8	0.5
		Exptl.	63 ± 7.5		
5)	Fat Body	Control	135 ± 5.6	5.5	.01
		Exptl.			

[1] The data represented here was obtained from 3 identical experiments.

SUMMARY

I. House fly DDT-ase has been purified to homogeneity by a fractionation procedure consisting of ammonium sulfate precipitation, molecular exclusion chromatography (Bio-Gel P-10 and P-150 polyacrylamide beads), and preparative disc gel electrophoresis. The procedure used 100 g of whole flies and yielded approximately 9 mg of pure enzyme with a specific activity of 8961 mumoles of DDT dehydrochlorinated per mg protein/hour.

II. Rabbit antiserum containing antibodies against the purified house fly DDT-ase prepared. Analysis of this antiserum by the quantitive precipitin reaction demonstrated that 3 moles of anti-body combined with 1 mole of antigen at the zone of equivalence.

It was also shown that incubating increasing amounts of antiserum with a constant amount of purified DDT-ase caused a progressive reduction in enzyme activity. At the equivalence point (Ab/DDT-ase = 3.06), the enzyme was 100% inhibited.

Ouchterlony double diffusion was performed by reacting the DDT-ase antiserum with the associated and dissociated forms of purified DDT-ase. It was shown that a precipitin band formed only between the antiserum and the associated form of the enzyme. This indicated that the antiserum was specific only for the tetrameric form of DDT-ase.

III. DDT-ase antiserum was used in the indirect fluorescent antibody technique for localizing DDT-ase in various tissues of the house fly. Specific fluorescent staining indicated that nerve and fat body tissue contained the highest titre of enzyme, the integument and muscle, intermediate levels, while the ovary and intestinal tract contained the lowest amounts of DDT-ase. More specifically, it was demonstrated that DDT-ase was localized in high concentrations in the epidermal and endocuticle layers of the integument, the chorionic membrane surrounding egg cells and the neural lamella of nerve cords.

IV. The fluorescent antibody technique was used to demonstrate induction of DDT-ase. A significant difference was observed in the quantitative fluorescence of identical tissues sectioned from DDT-treated and non-treated house flies. This experiment further supports the idea of an inductive phenomenon. Functioning in resistance, and demonstrates an original and valuable application of the fluorescent antibody technique.

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